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ScienceDirect
Mutation Research 611 (2006) 83–88
Genetic Toxicology and Environmental Mutagenesis
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Dominant lethal effects of 2,4-D in *Biomphalaria glabrata*

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Received 24 February 2006; received in revised form 23 June 2006; accepted 24 July 2006
Available online 12 September 2006

Abstract

Dominant lethal effects of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) were evaluated in the freshwater snail *Biomphalaria glabrata*. Wild-type snails were exposed during 10 days to 50, 75 and 100 ppm of 2,4-D dimethylamine salt (2,4-D DMA) and paired with non-exposed albino snails 1, 11, 25 and 40 days after the exposure. The offspring of the non-exposed albino snails was scored for lethal malformations.

One day after the exposure, a significant effect was observed at 75 and 100 ppm without a dose–response relationship. After 11 days, the effect was observed only at the highest dose. After 25 days, significant increases in the dominant lethal effects occurred at 50 and 75 ppm; effects were directly related to the doses. Background levels of lethal malformations were resumed after 40 days.

Although the major and direct measure of dominant lethal mutations is the rate of lethal malformations in the heterozygous offspring of the albino snails, the sensitivity of the assay was substantially increased with the evaluation of all non-viable embryos, that are the sum of those with lethal malformations, identified or not as wild-type.

Keywords: 2,4-Dichlorophenoxyacetic acid; Dominant lethal test; *Biomphalaria glabrata*; Embryonic malformations; Freshwater mollusk

1. Introduction

Phenoxyacetic herbicides constitute one of the largest groups of herbicides; among these 2,4-dichlorophenoxyacetic acid (2,4-D) is the one most widely used in the world [1]. These herbicides are synthetic structural analogues of the natural auxine, indole-3-yl-acetic acid, which plays a crucial role in the division, differentiation, and elongation of plant cells [2].

Given the potential environmental impact as a consequence of the intense use of pesticides, along with the large population potentially exposed, the determination of the effects of chronic exposure to these pollutants is urgently needed. Potential genetic hazards due to pesticides are now generally recognized [3], making mutagenicity data a part of the required information to support the current registration of a pesticide chemical in many countries [4–6].

An International Programme on Chemical Safety (IPCS) Task Group on Environmental Health Criteria has reviewed a series of studies carried out in different test systems to assess the potential mutagenic effects of 2,4-D and its derivatives. Mutagenicity studies included tests in rodents, cultured lymphocytes, Chinese hamster ovary (CHO) cells, yeast, and both somatic and germ cells of *Drosophila melanogaster*. The results ranged from induction of chromosomal damage and incidence

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doi:10.1016/j.mrgentox.2006.07.001

of malignant skin neoplasm to no effect at all. The working group concluded that it was not possible to make an evaluation of the mutagenic potential of 2,4-D on the basis of the available data [7].

Ensuing studies were not sufficient to establish the mutagenic potential of 2,4-D. In in vitro assays, 2,4-D induced sister chromatid exchanges (SCE) and DNA strand breaks in cultured CHO cells [8], and chromosomal aberrations and micronuclei in human peripheral blood lymphocytes [9], but did not induce mutations in *Salmonella* or unscheduled DNA synthesis in rat hepatocytes [10]. In a series of in vivo studies, 2,4-D induced SCE and chromosome aberrations in mouse bone marrow [11,12] and onion root tip [13]. 2,4-D was reportedly mutagenic in the micronucleus test in fish [14,15], but not in mice [10]. Mutagenic potential of 2,4-D to germ cells has not been established as well. In mice, 2,4-D was not mutagenic in the dominant lethal test [16], but induced chromosomal aberrations in spermatocytes, sperm head abnormalities [12], and sister chromatid exchanges in spermatogonial cells [11].

The indiscriminate use of pesticides may contaminate surface water. Residues of 2,4-D in aqueous systems can result from the deposition of spray drifts, the "washout" of the compound in the vapor or droplet phase from the atmosphere during rainfall, the run-off from treated fields, or following the application of the herbicide to water for the control of aquatic weeds. Industrial discharges, either from accidental spills or through sewage systems, may also contribute to the contamination of water. Despite its ready degradation by physical, chemical, and biological action, detectable residues of 2,4-D have been reported to persist for 4 weeks to up to 4 months in some situations [7].

To evaluate germ cell effects of mutagenic pollutants, the dominant lethal test has been established in the freshwater snail *Biomphalaria glabrata* [17]. This system was efficient, specific, and sensitive for the detection of germ cell mutations induced by reference mutagens. Results obtained with cyclophosphamide and mitomycin C showed that *B. glabrata* can absorb and activate chemical mutagens from the aquatic environment [17]. Dominant lethal effects induced by ionizing radiation in *B. glabrata* were quite similar to the highly conserved response observed in spermatogenic cells in widely different species [18].

There are few data on mutagenicity of 2,4-D in aquatic species. Notwithstanding the importance of this information in the assessment of the environmental impact of 2,4-D, most studies have focused on the effects to human health. Given the efficiency of the dominant lethal test in *B. glabrata* in the detection of reference mutagens from the aquatic environment, we thought it was pertinent to use this system to re-evaluate germ cell effects of 2,4-D.

2. Material and methods

2.1. Animals

B. glabrata is a hermaphrodite snail, in which female and male gametes are simultaneously and constantly produced in a hermaphrodite gland, the ovotestis, and transported through an unique narrow duct to the female and male tracts. An organ called genital intersection or "carrefour" can be found at the junction between the Hermaphrodite, male and female ducts of the reproductive system [19].

When isolated, these hermaphrodite snails reproduce by self-fertilization but, when paired, preferentially reproduce by outcrossing. These reproductive modes, together with the existence of pigmented and albino strains, make it relatively easy to study the genetics of this snail [20].

A pigmented wild-type homozygous strain of *B. glabrata* obtained from São Lourenço da Mata (State of Pernambuco, Brazil) and a non-pigmented albino strain, from Barreiro (State of Minas Gerais, Brazil), reared in the laboratory of the Departamento de Biofísica e Radiobiologia da Universidade Federal de Pernambuco (Brazil) were used in this study.

2.2. Animal selection

All snails used in the experiments were selected by previous analysis of background frequencies of embryonic malformations. Three or four egg-masses were collected from albino snails before the crossings and those with malformation frequencies above 5% were discarded. Wild-type snails were evaluated after a crossing with albino snails before the exposure. At least 50 phenotypically wild-type embryos per animal were analyzed among the progeny of the albino snails and those with frequencies of malformations above 3% were discarded.

2.3. Substance

A commercial formulation of 2,4-D [CAS 94-75-7] containing 806 g/L of the 2,4-D dimethylamine salt (2,4-D DMA), DMA 806BR-Dow Agroscience, corresponding to 670 g/L of the acid equivalent, was used in this study.

2.4. Exposure and crossings

Three groups of 11 snails were exposed to aqueous solutions of 2,4-D DMA at concentrations of 50, 75, and 100 ppm; 11 animals served as controls. The test compound was added directly to dechlorinate filtered water at 25 °C immediately before the exposure. Wild-type snails were individually exposed in disposable plastic flasks to the test substance for 10 days; dechlorinated filtered water was used in control groups. We used 110 mL flasks with perforated covers to allow breathing. 2,4-D

DMA solutions were renewed daily. Animals were fed daily with fresh lettuce during the exposure period.

Twenty-four hours after the end of the exposure, each wild-type snail was put together with a non-exposed albino snail for 24 h in individual aquaria (pairing). After this period, animals were isolated and egg capsules were collected from albino snails.

2.5. Analysis of embryonic malformations

The crossing between homozygous wild-type snails and albino snails produces the following types of embryos among the offspring of the albino snails: heterozygous wild-type embryos produced by cross-fertilization and albino embryos produced by self-fertilization or by cross-fertilization with other albino snails before the onset of the experiment. Wild-type embryos can be identified among the offspring of albino snails by the presence of pigmentation in the eyes, visible from the fourth day on of embryonic development. Thus, we analyzed only the wild-type embryos, originated from sperm of the exposed wild-type snails; albino embryos were discarded.

Egg capsules were collected daily. Plastic sheets were placed on the water surface to propitiate oviposition; egg capsules were then transferred to cell culture plates and maintained in climatic chambers at 25 °C until the end of the analysis. Animals were maintained isolated for at least 3 weeks before the crossings in order to increase the rate of cross-fertilization.

Embryos were observed for 8 days from the beginning of the development until nearly hatching using a stereomicroscope (Olympus). Only malformations leading to death were scored and embryos fell into two different categories: wild-type malformed and embryos in which the identification of the eyes is not possible. Non-viable embryos constitute the sum of those with lethal malformations identified or not as wild-type.

All analyses were carried out in coded scoring.

2.6. Statistical analysis

From our studies, we established, respectively, 5 and 3% as the thresholds of background frequencies of non-viable embryos and malformed heterozygous embryos in the offspring of the non-exposed albino snails. To test if the frequencies were larger than these limits, we used the binomial distribution to compare the hypothesis H: $p \geq 0.05$ against A: $p < 0.05$ for the first parameter and H: $p \geq 0.03$ against A: $p < 0.03$ for the second. Whenever we did not reject H, we compared the different doses using the standard χ^2-test.

3. Results

Data on dominant lethal effects of 2,4-D DMA are shown in Table 1. In all control groups, the frequencies of malformed and non-viable embryos were under the background levels established in previous experiments with *B. glabrata*. The frequencies of non-viable embryos were under 5%, and the frequencies of malformed embryos

Table 1
Dominant lethal effects of 2,4-D on *B. glabrata*

Crossing schedule (days)	Concentration (ppm)	No. of pairings	No. of crossings	Embryos			Wild-type embryos		
				Total	Non-viable	%	Total	Malformed	%
1	Control	27	27	4043	123	3.04	3850	46	1.19
	50	9	9	1080	23	2.13	1038	8	0.77
	75	11	10	1532	92[a]	6.01	1481	52[a]	3.51
	100	10	9	1271	94[a]	7.40	1100	30[a]	2.73
11	Control	28	27	2935	80	2.73	2775	13	0.47
	50	9	9	987	15	1.52	659	7	1.06
	75	11	10	1564	57	3.64	1493	18	1.21
	100	11	10	1361	69[a]	5.07	1166	18	1.54
25	Control	23	23	3302	40	1.21	2958	15	0.51
	50	7	7	822	33[a]	4.01	714	14[a]	1.96
	75	9	7	620	96[a]	15.48	980	5	0.51
	100	7	7	919	8[b]	0.87	748	1	0.13
40	Control	23	22	3275	63	1.92	3122	7	0.22
	50	6	5	617	9	1.46	611	4	0.65
	75	11	11	1721	41	2.38	984	12	1.22
	100	7	7	902	7	0.78	861	0	0

No. of pairings: number of pairs of wild-type snails and albino in individual aquaria; No. of crossings: number of albino snails producing heterozygous embryos.

[a] $p < 0.05$.

[b] Two affected animals exposed to 2,4-D died before 25 days crossing.

among the heterozygous offspring of the albino snails crossed with non-exposed were under 3%.

The dominant lethal effects of 2,4-D DMA were observed at the three doses, as shown by significant increases in the frequencies of lethal malformations in the heterozygous offspring of the albino snails crossed with exposed wild-type snails. These effects were observed in the crossings occurring at 1 and 25 days after the end of exposure. In the crossing occurring at 1 day after the exposure, the effect was observed at 75 and 100 ppm and there was no dose–response effect. At 25 days after exposure, the effect was observed only at 50 ppm. At 40 days, there was no effect of 2,4-D DMA in the frequencies of heterozygous malformed embryos.

The effect of 2,4-D DMA in the production of non-viable embryos was observed at the three doses: 1, 11 and 25 days after the exposure. After 1 day, the effect was observed at 75 and 100 ppm and there was no dose–response effect. After 11 days, the effect was observed only at the highest dose. After 25 days, the increase in the frequencies of non-viable embryos was observed at 50 and 75 ppm, and the effect was directly proportional to the doses.

4. Discussion

Results obtained in this study showed that 2,4-D can induce germ cell mutations in *B. glabrata*. The progeny of the non-exposed albino snails was analyzed for the rates of malformations in the heterozygous embryos and the rates of non-viable embryos. Dominant lethal effects were detected with the three test concentrations at 1, 11 and 25 days after 10 days of exposure. With 50 ppm, significant increase in both rates was observed after 25 days. With 75 ppm, the effects were detected after 1 day in both parameters and after 25 days, in the rates of non-viable embryos. With 100 ppm, the effects were observed after 1 day in both parameters and after 11 days, in the frequencies of non-viable embryos. The death of two animals, which had shown effects of the drug in the 11th day crossing, prevented the detection of further dominant lethal effects. Both the rates of heterozygous embryos with unspecific malformations and non-viable embryos resumed background levels 40 days after the exposure (see Table 1).

The major and direct measure of dominant lethal mutations in *B. glabrata* is shown by scoring the number of lethal malformations in the heterozygous offspring of the albino snails. Nevertheless, the sensitivity of the assay was substantially increased in this study with the evaluation of the frequencies of non-viable embryos. For example, the effect after 11 days exposure was detected only in the rates of non-viable embryos.

In previous studies, increases in the frequencies of heterozygous embryos with lethal malformations were accompanied by increases in all types of malformations, but the scoring of non-viable embryos did not increase the sensitivity of the test [17,18]. However, in this study, the analysis of non-viable embryos was crucial to the detection of the dominant lethal effects of 2,4-D. A possible explanation for this fact could be the occurrence of severe DNA damage leading to death prior to the stage when the pigmentation in the eyes can be observed. In the assay with rodents, loss of fertilized eggs before implantations reflects gross chromosomal aberrations. However, this effect cannot be distinguished from the loss of unfertilized eggs caused by a toxic effect that reduces the germ cells ability to fertilize [21]. In *Biomphalaria*, where no losses due to abortions and failures of implantation occur, even highly damaged embryos can be observed in the egg-masses. The degree of DNA damage would determine the stage of death; the higher the degree of damage, the earlier the time of death.

The origin of unidentified malformed embryos in our study could be ascertained, but their cross-fertilization origin is highly probable since: (i) as crossing rates were high, a minority of self-fertilized embryos was produced, (ii) frequencies of unidentified malformed embryos higher than the established background level of 5% were observed only in the exposed groups, and (iii) all animals were previously selected, thus, abnormal snails were excluded from the sample.

The sensitivity of the snail *B. glabrata* to 2,4-D in the dominant lethal test detected an effective concentration of 50 ppm of the commercial formulation, corresponding to 34 ppm of 2,4-D. In two studies on aquatic species, 2,4-D induced micronuclei in the freshwater fishes *Channa punctatus* [15] and *Clarias batrachus* [14] at 25 ppm. These two studies served as reference for the choice of test concentrations in our work. We performed preliminary tests to establish the range of concentrations on basis of acute toxicity; 25 ppm was defined as the lower limit according to the studies with fish and 100 ppm as the upper limit, since, at this level, some signs of acute toxicity were observed (data not shown).

Available data indicate that residues of 2,4-D rarely exceed 1 mg/kg in soil, several μg/L in water, several $\mu g/m^3$ in air, and a few tens of μg/kg in food sources. Exceptions may occur in the vicinity of 2,4-D herbicide spills, in water treated with aquatic 2,4-D herbicides, in berries and mushrooms grown in treated right-of-way areas, or when the herbicide is used in quantities far in excess of the rates applied in normal agricultural

or forestry practice. Although environmental concentrations are usually low, in some cases, high levels of 2,4-D may be found. For example, the use of 2,4-D for aquatic weed control may lead to contamination of sources of irrigation and drinking water. Also, environmental pollution arises through inadequate disposal practice: disposal of unused 2,4-D and washing of equipment may result in localized land pollution and pollution of water supplies through direct contamination or leaching from soil [7].

Single or multiple treatment schedules can be used in the dominant lethal test. In general, the multiple regimen has the advantage of simulating environmental exposure, as well as involving a smaller number of albino snails for crossing after treatment. Single treatment is useful for the determination of stage specificity of an agent in the test with rodents; for this regimen, multiweek crossing is required [21,22].

For the moment, multiweek crossings in *B. glabrata* can only provide an estimate of the affected stages of spermatogenesis. According to the estimate of chronology of spermatogenesis in *B. glabrata* [18], total duration of spermatogenesis is approximately 36 days, with the following distribution of developmental stages: 1–13 days, spermatogonia; 14–20 days, spermatocytes; 21–36 days, spermatids and spermatozoa. The treatment schedule consists of 10 days of exposure followed by four crossings at 1, 11, 25 and 40 days after the end of the exposure. Therefore, if the cell division of spermatogenic cells was not affected by the drug, in the crossing 1 day after exposure, we would have sampled spermatogenic cells exposed for 10 days at late spermatid and spermatozoa stage; after 11 days, cells exposed at spermatocyte and spermatid stage, and after 25 days, spermatogenic cells exposed for 10 days at spermatogonia stage. After 40 days, a new pool of spermatogenic cells, non-exposed to 2,4-D, would have been sampled.

As far as we know, this is the first study indicating dominant lethal effects of 2,4-D; a single study in rodents reported negative results [16]. On the other hand, 2,4-D and its metabolite 2,4-dichlorophenol (2,4-DCP) induced chromosomal aberrations in spermatocytes and sperm head abnormalities in mice [12]; they also increased the frequency of sister chromatid exchanges in spermatogonial cells of mice [11].

Mutagenic potential of 2,4-D has been very much discussed, since previous studies, using a wide range of assays for mutagenicity and genotoxicity, have produced conflicting results. Possible explanations for the controversial results obtained include the effect of impurities in samples and the differential sensitivities of the specific systems and endpoints used [2,8,9,11].

The dominant lethal test in *B. glabrata* [17] was established by using a similar approach to that used in the dominant lethal test in rodents [23]. Despite the lacking data on the timing of spermatogenesis and metabolizing systems in *Biomphalaria*, the test in this freshwater mollusk shows some advantages in comparison to the assay in rodents. Since the snail is a simultaneous hermaphrodite, effects on female germ cell lineages can be obtained from the same exposed animals. The brood size allows the scoring of a greater number of embryos: one adult snail lay egg-masses with 20–50 eggs almost daily and reproduces throughout the year.

Our findings indicate that 2,4-D produces dominant lethal mutations in *B. glabrata* provide additional evidence for the germ cell genotoxicity of these herbicides, and justify further studies on their environmental effects.

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