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Oxidative Stress as a Possible Mechanism of Toxicity of the Herbicide 2,4-Dichlorophenoxyacetic Acid (2,4-D)

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1. Introduction

Chlorophenoxy herbicides are widely used in agriculture and forestry, for the control of broad-leaved weeds in pastures, cereal crops, as well as along public rights of way. Structurally, these herbicides consist of a simple aliphatic carboxylic acid moiety attached to a chlorine-substituted aromatic ring via an ether linkage. One of the most commonly used herbicides of this type is 2,4-dichlorophenoxyacetic acid (2,4-D) (Fig. 1). In congruence with the similitude between its molecular structure and that of the plant hormone indole-acetic acid, 2,4-D acts as a plant growth regulator that can interfere with normal hormonal action and plant growth (Munro et al., 1992).

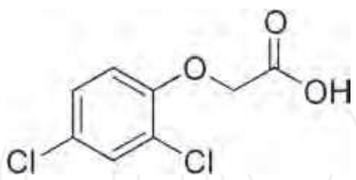

Fig. 1. Structure of the 2,4-Dichlorophenoxyacetic Acid.

2.4-D was synthesized for the first time in 1941 and commercially marketed in the United States (U.S.) in 1944 (IARC, 1986) and worldwide since 1950 (Munro et al. 1992). The widespread use of 2,4-D as a domestic herbicide and as a component of Orange Agent encouraged the study of its toxicity.

Human exposure to chlorophenoxy herbicides may occur through inhalation, skin contact or ingestion. The predominant route for occupational exposure to 2,4-D has been the absorption of spills or aerosol droplets through the skin.

Several studies have shown that doses of 50, 70 or 100 mg/kg body weight (bw)/day of 2,4-D produce a wide range of toxic effects on the embryo and on the reproductive and neural

systems in animal (mostly rat) and human models (Rosso et al., 2000; Barnekow et al., 2001; Charles et al., 2001). Doses of 50 mg/kg bw/day of 2,4-D have been reported to increase ventral prostate weight in rats. Treatment of human prostate cancer cell cultures with 10 nM 2,4-D enhanced the androgenic activity of dihydroxytestosterone (DHT) on cell proliferation and transactivation (Kim et al., 2005). In cultured chinese-hamster ovary cells, 2.0 to 10.0 μg/ml 2,4-D were reported to produce DNA damage and sister chromatid exchange (Gonzalez et al., 2005). Importantly, although the 2,4-D toxicity in low doses is controversial, the U.S. Environmental Protection Agency (U.S. EPA, 2006) established a LD50 of 639 mg/kg based on rat studies.

There could be particular situations in which the susceptibility of a population exposed to environmental pollutants can be dangerously enhanced. This may be the case for many rural populations subjected to some specific nutritional deficiencies, as often observed in developing countries. Such situation may be worthy of attention during the development stage, especially concerning the endocrine and nervous systems.

It has been recently found that 2,4-D administered to lactating rats can pass to suckling pups, an can also inhibit the suckling-induced hormone release in the mother. Thus, gestational and lactational periods –including the neonatal and prepubertal stages– seem to be particularly favorable for the induction of 2,4-D effects in rodents (Stürtz et al., 2000; 2006).

2. Adverse effects on developing nervous system

In human studies, prenatal exposure to 2,4-D was associated with mental retardation of the children (Casey, 1984). Comparable animal experiments in chicken and rats showed that prenatal exposure altered some behavioral patterns of the offspring (Sanders & Rogers, 1981; Sjoden & Soderberg, 1972).

In the rat, one critical period for normal maturation during growth seems to be that corresponding to the perinatal development of the brain—''the brain growth spurt''—spanning the first 3 or 4 weeks of life (Diaz & Samson, 1980). Therefore, exposure of rats to pesticides during the first weeks of life would have adverse effects on growth and behavior, as well as on the locomotor activity, as affected by anatomical changes. Noteworthy, the age at exposure is an important factor (Kolb & Wishaw, 1989).

This selective susceptibility of the developing nervous system may be due to several toxicokinetic factors and a partial lack of a blood–brain barrier (BBB) in the fetus. In humans, the BBB is not fully developed until the middle of the first year of life (Rodier, 1995).

Gupta et al. (1999) have shown that different classes of pesticides are able to change the permeability characteristics of the BBB in rats when administered during some susceptible periods of the BBB development, and that this effect may persist after exposure for variable periods. An altered BBB may render the nervous system more vulnerable to other toxics that would not be able to pass the BBB otherwise.

Therefore, although the developing nervous system has some capacity to adapt to or compensate for early perturbations, many chemical agents have been shown more toxic on the developing than on the adult nervous system (Tilson, 1998).

In the last two decades many different alterations have been reported in neonatal rats exposed to 2,4-D through breast milk, at a dose producing no overt signs of toxicity in dams. Alterations in astroglial cytoarchitecture and neuronal function (Brusco et al., 1997) as well as neuro-behavioral changes were observed in pups and adult rats after an early exposition to the herbicide (Bortolozzi et al., 1999, 2001). Other reported effects in neonate rats were a deficit in myelin lipid deposition (Konjuh et al., 2008) and changes in the ganglioside pattern in some brain regions (Rosso et al., 2000).

2.1 Metals and monoamines levels

Studies in well-fed or undernourished rat offsprings showed that the mechanisms for the induction of the above effects would include some changes in brain monoaminergic system (Ferri et al., 2000) and in iron (Fe), copper (Cu) and zinc (Zn) brain levels (Ferri et al., 2003).

Importantly, the combination of neonatal undernourishment plus mothers' exposure at 2,4-D low dose (70 mg/kg bw) induced a higher modification of the measured parameters than those induced by undernourishment or 2,4-D exposure alone. The data showed a different pup's brain areas susceptibility to the 2,4-D effects and an increased vulnerability to the herbicide, including an increased mortality at a higher dose (100 mg/kg bw), a feature which was not observed in well-nourished animals.

In addition, the results suggest that malnutrition or exposure to 2,4-D exert their effects independently (Tables 1 & 2) (Ferri et al., 2003) and the fact that the alterations observed are very different according to the area involved, reinforces the idea of a selective susceptibility for each brain region.

2.2 Oxidative stress

Different studies suggest some functional relationships between the oxidative status of the Central Nervous System (CNS) and the protecting level of catecholamines (Kumiko et al., 2001) and metals, like Fe and Cu, the major generators of reactive oxygen species –ROS- in Alzheimer's disease (Huang et al., 1999), related with a decreased glutathione (GSH) content (Dringer, 2000) and also involved in Fenton's and Haber Weiss' redox reactions . (Halliwell & Gutteridge, 1998; Milton, 2004). Other data have shown that 2,4-D affects the redox chain, thus altering cell energetic metabolism and redox balance (Palmeira et al., 1994; Sulik et al., 1998; Bukowska et al., 2003; Duchnowicz et al., 2002).

In rat pups, exposure to 2,4-D through breast milk induced a number of changes in different brain areas, such as disparate changes in the activity of some protective enzymes, an increase in reactive oxygen species (ROS) levels, and a depletion of reduced glutathione (GSH) content (Tables 3, 4 & 5, respectively) (Ferri et al., 2007).

Therefore, as long as a high oxygen consumption by the CNS increases its sensitivity to oxidative stress (Emerit et al., 2004), the observed changes in the levels of metal ions and neurotransmitters, particularly catecholamines, as well as the oxidative status imbalance, would point out oxidative stress as one possible mechanism of adverse 2,4-D effects on the CNS.

AREA	Treatment	NE	DA	DOPAC	HVA	TRP	5-HT	5-HIAA
PFc	**DMSO**	0.93 ± 0.04	3.20 ± 0.44	0.97 ± 0.09	0.28 ± 0.03	20.71 ± 0.61	1.07 ± 0.13	1.08 ± 0.07
	2,4-D	1.10 ± 0.06* (↑20%)	2.01 ± 0.30* (↓37%)	0.90 ± 0.17	0.25 ± 0.03	11.26 ± 0.51** (↓46%)	1.48 ± 0.09* (↑38%)	1.03± 0.05
Str	**DMSO**	4.24 ± 0.42	20.79 ± 1.61	9.37 ± 0.48	3.19 ± 0.12	27.46 ± 1.61	2.98 ± 0.31	2.84 ± 0.29
	2,4-D	2.36 ± 0.44* (↓44%)	17.03 ± 3.31	6.96 ± 0.69** (↓26%)	2.05 ± 0.32** (↓36%)	28.06 ± 1.76	1.85 ± 0.25* (↓38%)	2.81 ± 0.42
Hipp	**DMSO**	0.91 ± 0.12	0.70 ± 0.09	0.44 ± 0.07	0.30 ± 0.04	5.55 ± 0.35	0.74 ± 0.09	1.63 ± 0.11
	2,4-D	1.67 ± 0.24* (↑83%)	0.92 ± 0.09* (↑31%%)	0.58 ± 0.06* (↑31%)	0.50 ± 0.05* (↑66%)	3.68 ± 0.20** (↓34%)	1.10 ± 0.13* (↑49%)	1.75 ± 0.10
Hyp	**DMSO**	9.05 ± 1.19	1.58 ± 0.35	1.46 ± 0.21	1.08 ± 0.20	3.81 ± 0.26	1.52 ± 0.13	3.09 ± 0.59
	2,4-D	13.57 ± 1.44* (↑50%)	1.80 ± 0.33	1.03 ± 0.17	1.14 ± 0.18	2.96 ± 0.35	2.08 ± 0.31	2.68 ± 0.26
MB	**DMSO**	3.16 ± 0.57	1.54 ± 0.31	0.65 ± 0.14	0.36 ± 0.06	31.88 ± 1.21	2.79 ± 0.21	3.54 ± 0.40
	2,4-D	3.96 ± 0.17	1.96 ± 0.17	0.78 ± 0.12	0.24 ± 0.02	23.34 ± 0.97** (↓27%)	4.14 ± 0.19** (↑48%)	4.78 ± 0.37* (↑35%)
Cereb	**DMSO**	1.58 ± 0.12	0.17 ± 0.06	0.31 ± 0.01	0.09 ± 0.01	7.39 ± 0.55	0.46 ± 0.03	0.48 ± 0.03
	2,4-D	2.44 ± 0.08** (↑54%)	0.21 ± 0.04	0.29 ± 0.01	0.13 ± 0.01	4.86 ± 0.21** (↓34%)	0.43 ± 0.04	0.43 ± 0.02

Monoamine content is expressed as pMol/mg of tissue. Values indicate means ± SEM. Values between brackets are % of increase (↑) or decrease (↓), respectively, with respect to each DMSO control value.*p < 0.05; **p < 0.01; n= 6/group; 100 mg 2,4-D/kg cw of mother. PFc (Pre frontal cortex), Str (Striatum), Hipp (Hippocampus), Hyp (Hypothalamus), MB (Midbarin), Cereb (Cerebellum), NE (Norepinephrine), DA (Dopamine), DOPAC (3,4-Dihydroxyphenylacetic acid), HVA (Homovanillic Acid), TRP (Tryptophan), 5-HT (Serotonin) and 5-5-HIAA (Hydroxyindoleacetic acid); other abbreviations as indicated in the text.

Table 1. Monoamine levels in different brain areas of 25-day-old, 2,4-D-expossed pups.

Metal	Treatment	PFc	Str	Cereb	Hipp	MB	Hyp
Fe	**DMSO**	19.58 ± 2.36	17.07 ± 0.90	16.43 ± 1.27	14.23 ± 0.73	18.79 ± 2.03	19.48 ± 2.25
	2,4-D 70 mg/kg	24.48 ± 1.83* (↑25.05 %)	16.65 ± 2.65	17.53 ± 0.86	12.86 ± 1.20	15.54 ± 0.55	20.95 ± 1.76
	2,4-D 100 mg/kg	29.48 ± 2.19* (↑50.56 %)	15.75 ± 2.65	20.26 ± 0.68* (↑23.31%)	13.98 ± 1.80	14.95 ± 0.35	21.31 ± 2.68
Zn	**DMSO**	12.51 ± 1.20	34.10 ± 2.40	24.40 ± 1.90	25.40 ± 2.10	32.65 ± 1.10	29.80 ± 3.40
	2,4-D 70 mg/kg	14.64 ± 2.20	28.63 ± 2.40* (↓16.04 %)	21.50 ± 0.85	27.89 ± 1.60* (↑9.80%)	29.55 ± 1.74	27.11 ± 2.56
	2,4-D 100 mg/kg	17.93 ± 2.00* (↑43.32%)	13.10 ± 2.00** (↓61.58%)	24.40 ± 0.70	34.30 ± 3.50* (↑35.04%)	24.35 ± 1.54** (↓25.42%)	26.37 ± 3.20
Cu	**DMSO**	1.85 ± 0.08	2.25 ± 0.11	1.88 ± 0.07	1.84 ± 0.02	2.21 ± 0.19	1.95 ± 0.08
	2,4-D 70 mg/kg	1.97 ± 0.18	2.17 ± 0.13	2.00 ± 0.10	2.01 ± 0.20	2.16 ± 0.21	1.91 ± 0.16
	2,4-D 100 mg/kg	2.31 ± 0.21* (↑ 24.86%)	2.38 ± 0.21	2.20 ± 0.08** (↑17.02%)	2.23 ± 0.17* (↑21.19%)	2.14 ± 0.20	1.91 ± 0.15

Metal contents are expressed as micrograms per gram of wet tissue. Values indicate means ± SEM. Values between brackets are % of increase (↑) or decrease (↓), respectively, with respect to each DMSO control value. *p < 0.05 with reference to DMSO control values. **p <0 .01 with reference to DMSO control values; n= 6/group. 100 mg 2,4-D/kg cw of mother. PFc (Pre frontal cortex, Str (Striatum), Cereb (Cerebellum), Hipp (Hippocampus), MB (Midbarin), Hyp (Hypothalamus), and other abbreviations as in the text.

Table 2. Effects of 2,4-D on iron, zinc and copper levels in different brain areas of well-nourished pups.

Enzime	Treatment	Brain	PFc	Str	Cereb	Hipp	MB	Hyp
Cu,Zn-SOD	**DMSO**	2330 ± 119	1950 ± 200	2460 ± 150	2730 ± 330	2330 ± 200	1950 ± 120	2320 ± 160
	2,4-D	2400 ± 93	2450 ± 310* (↑ 25.6%)	2540 ± 220	2610 ± 240	2980 ± 320* (↑ 27.9%)	2140 ± 90	2400 ± 120
Mn-SOD	**DMSO**	250 ± 110	250 ± 80	310 ± 120	320 ± 130	270 ± 90	240 ± 120	370 ± 140
	2,4-D	284 ± 135	150 ± 70	280 ± 100	280 ± 90	390 ± 60	290 ± 110	350 ± 110
CAT	**DMSO**	2556 ± 150	2950 ± 250	2740 ± 200	2300 ± 130	2580 ± 160	2360 ± 200	2740 ± 150
	2,4-D	1978 ± 133* (↓ 22.5%)	2200 ± 200* (↓ 25.4%)	2250 ± 150* (↓ 17.9%)	2530 ± 170	2690 ± 210	1850 ± 120* (↓ 21.6%)	2810 ± 210
Se-GPx	**DMSO**	31.52 ± 1.24	30.00 ± 1.43	28.19 ± 2.10	25.62 ± 9.10	29.05 ± 1.14	31.93 ± 1.06	28.89 ± 1.85
	2,4-D	26.76 ± 1.14* (↓ 15.10%)	24.19 ± 1.90* (↓ 19.4%)	22.29 ± 2.86* (↓ 20.9%)	29.52 ± 2.10* (↑ 15.2%)	32.29 ± 1.00* (↑ 11.1%)	27.57 ± 1.03* (↓ 13.6%)	27.97 ± 1.56
noSe-GPx	**DMSO**	17.71 ± 0.69	20.80 ± 1.08	18.55 ± 0.62	18.18 ± 0.98	20.94 ± 0.69	22.65 ± 0.77	15.81 ± 0.69
	2,4-D	20.32 ± 0.94* (↑ 14.7%)	18.99 ± 0.99	15.65 ± 0.46* (↓ 15.6%)	17.95 ± 1.05	19.90 ± 0.87	19.35 ± 0.82* (↓ 14.6%)	17.01 ± 0.71

Enzyme activities are expresed as miliUnits per miligram of protein. Values indicate means ± SEM. Values between brackets are % of increase (↑) or decrease (↓), respectively, with respect to each DMSO control value. *p < 0.05, n= 6/group. 100 mg 2,4-D/kg cw of mother. PFc (Pre frontal cortex, Str (Striatum), Hipp (Hippocampus), Hyp (Hypothalamus), MB (Midbarin), Cereb (Cerebellum), Cu,Zn-SOD (Copper,Zinc superoxide dismutase), Mn-SOS (Manganese superoxide dismutase), CAT (catalase), Se-GPx (selenium-glutathione peroxidase), noSe-GPx (non selenium-glutathione peroxidase),and other abbreviations as in the text

Table 3. Protective Enzymes Activities in brain areas of 25-old-day pups lactationally exposed to 2,4-D.

	Brain	PFc	Str	Cereb	Hipp	MB	Hyp
DMSO	45.1±2.5	17.8±0.7	22.6±0.8	24.0±0.9	18.0±1.0	20.3± 1.0	22.6±1.2
2,4-D	38.0±1.4* (↓ 15.7%)	20.6±0.5* (↑ 15.7%)	25.1±0.7* (↑ 11.1%)	23.7±1.1	18.1±1.1	23.8±0.7* (↑ 17.2%)	21.1±1.3

ROS levels are expresed as IF per mg of protein. Values indicate means ± SEM. Values between brackets are % of increase (↑) or decrease (↓), respectively, with respect to each DMSO control value.; *p < 0.05, n= 6/group. 100 mg 2,4-D/kg cw of mother. PFc (Pre frontal cortex, Str (Striatum), Hipp (Hippocampus), Hyp (Hypothalamus), MB (Midbarin), Cereb (Cerebellum), other abbreviations as in the text.

Table 4. ROS levels in brain areas of 25-old-day pups lactationally exposed to 2,4-D.

	Brain	PFc	Str	Cereb	Hipp	MB	Hyp
DMSO	1.22±0.40	1.23±0.29	1.31±0.24	0.79±0,19	0.88±0.19	1.06±0.12	1.07±0.41
2,4-D	1.25±0.38	1.29±0.20	0.82±0.18* (↓ 37.4%)	0.80±0.22	0.94±0.24	0.70±0.15* (↓ 34.0%)	1.08±0.30

GSH levels are expresed as microgram per miligram of protein. Values indicate means ± SEM. Values between brackets are % of increase (↑) or decrease (↓), respectively, with respect to each DMSO control value. *p < 0.05, n= 6/group. 100 mg 2,4-D/kg bw of mother. PFc (Pre frontal cortex, Str (Striatum), Hipp (Hippocampus), Hyp (Hypothalamus), MB (Midbarin), Cereb (Cerebellum), other abbreviations as in the text.

Table 5. GSH levels in brain areas of 25-old-day pups lactationally exposed to 2,4-D.

3. Prostate, ovary and breast

The endocrine system of many vertebrate embryos seems to be particularly susceptible to a variety of substances or either natural or anthropogenic origin, including pesticides (Crews et al., 2000). However, there are few studies on developmental toxicology that focus on the 2,4-D's effects on hormone-sensitive organs such as the prostate, ovary and breast.

Free radicals are associated with oxidative stress and are also thought to play some significant roles in reproduction. Induction of oxidative stress by many environmental contaminants—such as pesticides—has also been pointed out during the last decade as a possible mechanism of some toxic effects on the reproductive system (Bagchi et al., 1992; Abdollahi et al., 2004). It is already known that reproductive cells and tissues will remain stable only when antioxidant and oxidant status are in balance (Lee et al., 2010). ROS levels are a double-edged sword, as long as they not only serve as key signal molecules in physiological processes, but also have a role in pathological processes involving the female reproductive tract (Agarwal et al., 2005).

On the other hand, there are diverse environmental chemical contaminants which can be potentially harmful to the mammary gland in association with estrogens. Oxidative catabolism of both estrogen and those compounds, a mechanism mediated by the same enzymes, generates reactive free radicals that can cause oxidative damage. Xenobiotic chemicals may exert their pathological effects through generation of reactive free radicals (Mukherjee et al., 2006).

There is growing evidence that free radicals can exert a wide spectrum of deleterious effects on the reproductive system and asocciated glands (Saradha et al., 2008). Thus, Pochettino et al. (2010) investigated the effect of 2,4-D on oxidative stress and antioxidative system and on some hormone-sensitive organs such as ventral prostate, ovaries and breasts, exposed to the herbicide during the pre- and the postnatal period, as described next (Pochettino et al., 2010).

3.1 Prostate

In rat ventral prostate, 2,4-D caused oxidative stress during the whole development, through a significant increase in lipid peroxides, hydroxyl radical levels and protein oxidation. Morevover, the antioxidant enzyme activity was increased at any age, as shown for Glutathione S-transferase (GST), catalase (CAT) and selenium-glutathione peroxidase (Se-GPx), with the exception of Se-GPx administered at the 90th postnatal day (PND 90). Nevertheless, at PND 90 a reduced activity of Glutathione Reductase (GR) was detected (Table 6).

GST is relevant to detoxification of endogenous compounds and xenobiotic substances such as environmental pollutants, drugs, and natural toxins (Pietsch et al., 2001; Padros et al., 2003; Cazenave et al., 2006). Several studies have demonstrated that enhanced GST activity by ROS in the testis could represent an adaptative response to oxidative stress, probably targeted to achieve a detoxification of peroxide-containing metabolites (Kaur et al., 2006).

As far as the testis is intimately related to the prostate, this interpretation looks coherent with the observed ROS-induced increase in GST activity in the prostate.

		PND 45	PND 60	PND 90
Hydroxyl radical	**Control**	3.25±0.34	2.97±0.39	1.09±0.13
	2,4-D	8.75±0.61* (↑169%)	6.53±0.09* (↑119%)	2.03±0.18* (↑85%)
Carbonyl groups	**Control**	3.54±0.12	10.66±1.07	7.02±0.88
	2,4-D	4.84±0.11* (↑37%)	15.01±1.32* (↑47%)	12.42±1.11* (↑77%)
Total Thiols	**Control**	491±12	642±86	341±24
	2,4-D	520±14	748±14	333±8
MDA	**Control**	29.09±0.32	27.74±3.74	38.27± 2.14
	2,4-D	41.91±3.05* (↑44%)	42.69±3.13* (↑54%)	47.48 ± 2.54* (↑24%)
GST	**Control**	8.93±0.67	10.53±2.53	13.37±2.09
	2,4-D	19.07±2,45* (↑113%)	15.95±1.04* (↑45)	18.14±0.26* (↑36)
CAT	**Control**	10.93±1,20	5.44±0.21	5.99±0.21
	2,4-D	14.74±1.26* (↑35%)	11.44±0.34* (↑110%)	7.77±0.39* (↑28%)
Se-GPx	**Control**	312±18	518±57	562±32
	2,4-D	436±33* (↑36)	880±41* (↑70%)	530±13
GR	**Control**	10.05±0.86	33.01±2.52	31.09±4.36
	2,4-D	10.47±1.78	37.75±3.48	10.57±0.05* (↓72%)

Hydroxy radical are expresed as 2,3 dihydroxybenzoic acid/salicilc acid rario; carbonyl groups and total thiols are expresed as micromol per miligram of protein; MDA is expresed as nanomol per microgram of protein. GST, CAT and GR activities are expresed as Units per miligram of protein; and Se-GPx is expresed as miliUnits per miligram of protein. Each value is the mean ± SEM. Values between brackets are % of increase (↑) or decrease (↓); *$p < 0.05$, n= 6/group. 70 mg 2,4-D/kg cw of mother. Abbreviations as in the text.

Table 6. Oxidative parameters in ventral prostate.

Therefore, the 2,4-D-induced increase in all ROS level, lipid peroxidation and protein oxidation may have caused some critical oxidative stress in ventral prostate. Nevertheless, the increased activity of some antioxidant enzymes in the prostate could have not been strong enough as to counteract the oxidative stress produced by the herbicide at different stages of rat development. Moreover, it is not a general rule that increase in oxidative species stimulates antioxidant activity (Celik & Tuluce, 2007).

3.2 Ovary

The complex ovarian structure varies widely during differentiation. Free radicals play important regulating roles during the ovarian follicular cycle, possibly through inhibition of steroid production (Behrman et al., 2001). There is also a delicate balance between ROS and antioxidant enzymes in the ovarian tissues (Agarwal et al., 2005). Non-physiological effects of free radicals include premature ovarian follicular atresia via cell apoptosis. Many pesticides— e.g. the xenoestrogen pesticide methoxychlor — can induce oxidative stress and apoptosis in the ovary (Gupta et al., 2006). Moreover, clinical studies have reported increased levels of reactive oxygen species associated to a decreased female fertility (Agarwal et al., 2006).

		PND 45	PND 60	PND 90
Hydroxyl radical	**Control**	3.65±0.26	1.89 ± 0.22	1.09 ± 0.13
	2,4-D	8.75±0.89	1.98 ± 0.13	4.35 ± 0.53* (↑93%)
Carbonyl groups	**Control**	14.77±2.75	5.74 ± 0.13	6.22 ± 0.94
	2,4-D	23.71±0.47* (↑60%)	8.80±0.72* (↑55%)	5.64±0.73
Total Thiols	**Control**	1462±162	672±24	519±38
	2,4-D	1360±176	676±39	537±22
MDA	**Control**	87.71±14.02	34.12±2.24	34.68±1.31
	2,4-D	192.5±17.8* (↑119%)	42.49±1.35* (↑24%)	39.39±0.89* (↑14%)
GST	**Control**	38.51±0.41	10.59±0.81	10.99±0.18
	2,4-D	25.15±1.37 (↓34.6%)	7.97±0.54* (↓24.7)	9.91±0.57
CAT	**Control**	42.89±3.14	27.86±1.08	16.08± 0.42
	2,4-D	43.41±0.67	15.34±0.43* (↓44.9%)	16.38±0.71
Se-GPx	**Control**	691±97	411±48	514±29
	2,4-D	1622±117* (↑135)	549±24* (↑33%)	593±23* (↑15%)
GR	**Control**	14.48±3.44	17.15±1.67	28.64±2.31
	2,4-D	12.67±2.61	16.88±1.45	19.62±1.75* (↓31%)

The parameters are expresed as in Table 7. Each value is the mean ± SEM. Values between brackets are % of increase (↑) or decrease (↓); *p < 0.05, n= 6/group. 70 mg 2,4-D/kg cw of mother. Abbreviations as in the text.

Table 7. Oxidative parameters in ovary.

On analyzing the 2,4-D toxic effects on the ovary, Pochettino et al. (2010) found an increase in lipid peroxide (LPO) evidenced by augmented levels of malondialdehyde (MDA) and decrease antioxidant enzyme activity. These effects differed with age, while an increase in Se-GPx activity was exceptionally observed at all ages (Table 7). These effects could reflect the natural diversity of rat ovarian cell types at different ages. Another explanation would be the well-known, protecting effect of estrogens against apoptosis and oxidative stress in a variety of tissues and cells (Spyridopoulos et al., 1997; Tomkinson et al., 1997; Garcia-Segura et al., 1998; Pelzer et al., 2000). Estrogens increase all ovarian weight, follicular growth, and the mitotic index of granulose cells, and also control granulosa cell apoptosis (Richards, et al., 1980; Bendell & Dorrington, 1991) and have exerted varied antioxidant effects (Chatterjee & Chatterjee 2009). Further studies are needed to analyze the time-course of the effects observed.

3.3 Breast

Pocchetino et al. (2010) observed that 2,4-D increased MDA levels at all ages (Table 8). It is known that MDA reflects the extent of oxidant status and is considered a good marker of oxidative stress (Wen et al., 2006). Both, singlet oxygen and hydroyl radicals have a high potential to initiate free-radical chain reactions in lipid peroxidation (Celik & Tuluce, 2007). As the hydroyl radical level was unchanged in that study, 2,4-D could have stimulated LPO by increasing singlet oxygen levels. In addition, 2,4-D inhibited the activity of anti-oxidative enzymes such as CAT, Se-GPx, GR and GST (Table 9).

		PND 45	PND 60	PND 90
Hydroxyl radical	**Control**	2.61±0.11	3.04±0.11	4.31±0.45
	2,4-D	2.65±0.44	3.49±0.52	4.49 ± 0.11
Carbonyl groups	**Control**	19.25±0.82	28.57±3.86	59.38±10.69
	2,4-D	21.34±5.47	23.31±5.49	57.37±14.89
Total Thiols	**Control**	942±5	1072±77	3551±757
	2,4-D	951±25	667±46* (↓62%)	1560±226* (↓56%)
MDA	**Control**	52.62±1.57	71.07±4.68	158.41±2.59
	2,4-D	70.65±7.48* (↑34%)	139.2±17.94* (↑96%)	217.8±18.95* (↑37%)
GST	**Control**	17.18±0.59	19.41±1.51	72.81±7.41
	2,4-D	10.41±1.91* (↓40%)	13.54±0.92*(↓30%)	32.35±5.98* (↓55%)
CAT	**Control**	59.38±3.03	137.62±10.73	358.21±36.31
	2,4-D	62.55±1.57	81.27 ± 2.55* (↓41%)	122.11±17.42* (↓66%)
Se-GPx	**Control**	538±44	1430±31	5596±1015
	2,4-D	198±19* (↓63%)	695±15* (↓51%)	2257±474* (↓60%)
GR	**Control**	15.94±0.91	90.75±5.51	228.81±14.31
	2,4-D	7.39±1.54* (↓53.6%)	60.02±9.05* (↓34%)	76.02±10.95* (↓67%)

The parameters are expresed as in Table 7. Each value is the mean ± SEM. Values between brackets are % of increase (↑) or decrease (↓); *p < 0.05, n= 6/group. 70 mg 2,4-D/kg cw of mother. Abbreviations as in the text.

Table 8. Oxidative parameters in breast

Therefore, the decreased activity of anti-oxidative enzymes may decrease the protection against oxidants (Amstad et al., 1991).

In that regard, Dimitrova et al. (1994) suggested that the superoxide radicals, either by themselves or after transformation to H_2O_2, stimulate cysteine oxidation and inhibit the activity of the enzymes. Furthermore, Regoli & Principato (1995) demonstrated that the flux of superoxide radicals inhibits CAT activity. Consequently, the decreased CAT activity might have reflected a flux of superoxide radicals promoted by 2,4-D. Moreover, GR also plays an important role in cellular antioxidant protection, catalyzing the reduction of glutathione disulfide (GSSG) to GSH (Kim et al., 2010).

Thus, the decrease in thiol groups could reflect GSH depletion in the breast. Therefore, 2,4-D produced oxidative imbalance, mainly during puberty and adulthood, probably because the gland is more sensitive to xenobiotics at these stages of development.

4. *In vitro* studies

It has been observed that 2.4-D concentrations of 1 to 2 mM impaired neurite outgrowth, disrupted the cytoskeleton, and disorganized the Golgi apparatus in cultured cerebellar granule cells (CGC) (Rosso et al., 2000). Futhermore, Kaioumuva et al. (2001b) have demonstrated that the dimethylamonium salt of 2,4-D (DMA 2,4-D) at 0.1 to 5 mM induces apoptosis in a dose- and time-dependent pattern in peripheral blood lymphocytes of healthy individuals and in Jurkat cells. Whereas, Tuschl & Schwab (2003) showed that 4 to 16 mM 2,4-D induces cytotoxic effects and apoptosis in HepG2 cells.

In rat CGC, either 1 or 2 mM 2,4-D induced similar increases of cellular death. The herbicide decreased significantly mean neuronal survival (46.4%) after 48 h, while no affect was observed after 24 h of treatment (Bongiovanni et al., 2007, 2011) (Fig. 2).

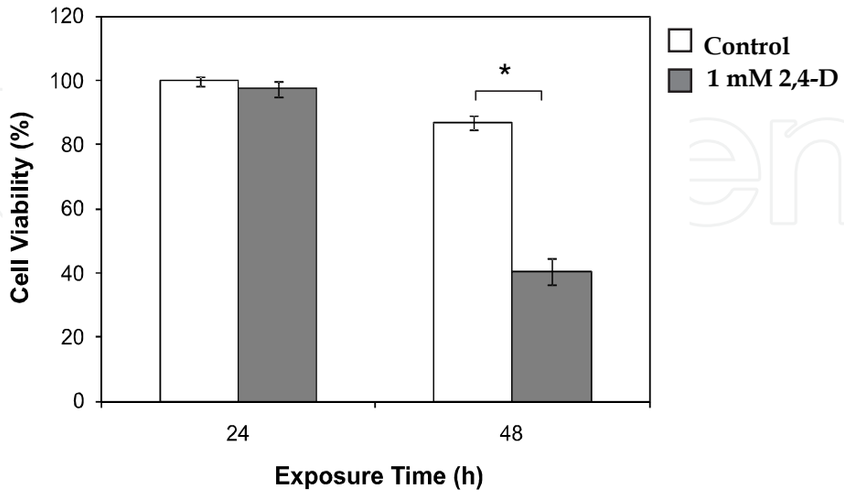

Fig. 2. Effect of 2,4-D on rat cerebellar granule cell viability. Cell cultures were incubated for 24 or 48 h in presence or ausence of 1 mM 2,4-D. Values are means ± SEM; * indicates $p < 0.001$ vs. control group; n= 10/group.

Bongiovanni et al. (2007, 2010) studied oxidative stress as a possible mechanism of toxicity aiming to elucidate the mechanism of death induction by 2,4-D. Oxidative stress parameters were altered: ROS level and Se-GPx activity increased whereas CAT activity decreased at both treatment times (24 and 48 h). GSH content was reduced only after 48 h of 2,4-D treatment. However, neither Mn-SOD nor Cu,Zn-SOD activities nor reactive nitrogen species (RNS) levels were affected (Tables 9 & 10). Interestingly, although the oxidative parameters evaluated were modified at the two time-limits studied, the cell viability only decreased at 48 h of treatment. This finding could be explained by a time dependency of this latter alteration.

Parameters	24 h		48 h	
	Control	1 mM 2,4-D	Control	1 mM 2,4-D
ROS	1.03 ± 0.25	2.30 ± 0,22* (↑ 123%)	2.28 ± 0.35	4.13 ± 0.32* (↑ 81%)
RNS	7.45 ± 1.13	8.23 ± 1.85	4.82 ± 0.27	6.05 ± 0.47
GSH	2.408 ± 0.09	2.225 ± 0.09	1.508 ± 0.061	1.125 ± 0.031* (↓ 25%)

Parameters are expresed as micrograms per miligram of protein. Values between brackets are % of increase (↑) or decrease (↓); *$p < 0.001$, n= 10/group. Abbreviations are indicated in the text.

Table 9. ROS, RNA and GSH levels (means ± SEM) in rat cerebellar granule cell in culture for 24 or 48 h in presence or ausence of 1 mM 2,4-D.

Enzimes	24 h		48 h	
	Control	1 mM 2,4-D	Control	1 mM 2,4-D
CAT	30.97 ± 1.26	15.80 ± 1.23* (↓ 49%)	15.82 ± 1.59	6.52 ± 0.83* (↓ 59%)
(Zn,Cu) SOD	10.43 ± 1.23	10.56 ± 1.45	8,49 ± 1,20	7.05 ± 1.65
(Mn) SOD	4.45 ± 0,88	5.97 ± 1.90	2,86 ± 1,90	1.98 ± 1.00
Se-GPx	9.71 ± 1.20	39.75 ± 2.90* (↑ 309%)	12.73 ± 1.75	33.73 ± 4.31* (↑165%)

Parameters are expresed as Units per miligram of protein. Values between brackets are % of increase (↑) or decrease (↓); *p < 0.001, n= 10/group. Abbreviations are indicated in the text.

Table 10. CAT, SODs and GPx activities (means ± SEM) in rat cerebellar granule cells in culture for 24 or 48 h in presence or ausence of 1 mM 2,4-D.

On using a PC-12 cell model, other authors have been previously shown that a depletion of mitochondrial and cytoplasmatic GSH results in increased ROS levels, disruption of the mitochondial transmembrane potential, rapid loss of mitochondial function, decrease in the ATP concentration, and eventually a higher cell death rate (Nieminen et al., 1995; Wüllner et al., 1999).

Therefore, the alteration in oxidative parameters suggest that the possible mechanisms of chlorophenoxy herbicide toxicity could involve dose-dependent cell membrane damage, uncoupling of oxidative phosphorylation, acetylcoenzyme disruption (Bradberry et al., 2000), and an indirect disruption of mitochondrial transmembrane potential which may lead to caspase inactivation (Kaioumova et al., 2001a). Mitochondrial structural modifications and increased permeability of the pores were also reported in association with a ROS increase (Belizário et al., 2007). In contrast, other studies suggest that 2,4-D cytotoxic effects are exerted by apoptosis induction via a direct effect on mitochondria (Tuschl & Schwab, 2003).

In this regard, Bongiovanni et al. (2011), in agreement with De Moliner et al. (2002), demonstrated that 2,4-D induces apoptosis and necrosis in CGC. While De Moliner et al. (2002) showed that 2,4-D-induced apoptosis is associated with and increase in caspase-3 activity preceded by cytochrome-c release from mitochondria, the quantification of ultrastructural changes showed that 1 mM 2,4-D stimulated neuronal death. As much as 49% of necrotic cells and 20% of apoptotic cells were observed, while only 31% of CGC presented normal growth with respect control group ($p<0.001$; Fig. 3 compared with Fig. 4) (Bongiovanni et al., 2011).

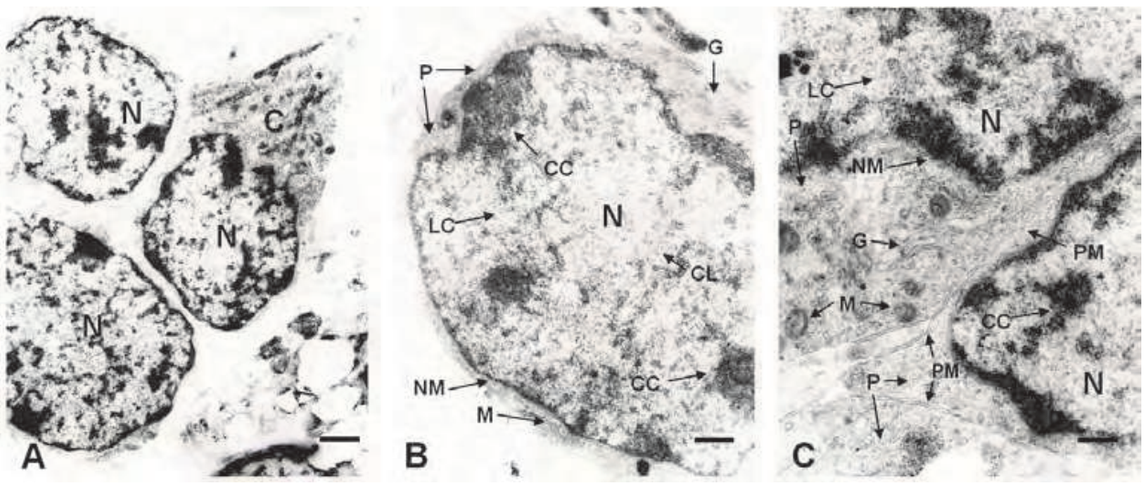

Fig. 3. Electron photomicrographies showing cerebellar granular neurons cultured in a control medium (NaCl 0.9%) for 48 h. a–b. Cell morphology is preserved (nucleus with laxe chromatin, dense chromatin patch close to the nucleus envelope, scarce cytoplasm, and the presence of neurites). Bars correspond to 1 µm in (a) and 160 nm in (b); c. Cells show preserved ultrastructural characteristics (Golgi apparatus, polyribosomes and mitochondrial characteristics of normal granular cerebellar cells). Bars correspond to 320 nm in (c). C cytoplasm, CC dense chromatin, G Golgi apparatus, LC laxe chromatin, M mitochondria, N nucleus, NM nuclear membrane, P polyribosome, PM plasmatic membrane.

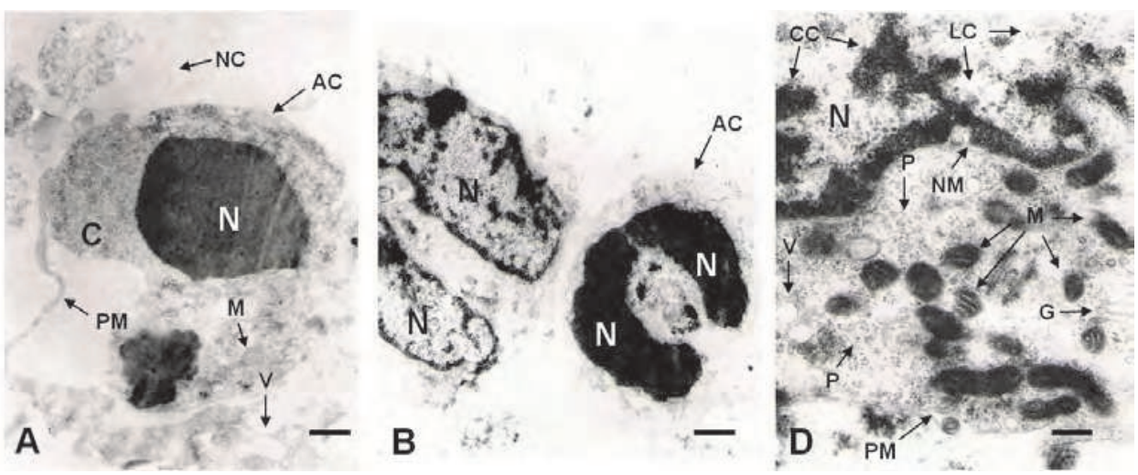

Fig. 4. Electron photomicrographies showing the ultrastructural cytoplasmatic characteristics of cerebellar granular cells after 2,4-D addition to the medium for 48 h. a–b. An apoptotic cell (nuclear fragmentation and very dense chromatinic accumulus), a necrotic cell (cytoplasm very scarce, no nucleus), and cells with scarce cytoplasm and small nucleus are shown, allowing comparison with the control group (Cf Figs. 3a, b). Bars correspond to 1 µm. c. A cell with cytoplasmatic protutions, vacuoles, disorganization of the cytoplasmatic reticulum, distended cisterns of the Golgi apparatus, and mitochondrial swelling. Bars correspond to 400 nm. AC apoptotic cell, NC necrotic cell, V vacuole, and other abreviations in Fig. 3.

In these studies, melatonin and amphetamine were used as phamacological tools aiming to improve the analysis of oxidative stress as a mechanism of toxicity, by assessing whether these compounds could be effective in preventing the toxic effect of 2,4-D in the redox balance of CGC *in vitro* (Bongiovanni et al., 2007, 2011).

A remarkable body of evidence indicates that melatonin exerts antioxidative protection in cell culture and *in vivo* systems (Pandi-Perumal et al., 2006). Regarding to 2,4-D toxicity, the oxidative stress induced by 1 mM 2,4-D was counteracted by the concomitant addition of 0.1 or 0.5 mM melatonin in CGC cultures (Bongiovanni et al., 2007).

On the other hand, amphetamine has constistently been reported to accelerate the recovery of several functions in animals and humans with brain injury (Goldstein, 2000; Martinsson & Eksborg, 2004). Amphetamine was also shown to stimulate both the dendritic growth in the ventral tegmental area (Mueller et al., 2006) and the neurotrophic and neuroplastic responses after brain damage (Moroz et al., 2004; Adkins & Jones 2005). However, few data are available regarding any possible protective effect of amphetamine. In this regard, Bongiovanni et al., (2011) demonstrated that 1 or 10 µM amphetamine reverted the 2,4-D-induced apoptosis and oxidative stress in CGC. Nevertheless, amphetamine alone induced no significant changes with respect to the control culture. Noteworthy, at 1 µM AMPH plus 2,4-D, 39% of the cells were normal; 53% were necrotic, and 8% showed apoptosis. At 10 µM AMPH plus 2,4-D, 57% of the cells were normal, 43% were necrotic, and no apoptotic cells were observed by electron microscopy (Fig. 4 compared with Fig. 5).

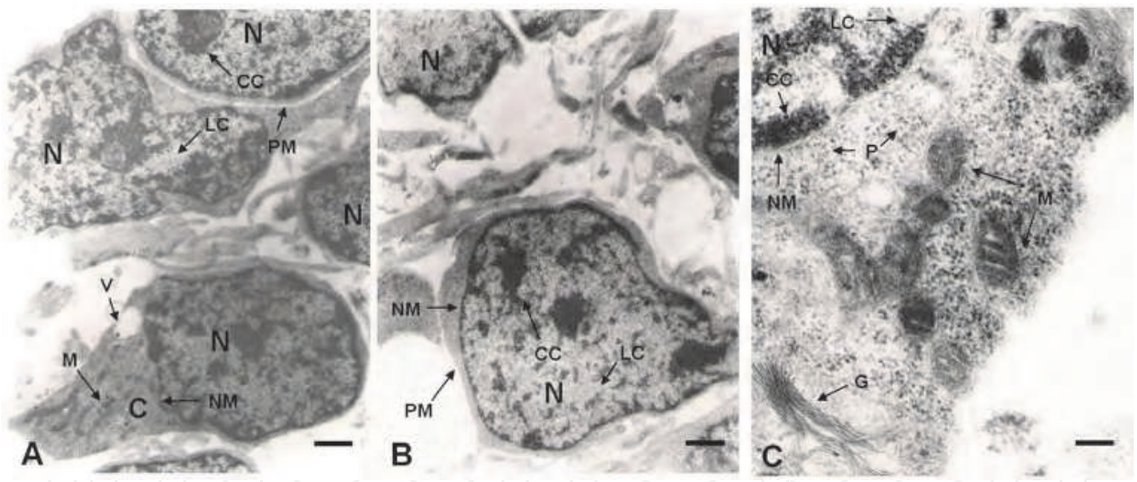

Fig.5. Electron photomicrographies showing the ultrastructural cytoplasmatic characteristics of cerebellar granular cells after 2,4-D and 10 µM AMPH addition to the medium for 48 h. a–b. Cells present more conserved morphology (nucleus and cytoplasm) than those treated with 2,4-D alone (Cf Figs. 4a, b). Bars correspond to 1 µm. c. The cell shows mitochondria and Golgi cisterns more preserved than those of the cells treated with 2,4-D alone (Cf Fig. 4c). Bars correspond to 600 nm. AC apoptotic cell, NC necrotic cell, V vacuole and other abreviations in Fig. 3.

The collected evidence would indicate a protective effect of melatonin and amphetamine against 2,4-D-induced cell death, possibly due to an inhibition of the oxidative mechanisms, as judged by the close relationship between ROS and apotosis induction (Carmody &

Cooter, 2001). While apoptosis and necrosis present some early features that may be common to both, mithocondrial disorders could be irreversibly compromised in necrotic, but not in apoptotic neurons (Nicotera & Leist, 1997). This could explain why amphetamine decrease apoptosis but not necrosis in 2,4-D-treated cells.

In summary, 2,4-D would induce necrosis and apoptosis, the latter being possibly mediated by an oxidative imbalance.

5. Concluding remarks

A great body of evidence suggests that exposure to 2,4-D or to its ester or salt formulations is associated with a wide range of adverse effects in human and different animal species (Berkley & Magee, 1963; Bortolozzi et al, 2001, 2003; Ferri et al., 2003, 2007; Konjuh et al., 2008; Stürtz et al., 2010).

Oxidative stress may affect the cells as a result of imbalance between the (physiological) production of potentially toxic ROS and some (physiological) scavenging activities (Park et al., 1999). Xenobiotics that interact with one or several complexes of the mitochondrial electron transport system, impairing the normal electron flow, may enhance ROS generation, leading to an imbalance between prooxidant species and cellular antioxidants (Jurado et al., 2011).

This review has analyzed the oxidative stress as a possible mechanism of toxicity by the herbicide 2,4-D. The collected evidence confirms that 2,4-D is an environmental pollutant that induces oxidative stress and could determine important deleterious changes in the development of the neural and reproductive systems in the studied models (Ferri et al., 2007; Bongiovanni et al., 2007, 2011; Pocchettino et al., 2010).

While the reported results showed that 2,4-D induces both necrosis and apoptosis, the evidence suggests that apoptosis would be mediated by or associated to an oxidative imbalance (Bongiovanni et al., 2011). Then, the oxidative stress would produce cytochrome-c release from mitochondria and a consequent activation of caspase-3 in the affected cells (De Molliner et al., 2002). However, as mitochondria contribute to both apoptosis and necrosis, intracellular ATP and GSH could determine cell death by one or both of these mechanisms (Leist et al., 1997; Yutaka et al., 1997; Qian et al., 1999; Nieminen, 2003; Bongaerts, 2008). Therefore, the 2,4-D cytotoxic actions may involve some permissive effect on either necrosis or apoptosis induction.

Finally, the experimental evidence reported that 2.4-D can not only affect the nervous system or other hormone-sensitive organs, but also exert a very important, deleterious effect on embryonic and fetal development.

6. Acknowledgment

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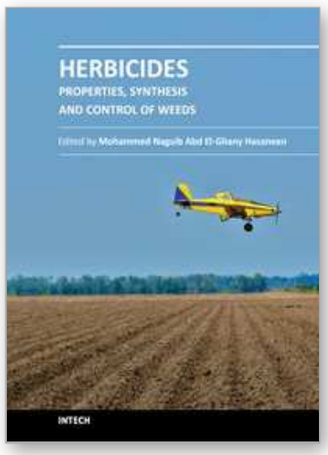

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This book is divided into two sections namely: synthesis and properties of herbicides and herbicidal control of weeds. Chapters 1 to 11 deal with the study of different synthetic pathways of certain herbicides and the physical and chemical properties of other synthesized herbicides. The other 14 chapters (12-25) discussed the different methods by which each herbicide controls specific weed population. The overall purpose of the book, is to show properties and characterization of herbicides, the physical and chemical properties of selected types of herbicides, and the influence of certain herbicides on soil physical and chemical properties on microflora. In addition, an evaluation of the degree of contamination of either soils and/or crops by herbicides is discussed alongside an investigation into the performance and photochemistry of herbicides and the fate of excess herbicides in soils and field crops.

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