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ScienceDirect
Toxicology 237 (2007) 104–110
TOXICOLOGY
www.elsevier.com/locate/toxicol

Lack of effects of 2,4-dichlorophenoxyacetic acid administration on markers of oxidative stress during early pregnancy in mice

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Received 2 March 2007; received in revised form 27 April 2007; accepted 1 May 2007
Available online 6 May 2007

Abstract

Induction of oxidative stress by 2,4-dichlorophenoxyacetic acid (2,4-D) both as a pure compound and in commercial formulation was investigated during early pregnancy in mice. Pregnant animals were exposed to increasing doses of the herbicide (0.01, 0.1 and 100 mg/kg/d) during gestation days 0–9, after which animals were euthanized and their blood analyzed for catalase activity, thiobarbituric acid reactive substances (TBARs) and total antioxidant capacity (TAC). Number of corpora lutea and uterine implantations and resorptions were also determined. Herbicide exposure did not cause any overt signs of maternal toxicity at any of the doses administered; neither did it cause an effect on developmental parameters. Catalase activity and TBARs were not modified by herbicide exposure although TAC was significantly decreased at 100 mg/kg/d of both pure and formulated compound. Thus, 2,4-D does not seem to induce oxidative stress during early pregnancy in mice at the doses administered, indicating that this mechanism is probably not involved in mediating herbicide toxicity at these dose levels. Furthermore, since no manifestations of developmental toxicity were observed after administration of the herbicide, it is also possible that 2,4-D may not produce any early developmental toxicity at the low environmentally relevant doses tested in this animal model.

Keywords: 2,4-D; Oxidative stress; Developmental toxicity; Phenoxyacetic herbicides

1. Introduction

2,4-Dichlorophenoxyacetic acid (2,4-D) is a widely used herbicide, chemically-derived from phenoxyacetic acid. Its herbicidal activity is mediated by an auxin-like capacity to alter normal protein synthesis and cell division in plant meristems and leaves (Stevens and Breckenridge, 2001) although it has recently been suggested that its herbicidal activity may also be due to an increase in the production of oxygen reactive species leading to the generation of oxidative stress in the weed (Romero-Puertas et al., 2004).

Several reports have shown that 2,4-D produces oxidative stress and/or depletes antioxidants both *in vitro* and *in vivo*. *In vitro* studies have mainly looked at the effect of the herbicide on hepatocytes and red blood cells (Palmeira et al., 1994, 1995; Oakes and Pollak, 1999; Bukowska et al., 2000; Duchnowicz and Koter, 2003, among others) while *in vivo* oxidative activity has been shown in different species including yeast (Teixeira et al., 2004), plants (Romero-Puertas et al., 2004), fish (Ozcan and Uner, 2000; Ozcan et al., 2004) and rats (Celik et al., 2006).

2,4-D has been reported to be teratogenic and embryo and fetotoxic in rats but only at doses that exceed

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doi:10.1016/j.tox.2007.05.002

maternal renal clearance. Early work indicated a decrease in litter size and fetal weight in addition to skeletal malformations (Schwetz et al., 1971; Khera and McKinley, 1972) while newer data have indicated that the herbicide only produces adverse fetal effects in concomitance to maternal toxicity at doses above 90 mg/kg/d acid equivalent (Charles et al., 2001), with no reproductive parameters such as number of uterine implantations or resorptions being altered. On the other hand, Cavieres et al. (2002) observed a decrease in litter size in mice administered increasing doses of a commercial formulation containing a mixture of 2,4-D and other phenoxy and acetic acid-derived herbicides during organogenesis. At lower doses a reduction in the number of implantation sites accounted for the decrease in litter size.

Some epidemiological data show that phenoxyacid herbicides may be related to the production of birth and reproductive defects in exposed populations. For instance, rates of adverse birth outcomes in rural counties of four US agricultural states with high phenoxyacid herbicide use showed significant increases for the circulatory/respiratory birth defect category during 1995–1997 (Schreinemachers, 2003). Additionally, Arbuckle et al. (1999, 2001) reported increased risk of abortion at less than 12 weeks of gestation for preconception exposure to phenoxyacid herbicides. On the other hand, time to pregnancy was used by Curtis et al. (1999) to assess a relationship between pesticide exposure and fecundity showing that specific pesticides including phenoxyacid herbicides were associated with a decrease in fecundity when women engaged in pesticide-related activities.

Given that oxidative stress may contribute to implantation failure and early embryo death, we have investigated if exposure to 2,4-D produces such defects in mice. We administered the herbicide both as a pure compound and as a commercial formulation in order to study any possible influences of other ingredients included in a commercial herbicide product. Results show that 2,4-D does not induce oxidative stress at the low, environmentally relevant doses tested, thus indicating that this mechanism of toxicity is probably not involved in early developmental toxicity at these dose levels.

2. Materials and methods

2.1. Pesticide exposure

2,4-D either in a commercial herbicide formulation available in Chile (CF) or as a pure compound (PC) were diluted into the drinking water at the three dose levels indicated in Table 1. The lowest dose corresponded to the reference dose (RfD) of the herbicide as stablished by the USEPA drinking water standards and health advisories, with the RfD being an estimation of the daily exposure to an agent that is assumed to be without and adverse health impact on the human population. Concentrations were confirmed by HPLC.

Table 1
Concentration (ppm) and dose of 2,4-D in drinking water (mg/kg/d) administered to mice

Treatment[a] (count)	Concentration (ppm)	Dose (mg/kg/d)
Control (13)	0	0
0.01 PC (13)	0.042	0.01
0.1 PC (13)	4.18	0.1
100 PC (11)	418	100
0.01 FC (12)	0.046	0.01
0.1 FC (10)	4.62	0.1
100 FC (11)	462	100

[a] PC: pure compound, CF: commercial formulation.

All experiments were conducted at the animal facilities of the University of Valparaíso, Chile with the approval of the Faculty of Pharmacy Ethics Committee. Six-week old, ICR/Jcl mice were purchased from the University of Chile, Faculty of Dentistry in Santiago and housed for 2 weeks in the same room where the experiments were conducted for adjustment to the light–dark cycle and temperature of the room. The mice were then mated at 8 weeks of age with the presence of a copulatory plug indicating gestation day (GD) 0. After mating, the males were removed and euthanized while pregnant females were maintained in hanging stainless steel cages with free access to food (Champion Laboratory Rat Food, Santiago) and herbicide-containing water. Water was delivered in aluminum-foil-covered glass water bottles during gestation days (GD) 0–9. Water bottles were weighed daily to determine water consumption and thus control exposure to the right dose. Weights of pregnant females were recorded on GD0, GD6 and GD9. Maternal observations such as changes in food and water consumption, behaviour and the presence of toxicity signs were also made to ensure pregnancy was proceeding normally.

2.2. Determination of oxidative stress

Induction of oxidative stress was assessed by the determination of catalase activity, thiobarbituric reactive species (TBARs) and total antioxidant capacity (TAC). Positive control treatment consisted of the subcutaneous administration of 3 ml/kg carbon tetrachloride, on GD8 (24 h before euthanization). On GD9, females were euthanized by cervical dislocation. Blood samples were collected in heparin vacutainer tubes and treated as follows: 2 mL were transferred into silicone-coated centrifuge tubes to avoid haemolysis and centrifuged for 20 min at 1500 rpm. Plasma was labelled and kept at −18 °C for later analysis. Leukocyte layer was discarded

and erythrocytes were rinsed three times with phosphate saline buffer (pH 7.4) before dividing into two portions, one of which was haemolysed with a hypotonic solution (6.7%, v/v 0.088 M $NaHPO_4$ and 0.022 M NaH_2PO_4, pH 7.0). The second portion of red blood cells was immediately used for TBARs determination.

2.2.1. Catalase activity

Enzyme activity was determined from the kinetics of H_2O_2 degradation according to Aebi (1984). Briefly, 5 μl haemolysed erythrocytes were added into a spectroscopic cell containing 2.9 ml pH 7.0 phosphate saline buffer and 100 μl 0.3 M H_2O_2. Kinetics were measured at 240 nm every 15 s for 60 s, against PBS. Activity was corrected for haemoglobin concentration which was determined spectrophotometrically according to Winterbourne (1990).

2.2.2. TBARs

One millilitre red blood cells were added to a centrifuge tube with 1 mL 30%, p/v trichloracetic acid and centrifuged for 10 min at 3000 rpm. One millilitre 0.67% thiobarbituric acid was added to 1 mL of supernatant and kept in a boiling water bath for 30 min after which reaction was stopped by putting in an ice bath. Absorbance range was determined spectrophotometrically between 400 and 600 nm against water.

2.2.3. TAC

Total antioxidant capacity was determined as described in Romay et al. (1996). In brief, a mixture of 1:1, 10 mM ABAP (2,2-azobis-(2-amidinopropane)) and 150 μM ABTS (2,2-azino-di-3-ethylbenzthiazoline sulfonic acid) was incubated at 45 °C for 30 min. Then, 1 mL of the mix was added to 10 μL of plasma. Absorbance was determined at 734 nm at 0, 10, 30 and 50 s against ABTS.

2.3. Determination of reproductive parameters

After blood collection, uterine horns and ovaries were dissected through a caesarean section. Number of corpora lutea was counted under a dissecting scope. Uterine horns were analyzed for number of implantation sites, resorptions and live embryos.

2.4. Statistical analysis

ANOVA was used to determine if oxidative stress parameters and reproductive parameters were influenced by herbicide exposure while maternal weight was analyzed by MANOVA. The analysis was performed using the, MINITAB 2000 v 13.31 software.

3. Results

Herbicide exposure did not cause any overt signs of maternal toxicity at any of the doses administered. No differences in weight gain were detected among treatments. Average number of corpora lutea, implantation sites, resorptions and live embryos were also similar among treatments and not influenced by dose of herbicide (Table 2, $p > 0.05$).

The administration of 100 mg/kg/d of both, PC and CF produced an increase in catalase activity which although not statistically significant (ANOVA, $p > 0.05$) indicated a trend towards an induction of enzyme activity with increasing herbicide dose (Fig. 1). Similarly, TBARs concentration was not different among treatments (Fig. 2). On the other hand, TAC values were significantly decreased only after administration of 100 mg/kg/dose 2,4-D for both PC and FC ($p = 0.001$), indicating that non-enzymatic antioxidant defences are depleted at that dose level (Fig. 3).

In all cases, the difference between the response to herbicide and to CCl_4 was obvious, indicating that any induction of oxidative stress by herbicide is much lower than that induced by the positive control treatment.

No differences between the responses induced by administration of 2,4-D as PC and as CF, indicating that the components of the CF did not influence the potential of 2,4-D as an oxidative stress inducer.

Table 2
Reproductive and fetal parameters in pregnant GD9 mice exposed to 2,4-D

	Treatment[a,b]							
	Control	0.01 PC	0.1 PC	100 PC	0.01 CF	0.1 CF	100 CF	CCl_4
Count	13	13	13	11	12	10	11	10
Weight gain (g)	5.3 ± 2.6	4.0 ± 1.3	3.4 ± 0.7	4.6 ± 1.1	4.8 ± 1.5	4.4 ± 1.8	5.1 ± 1.3	na
No. of corpora lutea	12.9 ± 3.3	12.8 ± 2.6	12.3 ± 2.0	12.4 ± 2.1	13.0 ± 1.8	11.9 ± 3.2	11.7 ± 3.4	13.3 ± 2.2
No. of implantation sites	12.1 ± 4.2	12.8 ± 2.8	12.5 ± 1.7	12.2 ± 2.4	12.3 ± 2.0	10.9 ± 3.2	10.8 ± 4.0	12.5 ± 2.2
No. of resorptions	0.3 ± 0.5	0.2 ± 0.4	0.4 ± 0.5	0.8 ± 0.8	0.4 ± 0.5	0.4 ± 0.8	0.5 ± 0.7	0.6 ± 0.8
No. of live embryos	11.9 ± 3.8	12.6 ± 2.5	12.3 ± 2.7	11.9 ± 2.0	12.2 ± 3.0	10.5 ± 2.6	10.7 ± 3.4	12.1 ± 1.9

Data are expressed as mean ± S.D.; na: not applicable.

[a] Each column indicates dose of herbicide (mg/kg/d) either as pure compound (PC) or in commercial formulation (CF).

[b] No significant differences in any of the parameters were found.

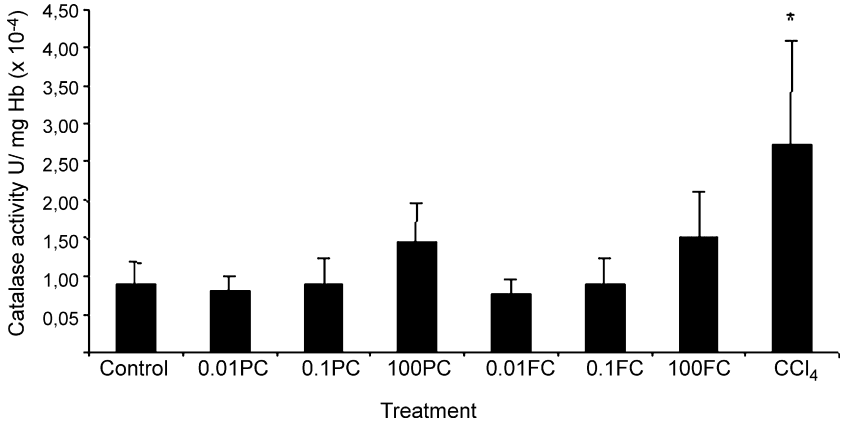

Fig. 1. Catalase activity (U/mgHb) after exposure to increasing doses of 2,4-D in pregnant GD9 mice. Bar labels indicate dose of herbicide (mg/kg/d) either as pure compound (PC) or in commercial formulation (CF). Control = 0 mg/kg/d. Data is expressed as means ± S.D. (*$p = 0.042$).

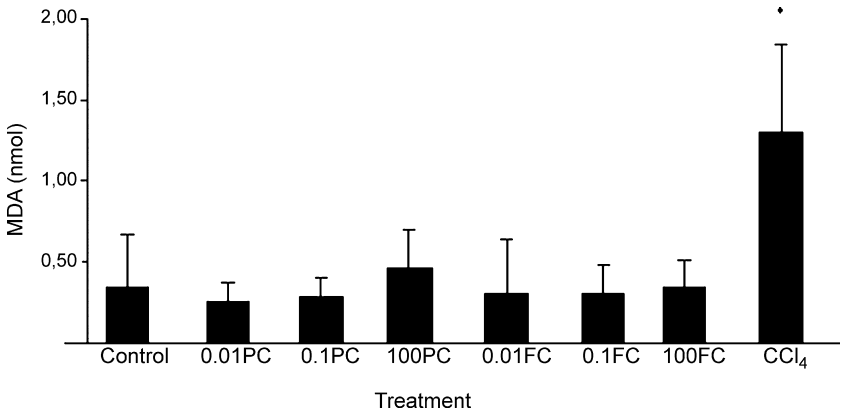

Fig. 2. TBARs concentration (nmol MDA) in red blood cells of pregnant GDP mice exposed to increasing dose of 2,4-D. Bar labels indicate dose of herbicide (mg/kg/d) either as pure compound (PC) or in commercial formulation (CF). Control = 0 mg/kg/d. Data is expressed as means ± S.D. ($^{*}p = 0.001$).

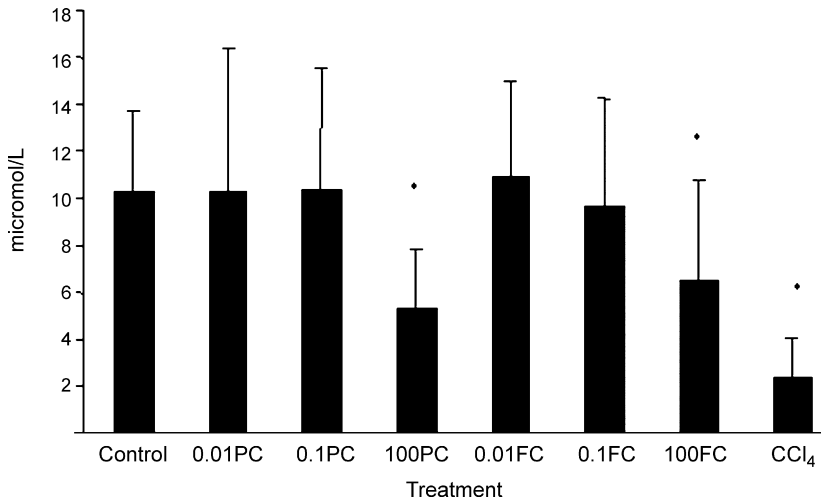

Fig. 3. Total antioxidant capacity (μmol/L) in blood of pregnant GD9 mice exposed to increasing doses of 2,4-D. Bar labels indicate dose of herbicide (mg/kg/d) either as pure compound (PC) or in commercial formulation (CF). Control = 0 mg/kg/d. Data is expressed as means ± S.D. ($^{*}p = 0.001$).

4. Discussion

2,4-D is an herbicide widely used throughout the world. Acute poisoning is uncommon although ingestion of high doses may lead to death. The precise mechanism of acute toxicity is not elucidated but may involve disruption of plasma and intracellular membranes or uncoupling of oxidative phosphorylation (Bradberry et al., 2000). This last mechanism may be involved in the generation of oxidative stress and subsequent induction of cytotoxic and genotoxic effects which may lead, among other to reproductive and developmental toxicity. No studies supporting this mechanism for subchronic, chronic or gestational exposure to low doses are found in the literature. Here, we have tested the *in vivo* capacity to induce oxidative stress by integrating into the experimental design a gestational rodent model and environmentally relevant doses, such as 0.01 mg/kg/d, which corresponds to the RfD for 2,4-D.

We administered the herbicide both as a pure compound and as part of a commercial formulation, since several reports have suggested that formulations may be more toxic than the active principle due to the different compounds that are included as inert ingredients. Surfactants and detergents may increase pesticide bioavailability and enhance biological responses. For instance, Oakes and Pollak (1999) concluded that the surfactant in a commercial formulation of 2,4-D was implicated in the alteration of mitochondrial processes since the formulation induced changes at a lower dose than 2,4-D. In the present study, there were no differences between the effect of the pure compound and the commercial formulation which may be due to toxicokinetic and toxicodynamic factors absent in an *in vitro* study such as the one by Oakes and Pollak (1999) or that the inert ingredients in our commercial formulation did not induce biological responses at the doses administered. Since in most cases, the nature of the inert ingredients in a pesticide commercial formulation is unknown, the definition of their contribution to the induction of toxicity becomes an extremely difficult, although very necessary issue.

Recently, Celik et al. (2006) studied the effects of plant growth regulators, including 2,4-D, on serum marker enzymes and erythrocyte and tissue antioxidant defense and lipid peroxidation in rats. The authors found that the administration of 1.5 and 3 mg/d of 2,4-D during 25 days induced, among other changes, an increase in erythrocyte catalase activity with no statistically significant changes in TBARs. These results agree with ours in that 2,4-D seems to induce *in vivo* oxidation only with increasing doses and with increasing length of administration period.

Other investigators have looked at the *in vitro* effects of 2,4-D on the generation of oxidative stress, either at the mitochondrial level in hepatocytes or in red blood cells (Palmeira et al., 1994, 1995; Oakes and Pollak, 1999; Bukowska et al., 2000; Duchnowicz and Koter, 2003). In both cases, the incubation of cells with the herbicide produces depletion of antioxidants and increases in oxidation markers, which does not agree with the general lack of response observed in this study. Toxicokinetic factors, not applicable to *in vitro* studies may explain this difference. For instance, 2,4-D is highly bound to proteins and since it is ionized at physiological pH, it is quickly eliminated by the kidneys, both factors that limit its distribution to tissues. On the other hand, hepatic biotransformation of 2,4-D is poor and does not generate reactive metabolites which may directly participate in the induction of oxidative stress (Munro et al., 1992).

2,4-D did not appear to induce any oxidative response in this study, although at 100 mg/kg/d, the non-enzymatic antioxidant first line of defense was markedly decreased. It is possible that above 100 mg/kg/d the induction of oxidation reaches a level that surpasses antioxidant defenses. This could be due to a decrease in the excretion of 2,4-D at this dose level. It is known that phenoxyacetic acid herbicides are eliminated by a renal anion transport system which is saturated as plasma concentration increases (reviewed in Timchalk, 2004). Since saturation of the rodent renal transporter is reported to occur at doses in excess of 50 mg/kg/d (Gorzinski et al., 1987), then the rise in blood concentration as dose of herbicide increases may lead to the distribution of the compound into cells and tissues which then become susceptible to oxidative stress. This may account for our current observations of oxidation being induced in red blood cells.

Li et al. (2002) have proposed that acylation of 2,4-D with AcylCoA leads to the formation of 2,4-D-CoA, an electrophylic compound that decreases the GSH/GGSG ratio and which covalently binds to hepatic proteins, which agrees with the description of Di Paolo et al. (2001) of a 52 kD protein that binds the herbicide after the I.P. administration of 70 mg/kg/d for 30 days to rats. Thus, it is tempting to propose that with increasing doses, saturation of the renal transporter leads to 2,4-D entry into cells where it covalently binds to mitochondrial proteins affecting electron transport. Mitochondrial impairments would increase the production of partially reduced oxygen species such as superoxide anion which dismutates to hydrogen peroxide and which in turn causes a dose-dependent increase in catalase activity.

Oxidative stress is defined as an imbalance between the production of free radicals and antioxidant defences.

According to this definition and based on our observations, we conclude that the herbicide does not produce oxidative stress at the doses used in this study. Johnson and Wattenberg (1996) have reported that the highest levels of exposure to 2,4-D occur in occupational settings, the amount of herbicide absorbed depending on the type of work and safety measures used. Farmers who do not wear protective gear may receive an average dose of 5.78 μg/kg/day while the general population in herbicide use areas or home gardeners usually would not receive doses greater than 2 μg/kg/day (Johnson and Wattenberg, 1996), doses well below those usually administered in animal toxicity studies. Thus, induction of oxidative stress by 2,4-D may only be produced after exposure to high, toxicological doses but not to low, environmentally relevant ones.

Conflict of interest

All authors declare they do not have any conflict of interest.

Acknowledgements

The authors gratefully acknowledge M.Sc. Enrique Cabrera for his assistance with statistical analysis. This work was supported by a grant from the University of Valparaíso, Chile (DIPUV REG No. 22/2002).

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