ejpmr, 2018,5(12), 121-127

SJIF Impact Factor 4.161

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

EFFECTS OF GESTATIONAL EXPOSURE TO 2, 4-DICHLOROPHENOYACETIC ACID ON EMBRYOTOXICITY AND FETOTOXICITY IN FEMALE WISTAR RATS

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Article Received on 17/10/2018 **Article Revised on 07/11/2018** **Article Accepted on 28/11/2018**

ABSTRACT

2,4- dichlorophenoxyacetic acid (2,4-D), a plant growth regulator, has been used worldwide as an herbicide. It has been shown to produce a wide range of adverse effects from embryotoxicity and teratogenicity to neurotoxicity on animal and human health. In the present study we investigated the effect of 2,4- D on embryo- and fetal development in female rats. Female Wistar rats received orally by intra-gastric gavage (i.g.) 100 and 200 mg of 2,4-D/kg body weight (bw) during organogenesis (days 6 to 15 of gestation). Then the reproductive parameters were determined in animals, fetuses were examined for external and skeletal malformations. Placenta was examined histologically. Reduced fetal weight, retarded fetal development, number of fetuses per mother and high incidences of dead fetuses and resorptions in treated mothers were observed. Gross morphological abnormalities, such as displayed form of oedema, lack of tail, hypotrophy, severs subdermal haemorrhage patches and hypotrophy of placenta were observed in fetuses after 2,4-D-treated mothers. A skeletal development of fetuses presented an incomplete ossification in nasal, cranium, abdominal or caudal bones in rats treated with 100 mg/kg of 2,4-D, whereas rats treated with 200 mg/kg showed absence of ossification of the sacral vertebrae compared with the control. Placental histological observations revealed a pronounced morphological alteration, with atrophy of decidual cells, a degenerated of chorionic villi and hypertrophy of blood lacuna. The present study suggests a risk to the developing embryo when the mother is exposed to a high concentration of 2,4-D during organogenesis.

KEYWORDS: Embryotoxicity, 2,4-Dichlorophenoxyacetic acid, Malformations, Skeletal, Placenta.

INTRODUCTION

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.[1] 2,4-diclorophenoxyacetic acid (2,4-D) is an herbicide widely used in broadleaf and woody plant production, which disrupts the mechanisms of biological responses to hormones in plants.[2] 2,4-D mimics the effect of the auxins, or plant growth-regulating hormones, stimulating growth and rejuvenating old cells, and overstimulating young cells, which leads to an abnormal growth pattern and death in some plants.[2] In addition, 2,4-D has been reported to induce several adverse alterations in human cell lines and other mammalian cells.[3,4,5] In fact, 2,4-D is adsorbed into the mammalian organism from skin and the alimentary tract being then excreted in the urine.[6] The exposure to 2,4-D is known to impair some important cellular functions, particularly those related to the central nervous system,[3,7] although the mechanism by which 2,4-D acts are not fully disclosed. Nonetheless, it has been proposed that the acute toxicity of this endocrine disruptor involves intracellular membrane disruption and oxidative phosphorylation decoupling,[8] but the overall cellular metabolic alterations induced by 2,4-D are often overlooked. Also, the teratogenic, neurotoxic, immunosuppressive, cytotoxic and hepatoxic effects of 2,4-D have been well documented.[9,10] Herbicide 2,4-D increases lipid peroxydation in animal and human cells in vitro.[11,12] 2,4-D has been shown to cause cellular mutations which can lead to cancer. This mutagen contains dioxins, a group of chemicals known to be hazardous to human health and to the environment.[13] Exposure to 2,4-D induce nephrotoxicity in rats during late pregnancy and early postnatal periods.[14] Mikov *et al.*[15] reported that 2,4-D has a hypoglycemic effect in mice. In rodents, this chemical also increases levels of the hormones progesterone and prolactin, and causes abnormalities in the estrus cycle.[16] In Minnesota, higher rates of birth defects have been observed in areas of the state with the highest use of 2,4-D and other herbicides of the same class. This increase in birth defects was most pronounced among infants who were conceived in the spring, the time of greatest herbicide use.[17] In chinese-

hamster ovary cells, 2,4-D produced DNA damage and sister chromatid exchange.[18] Recently, it has been reported that 2,4-D can pass from dams to suckling pups, and inhibit the suckling-induced hormone release in lactating rats.[19,20] Thus, gestational and lactational periods including the neonatal and prepubertal stages seem to be particularly favourable for the induction of 2,4-D effects in rodents. However, the toxic effects of 2,4-D on embryo-fetal development in rats are still unclear. The present study was carried out to determine the effect of 2,4-D given orally by gavage during the period of organogenesis on embryo- and fetal development, as well as its effects on various regions of fetuses bone. Moreover, the morphology and the histology of placenta and fetuses were investigated.

MATERIALS AND METHODS

Animals and reagents

Two-month-old Wistar female rats (150g) were used in this study. Rats were housed under controlled conditions of temperature (22 ± 1°C), with a 14-h light/dark cycle. 2, 4-D (purity 98%) was purchased from Sigma-Aldrich (St. Louis, MO). Female rats of proven fertility were kept for mating (2:1) with normal healthy adult males overnight. The day at which sperms were found in the vaginal smear was designated as day 0 of gestation. These pregnant females were divided into three equal groups (n = 8) of approximately similar weight as follows: (1) animals received orally by intra-gastric gavage (i.g.) 100 mg of 2,4-D/kg body weight (bw), (2) animals received orally by i.g. 200 mg of 2,4-D/kg bw, (3) control group received equal volumes of vehicle from days 6 to 15 of gestation. Dilution of 2, 4-D was so made that the volume of each injection was maintained to 1ml/100g bw of rat.[21] Doses of 2,4-D were chosen in accordance with the LD50 (acute oral toxicity) doses in rats, which was 375 mg/kg/day.[22] The animals were kept in polycarbonate cages individually and were given feed and water *ad libitum*. The dams were observed daily for change in body weight and physical signs of toxicity, if any. After 19 days of gestation, all animals were killed by decapitation; the number of fetuses/litter, number of live/dead fetuses, crown-rump length, number of resorptions, number of implantations, weight of uteri, of fetuses and their respective placenta were recorded. Post-implantation loss was calculated on the basis of previous report:[23]

$$\text{Post-implantation} = [(\text{total implantations} - \text{live fetuses})/\text{total implantations}] \times 100.$$

Morphological evaluation of the offspring

The left and the right uterine horns were removed and the rat was terminated. The first implantation site next to the ovary was denoted following the convention position 1 in order to the last position in the uterine horn next to the cervix. Each embryo, from right and left uterine horn regarding to intrauterine position, was removed to examine the gross malformations. Structural deviations from normal development were considered as denoted malformations, i.e. facial defects, body malrotation and neural tube defects. Malformed and non-malformed offspring were subsequently examined for skeletal anomalies. A skeletal radiographic (General Electric; voltage 27 kV, intensity 5 mA) was done to observe the effect of chromium VI on various regions of bone (nasal bone, cranium, abdominal, caudal) in fetuses.

Histological analysis

Placenta was fixed overnight at room temperature by direct immersion in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The samples were dehydrated with an ethanol and toluene series and embedded in paraffin. Serial sections (4µm) were mounted on gelatin-coated glass slides cut and stained with haematoxylin and eosin.

Statistical analysis

Data were analyzed using Stat View 512+ Software (Abacus Concept, Inc. Calabasas, CA, USA). Differences among groups were measured using oneway analysis of variance (ANOVA) followed by the Student–Newman–Keuls as post hoc test. The results were expressed as means ± SEM and differences were considered statistically significant at $p<0.05$.

RESULTS

No notable changes in behaviour or clinical signs were observed in the control or in the treated dams. No mortality was observed during the experimental period. The dams treated with 100 and 200mg of 2,4-D/kg b.wt day-1 presented a significant reduction in body weight gain during treatment period (Table 1). This decrease was about 15% and 28.5% compared with the control group. The relative weight of uterus was also decreased after exposure to 2,4-D during the organogenesis period, this decrease reached 11.7% and 35.6% of controls for 100 and 200mg of 2,4-D/kg b.wt day^{-1}, respectively. The number of fetuses/litter was significantly reduced in treated groups when compared with controls (8.71 ± 0.91 and 8.57 ± 0.48 respectively with 100 and 200mg of 2,4-D/kg b.wt versus 11.29 ± 0.81). Fetal weight and crown rump length were also significantly decreased in groups treated with 2,4-D. This decrease was about 48% and 53.2% in fetal weight and 19% and 31.6% in crown rump length respectively in rats treated with 100 and 200mg of 2,4-D/kg compared with the control. Our data indicate that the placenta weight was significantly reduced in groups treated with 2,4-D (0.44 ± 0.01 and 0.43 ± 0.01 respectively with 100 and 200mg 2,4-D/kg b.wt versus 0.46 ± 0.006). The incidences of dead fetuses were high in groups treated with 2,4-D compared with the control group. The number of resorption sites was found to be dose dependent. The higher dose caused more incidences of resorption sites than the lower one (0.71 ± 0.26 and 1.42 ± 0.34 respectively with 100 and 200mg 2,4-D/kg b.wt versus 0.14 ± 0.15). In addition, maternal exposure to 2,4-D during the organogenesis period decreases significantly the number of implantations. This decrease was about 12.7% and 17.8% respectively in rats treated with 100 and 200mg

2,4-D/kg compared with the control. Controversy, the data revealed significantly higher incidences of post implantation loss in treated groups (397% and 659%, respectively with 100 and 200mg 2,4-D/kg b.wt) (Table 1). Gross structural abnormalities in the form of edema and subdermal haemorrhagic patches were observed on abdominal regions in fetuses of pregnant rats treated with 100 mg of 2,4-D/kg when compared with controls (data not shown). The changes were more significantly in groups treated with a high dose of 2,4-D (200mg/kg) compared with the preceding lower-dose group. Morphological study of the fetuses, from treated rats with 200 mg of 2,4-D/kg, showed gross malformations like edema, facial defect, lack of tail, hypotrophy and severe subdermal haemorrhage patches compared with the control (Figure 1). A skeletal development of fetuses showed dose-related effects (Figure 2). In the 100 mg/kg treated group, fetuses presented only an incomplete ossification in nasal, cranium, abdominal or caudal bones compared with the control. Whereas, the most striking abnormality seen in X-rays was absence of ossification of the sacral vertebrae: this situation occurred in the fetuses from the pregnant rats treated with 200 mg of 2,4-D/kg during the organogenesis period (Figure 2). Placentas from dams given 2,4-D during the organogenesis period showed some morphological alterations when compared with the control group (Figure 3). The placentas exhibited dose-dependant hypotrophy macroscopically in rats exposed to 100 and 200mg of 2,4-D/kg. Histopathologically, in the treated group, increased dose-dependant thickness was noted significantly in the decidua basalis with atrophy of decidual cells. Furthermore, we observed degeneration of chorionic villi and hypertrophy of blood lacuna that was particularly remarkable in groups treated with a high dose (Figure 4).

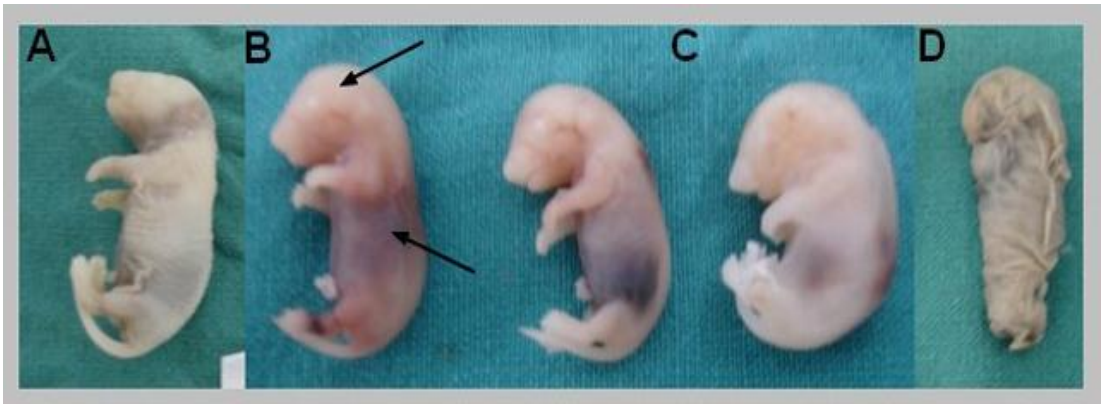

Figure 1: Type of malformations in fetuses of pregnant rats in 19 days of gestation.

Legend

Rats were gavaged with 200mg of 2,4-D/kg body weight during the organogenesis period. (A) Normal; (B) edema and presence of haemorrhage areas (arrowed); (C) hypotrophy and severe subdermal haemorrhage patches; (D) facial defect and lack of tail.

Figure 2: A skeletal radiographic of fetuses from control (*A*) and 2,4-D-treated rats with 100 mg/kg (*B*) and 200 mg/kg (*C*) during the organogenesis period.

Legend

(A) Fetuses showed the presence of four sacral vertebrae (a: nasal bones, b: cranium bones, c: abdominal bones, d: caudal bones). (B) Fetuses showed incomplete ossification (arrowed). (C) Fetuses showed the absence of any vertebrae.

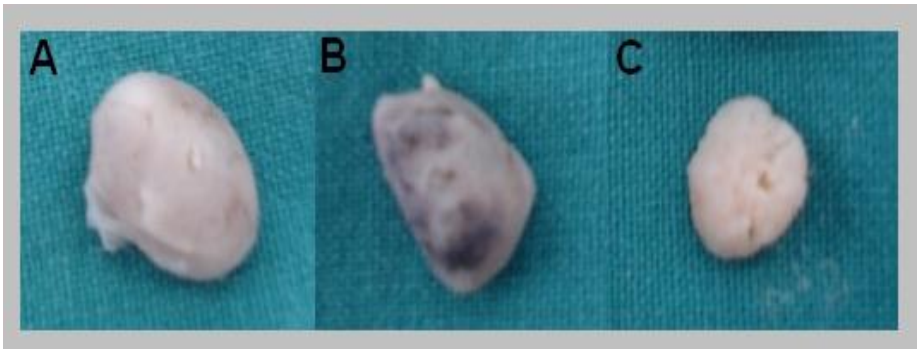

Figure 3: Gross appearance of the placenta of the control (*A*) and 2,4-D-treated group with 100 mg/kg (*B*) and 200 mg/kg (*C*).

Legend

Rats were gavaged with 100 and 200mg of 2,4-D/kg body weight during the organogenesis period body weight during organogenesis period. (B, C) Marked hypotrophy in the 2,4-D-treated placenta.

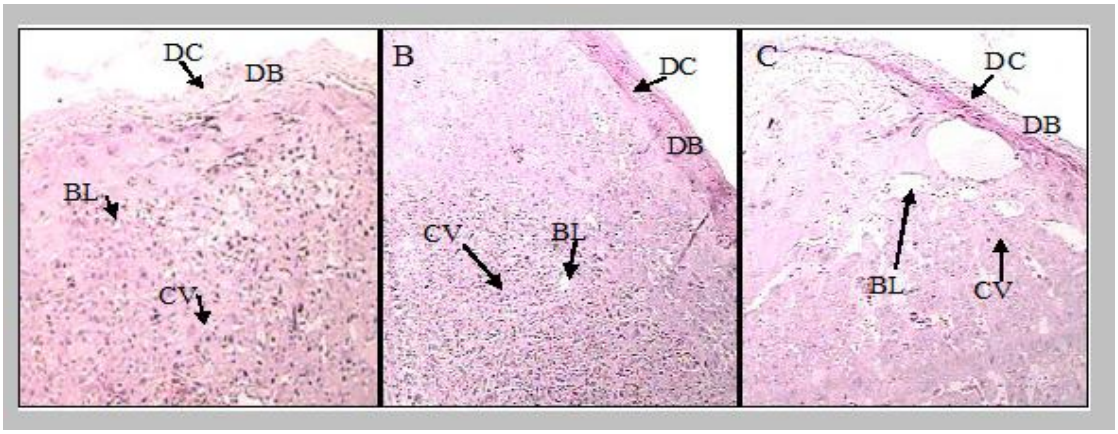

Figure 4: Photomicrographs of sections of placenta from control (*A*) and 2,4-D-treated rats with 100 mg/kg (*B*) and 200 mg/kg (*C*) during the organogenesis period.

Legend

Placentas were fixed by direct immersion in paraformaldehyde solution. Serial (5 μm) sections were mounted on gelatin-coated glass slides and stained with hematoxylin and eosin. DB, decidua basalis; DC, decidual cells; BL, blood lacunas; CV, chorionic villi. Magnification: ×200 (A–C).

Table 1: 2,4-D-induced embryotoxicity and fetotoxicity in rats treated during organogenesis.

Parameters	Control	100 mg/kg	200 mg/kg
Weight gain in mother (g)	63 ± 4.24	53.6 ± 1.10[a]	45 ± 2.02[b]
Relative weight of uterus (g/100g)	3.4 ± 0.13	3 ± 0.14[a]	2.19 ± 0.50[a]
No. of fetuses (dead and live)/ litter	11.29 ± 0.81	8.71 ± 0.91[a]	8.57 ± 0.48[b]
No. of dead fetuses (no. of litters)	All live	2(2)	4(4)
Fetal weight (g)	3.91 ± 0.07	2.04 ± 0.12[b]	1.83 ± 0.07[b]
Placental weight (g)	0.46 ± 0.006	0.44 ± 0.01[b]	0.43 ± 0.01[b]
Crown rump length (cm)	3.25 ± 0.09	2.63 ± 0.04[b]	2.22 ± 0.05[b]
No. of resorption sites	0.14 ± 0.15	0.71 ± 0.26	1.42 ± 0.34[b]
No. of implantations	11.29 ± 0.81	9.85 ± 0.81	9.28 ± 0.38[a]
Post implantation loss (%)	1.59 ± 1.58	7.91 ± 3.04[a]	14.12 ± 4.88[a]

Values represent mean ± SEM of 8 rats in each group. 2,4-D treatment was preformed as described in the methods section. [a] *$p < 0.05$ compared with controls.* [b] *$p < 0.01$ compared with controls*

DISCUSSION

2,4-D, a worldwide-used herbicide, has been shown to produce a wide range of adverse effects from embryotoxicity and teratogenicity to neurotoxicity in animals and humans. In the present study, orally exposed to 2,4-D revealed fetal development retardation and embryo fetotoxicity as evidenced by the reduction in fetal weight, crown rump length, number of fetuses per dam, number of implantations and higher incidences of resorptions sites, embryonic death and post-implantation loss in animals treated with 100 and 200mg/kg of 2,4-D. Furthermore, our results indicated that the treatment of pregnant rats with 2,4-D induced a decrease in weight gain in mother and in their uterus's relative weight. Our

findings were in accordance with other studies carried out in pregnant rats and have demonstrated that orally administration of 2,4-D causes fetotoxic effects with significant reduction of implantation rate, weight of fetuses and an increase in the resorptions number, pre-implantation and post- implantation loss for mothers treated with 2,4-D.[9,24] Charles and co-workers demonstrated that administration of 2,4-D by i.g. to pregnant rats from 6 to 15 days severely affects feed consumption and maternal body weights.[9] Therefore, the poor nutrition in pregnant rats leads to intra-uterine growth retardation, post-natal growth failure, changes in the endocrine parameters of the somatotrophic axis, and to increased blood pressure in later life.[25, 26] The relation ship between maternal and developmental toxicity is important in making regulatory decisions regarding the developmental toxicity of a chemical. Accordingly, a systematic and critical approach is needed to characterize the role of maternal toxicity in laboratory animal studies with adverse developmental outcome.[27] The decrease in maternal weight gain during pregnancy clearly indicated that 2,4-D was maternally toxic. Previous study[9] reported that administration of 100 and 175 mg of 2,4-D/kg from 6 to 15 days of gestation affects the developing rat fetus associated with clinical signs of toxicity and mortality in the maternal animals. The fetal development retardation and the embryo- fetotoxicity observed in our study may be explained by the fact that pre-implantation blastocysts are being negatively affected or that the process of implantation is altered, or that a combination of both mechanisms is occurring. In rodents, uterine receptivity to embryos is modulated by ovarian estrogen and progesterone.[28] Previous study reported that 2, 4-D could disrupt the length of hormone-dependent reproductive cycles in female rats.[29] Luteinizing hormone controls progesterone release, and estrogen not only prepares the uterine endometrium but also activates blastocysts for implantation.[30] Additionally, copulation in rodents produces surges of prolactin from the pituitary gland, which stimulate the production of uterotrophic progesterone.[31] Previous study supposes that some sort of endocrine modulation is mediating the effects of the herbicide on litter size.[24] The present study showed clearly that 2,4-D exposure to pregnant rats during the period of organogenesis revealed marked teratological changes in treated fetuses. The malformed offspring in the uterine harms of treated animals displayed a form of edema, lack of tail, hypotrophy and severe subdermal haemorrhage patches compared with the control. Similarly, previous studies showed that exposure to 2,4-D increased the incidence of malformed fetuses and induced skeletal and urogenital malformations in rat fetuses.[9, 32, 33] These findings could be explained by the reduction in the uterine vascularization, which induced less blood flow to the implantation sites, consequently inducing fetal- placental growth retardation.[32] It was reported that acute interruption of blood flow to the uterine horn was shown to be teratogenic in the rat embryo on gestational days 8–10.[34] On the other hand, studies reported that interrupted uterine blood flow caused growth retardation and fetal mortality, but not somatic malformations.[32] Even more interesting, at these stages when the skeleton could be observed, was the presence of many abnormalities with incomplete or absent ossification of sacral vertebrae, which demonstrated that 2,4-D is responsible for the observed vertebral anomalies. Our results were in accordance with other studies carried out in pregnant rats and have demonstrated that the administration of 2,4-D (100 mg/kg), from gestation days 1 to 19, significantly increased the incidence of morphological and skeletal defects in fetuses from treated groups.[35, 36] The higher incidence of skeletal abnormalities could be caused by the generation of reactive intermediates which was related to 2,4-D toxicity. Previous study showed that indicators of oxidative stress were increased and antioxidant enzyme levels were reduced in the hemolysate and bone homogenates from offspring from rats exposed to 2,4-D.[37] In yet another study, exposure of rats to 100 mg of 2,4-D/kg, from gestation days 1 to 19, resulted in increased levels of malondialdehyde and reduced levels of antioxidant enzymes in the liver of dams and fetuses.[36] However, fetal growth is greatly associated with placental development. The placenta is a highly specialized tissue supported by several cell systems involved in both its structure and function in fetal-maternal exchange that suggests a role in regulation of fetal growth during pregnancy. In the present study, we observed that 2,4-D caused histological alterations in placenta, such as thickness of decidua basalis, atrophy of decidual cells, a degeneration of chorionic villi and hypertrophy of blood lacuna. Therefore, the retention of 2,4-D in placenta in the treated group have impaired placental physiology resulting in embryo and fetotoxic effects. Zahm *et al.*[38] found a trace of 2,4-D in the milk of lactating animals for 6 days following exposure to pregnant rats and about 20% of dose was detected in uterus, placenta, fetus and amniotic fluid. Thus, 2, 4-D passes through the placenta in rats which easily transferred to the embryo and fetus and may directly affected the embryonic structures. The toxicity of 2, 4-D and other related compounds is attributed to the free acid form of the chemicals[2] and may be mediated by effects associated with the plasma membrane, interference in cellular metabolic pathways involving acetyl coenzyme A, or uncoupling of oxidative phosphorylation,[8] this might explain the greater degree of toxicity of 2,4- D. The present study indicates that orally administration of 2,4-D by intra-gastric gavage during the period of organogenesis may cause a risk to the developing embryo and fetus. However, to elucidate his pathway of action require further studies.

ACKNOWLEDGEMENTS

The authors thank B Azib for his excellent technical assistance. This work is supported by a grant from the Tunisian Ministry of Higher Education and Scientific Research.

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