Rev. Int. Contam. Ambient. 5, 49-58, 1989.

# MUTAGENIC AND TERATOGENIC EFFECTS OF DIAZINON

Mario A. ALTAMIRANO-LOZANO, Ma. del Carmen CAMACHO-MANZANILLA, Raymundo LOYOLA-ÁLVAREZ and Elia ROLDAN-REYES

Laboratorio de Citogenética y Mutagénesis, Escuela Nacional de Estudios Profesionales-Zaragoza, Campo II, UNAM. Apdo. Postal 9-020, México 15000 D.F.
(Recibido octubre 1989, aceptado diciembre 1989)

## ABSTRACT

Cytogenetic effects of the diazinon were analyzed in bone marrow cells of mice injected intraperitoneally (i.p.). Five different endpoints were examined: sister chromatid exchanges (SCE's), chromosomal aberrations (CA), mitotic index (MI), cell cycle kinetics, and average generation time (AGT). Results show a significative increased in both, SCE's and CA. Diazinon at 43 and 54 mg/Kg produce a significative reduction in MI, a increase in first-cycle metaphase and decrease in second- and third cycle metaphases, while the AGT were significantly increased.

For the analysis of teratogenic effects, diazinon was administered i.p. to pregnant rats. Data shows no effects in the total number of implants at two dose levels tested, however a dose-related increase of resorptions, and significant fetal body weight depression was observed in all doses. Examination of the fetuses revealed significant incidences of some gross and skeletal alterations. These results indicates that diazinon may be considerated as potentially mutagenic, embryotoxic, and teratogenic *in vivo*.

## RESUMEN

Los efectos citogenéticos del diazinón fueron analizados en las células de la médula ósea de ratones tratados por vía intraperitoneal (i.p.). Para este hecho se evaluaron cinco parámetros: intercambio de cromátidas hermanas (ICH), aberraciones cromosómicas (AC), índice mitótico (IM), cinética de división celular y tiempo de generación promedio (TGP). En los resultados se notó aumento significativo en las frecuencias de ICH y de AC. El diazinón en las dosis de 43 y 54 mg/Kg produjo reducción en el IM, incremento en las células en primer ciclo y disminución en las células de segundo y tercer ciclos, mientras que el TGP fue significativamente elevado.

Para el análisis de los efectos teratogénicos del diazinón, este fue administrado i.p. a ratas preñadas en concentraciones de 25 y 50 mg/Kg en los días 9 y 11 de preñez. Los datos mostraron que no hay efecto en el número de implantes, sin embargo, en todas las dosis se observó incremento en la frecuencia de reabsorciones y disminución significativa de! ,)eso fetal. El análisis de los fetos evidenció aumento significativo de algunas alteraciones macroscópicas y esqueléticas. Los resultados indicaron que el diazinón puede ser considerado como un agente potencialmente mutagénico, embriotóxico y teratogénico *in vivo*.

## INTRODUCTION

Of the some 1500 chemicals used in agriculture, pesticides are the most important. They are known to be potentially dangerous to the environment, nature, and for the human beings (Grant 1982). Pesticides are chemical sustances used to control any type of unwanted organisms. Consequently the term include a wide variety of chemicals, applied in many places, however they are an integral and necessary component of the technological world in which we live.

Organophosphorous (OP) insecticides represent a major class of pesticides in use today. They exhibit less persistence than the organochlorine pesticides and exhibit greater toxicity to mammals because they are cholinesterase inhibitors (Menn *et al.* 1976, López *et al.* 1986).

The mutagenic and teratogenic potentials of anticholinesterase organophosphate insecticides have been investigated extensively (Wild 1975, Fishbein 1976, Nishio and Uyeki 1981). Some of the effects in man include hereditable genetic diseases, cancer, reproductive dysfunctions, and birth defects (Kushaba-Rugaaju and Kitos 1985, Waters *et al.* 1982).

Diazinon (O, O- diethyl- O-[2- isopropyl- 6- methyl- 4-pirimidinyl]phosphorothioate is a widely used OP insecticide, both in agricultural and the home (Mucke *et al.* 1970, Leidy *et al.* 1982, 1984, Branham and Wehner 1985), however the results of cytogenetic, mutagenic and teratogenic tests using this pesticide were equivocal and controversial (Tsoneba-Maneya *et al.* 1969, Wild 1975, Fishbein 1976, Matsouka and Hayahi 1979, Chen *et al.* 1981, Kushaba-Rugaaju and Kitos 1985, López *et al.* 1986). It is for this reason, in the present study, the ability of a diazinon to produce embryotoxic and teratogenic responses, and its ability to induce chromosomal aberrations (CA) and sister chromatid exchanges (SCE's) has been investigated.

## MATERIAL AND METHODS

### *Chemical*

Diazinon, a commercial formulation manufactured by Labs. Helios, México, containing 235 g of active ingredient/l was obtained from a local market and used in all experiments.

*Cytogenetic studies*

Animals

Male CD-1 mice (2-3 months old, 25-30 g) were housed in groups (experimental and control) of 4 (according with Latt *et a.* 981) in hanging plastic cages under controlled conditions. They were fed with a rat pellets and water *ad libitum*.

Treatments

The working solutions of diazinon were prepared in physiological saline solution (PSS) (0.95% NaCl) and immediately injected intraperitoneally (i.p.) in appropriate volumes containing 32, 43 or 54 mg of Diazinon/Kg body weight (in our laboratory the i.p. $LD_{50}$ was 108 mg/Kg body weight). The control group received the largest volume of PSS.

Collection of bone marrow cells and staining procedure

Twenty-two hours after treatment with diazinon and a single i.p. injection of Bromodeoxyuridine (BrdU, Sigma) adsorbed to activated charcoal (Morales 1980), the animals were then given cochicine (Merck, 1.5 $\mu$g/g body weight, i.p.). Two hours later, they were killed by cervical dislocation, and the bone marrow cells from the femurs were flushed with 5 ml of 0.075 M KCl prewarmed at 37 °C. The cells were fixed in methanol-acetic acid (3:1), aliquoted onto chilled slides, and air dried at least 24 hours.

On the following day, the slides were immersed in 0.075 M KCl solution and exposed to UV light, at a distance of 10 cm, for 20 min, incubated for 20 min at 60 °C in 2XSSC, and 5 min in 5 N HCl. The slides were then washed with distilled water and stained in Giemsa solution (1:30 in tap water) for 10 min.

For each animal 100 metaphases in first division were analysed for structural aberrations (the types of aberrations scored included chromatid-type aberrations such as chromatid breaks, and chromatid interchanges, and chromosome-type aberrations such as breaks and rings). One hundred consecutive metaphases for cell cycle kinetics, 100 cells in second division were studied for evidence of SCE's and 1000 cells counted to estimate the mitotic index (MI). Cell cycle data were analysed by the method of Ivett and Tice (1982) for estimated average generational time (AGT).

*Teratological studies*

Animals

Sexually mature (250-300 g) virgin, female CII-ZV rats were housed in hanging plastic cages under controlled conditions and with a 8/16 light/dark cycle. They were fed with a rat pellets and water *ad libitum*.

Following an acclimation period, the female rats were paired (1:1) with mature males of the same strain overnight (6 p.m. - 8 a.m.). Successful copulation was assumed to have ocurred if a copulatory plug and/or sperm was present at the end of the mating period (day 0 of gestation).

Treatments

Fifteen pregnant females were injected i.p. with a single dose of diazinon(prepared in PSS) at 25 or 50 mg/Kg body weight, on days 9 and 11 of gestation. Ten control animals were treated with PSS only.

On day 19 of pregnancy the dams were sacrificed and the uterine horns were exposed and the number of implants, resorptions and live fetuses were counted. Live fetuses were removed from uterus, blotted dry, weighted and examined for external malformations. A 2/3 of fetuses from each litter were fixed in 70 % ethanol, cleared and stained with Alizarin Red S and examined for eskeletal defects. The remaining fetuses were fixed in Bouin's fluid for subsequent visceral examination by razor-blade sectioning as described by Wilson (1965).

## RESULTS AND DISCUSSION

*Cytogenetics*

Conventional chromosome analysis of methaphase spreads were done to detect the presence of chromosome aberrations and SCE's. Results show a significative increased in CA in relation to the dose, also SCE's increased were dose-related (Table I).

The structural element common to all the OP show two electrophilic sites, P and C, with permit attachment to nucleophiles. These latter can bind to either P sites and became phosphorylated, or C site, were they became alkylated (López *et al.* 1986).

Most OP are chemical alkylating agents which may lead to a mutagenic potential by binding to DNA regions rich in GC pairs causing these to become unstable (Lawley and Brooks 1963, Kaur and Grover 1985a). Wild (1975) report that all methyl esters tested were mutagenic in microorganisms and the ethyl esters such parathion and diazinon were nonmutagenic. In cell cultures diazinon does not increase the percentage of structural CA and SCE's (Chen *et al.* 1981, Nishio and Uyeki 1981, López *et al.* 1986), however diazinon in barley increased the frequency of structural and numerical aberrations in root meristems and pollen mother cells (Kaur and Grover 1985a, b). Matsouka and Hayahi (1979) found that diazinon causes a high rate of chromosomal aberrations in cultured Chinese hamster cells only after it was metabolically actived with S9 mix. Our results confirm the mutagenic activity of diazinon *in vivo*.

The method of BrdU-labeling for SCE's analysis can be used simultaneously for

TABLE I. SISTER CHROMATID EXCHANGES (SCE), CHROMOSOMAL ABERRATIONS (CA), CELL CYCLE KINETICS, AND AVERAGE GENERATION TIME (AGT) INDUCED BY DIAZINON IN BONE MARROW CELL OF MALE CD–1 MICE

| *TREATMENT (mg/kg)* | *SCE/CELL ± S.E.*** | *CA* (%)**** | *MI (%)***** | *CELL CYCLE (%)* | | | *AGT (Hrs)* |
|---|---|---|---|---|---|---|---|
| | | | | *M1* | *M2* | *M3* | |
| CONTROL | 2.94 ± 0.17 | 0 | 5.0 | 24 | 72 | 4 | 13.3 |
| 32 | 3.21 ± 0.37 | 3 | 4.6 | 63 | 35 | 2 | 17.2(b) |
| 43 | 4.18 ± 0.30(a) | 5(a) | 3.2(a) | 73 | 27 | 0 | 18.9(b) |
| 54 | 7.66 ± 0.63(a) | 12(b) | 1.0(b) | 84 | 16 | 0 | 20.7(b) |

* only chromatid breaks were observed. Statistical analysis with: ** = "t" test; *** = Chi-square test; **** = "z" test; a = $P < 0.05$; b = $P < 0.001$

investigation of cellular kinetics with great sensitivity (Tice *et al.* 1976, Beek and Obe 1979). Cell cycle delay, in addition to the production of SCE's represent an other level of biological damage incurred due to the treatment of an OP (Chen *et al.* 1981).

Data shows that diazinon at 43 and 54 mg/Kg produce a significant reduction in MI with respect to control, and in all treatments, a increase in first-cycle metaphases and decrease in second and third-cycle metaphases was observed. These kinetics changes are reflected in the AGT duration (13.3 h of control vs 17.2, 18.9 and 20.7 h for 32, 43 and 54 mg/Kg respectively) (Table I).

Diazinon exerts a definite cytotoxicity action on bone marrow cells, as may be seen from the decrease observed in the MI. This cytotoxic action observed is common to all OP pesticides (Chen *et al.* 1981).

Our results show a relationship between cell cycle delays, SCE's induction and cytotoxicity, which agrees with those reported by Craig-Holmes and Shaw (1977) and Chen *et al.* (1981). Their studies show the effects of many chemicals on SCE's and cell kinetics in mammals cells *in vitro*, and found that some of these compounds produce significant changes in both cell kinetics and SCE's frequencies.

*Teratogenic*

The potential for adverse effects on man is determined by both the inherent toxic properties of the pesticides and the extent of exposure to the pesticide (Severn 1982).

Interest in the possible teratogenic potential of OP insecticides in mammals was investigated and reports show that these chemicals caused nervous system lesions as well skeletal abnormalities (Meiniel *et al.* 1970).

TABLE II. EFFECTS OF DIAZINON ON IMPLANTATION, RESORPTION, LIVE FETUSES, AND FETAL BODY WEIGHTS ON DAY 9 AND 11 OF GESTATION

| | | *No. Implants* | | *Resorptions* | | *Live Fetuses* | | *Fetal Body WT* |
|---|---|---|---|---|---|---|---|---|
| *Treatment (mg/Kg)* | *No. of Litters* | *Total/ Group* | *Average/ Litter* | *Total/ Group* | *% Implants* | *Total/ Group* | *% Implants* | *Average/ Group* |
| 0 | 10 | 108 | 10.8 ± 0.7 | 3 | 2.8 | 105 | 97.2 | 2.0±0.06 |
| 25 | 15 | 178 | 11.9 ± 1.2 | 14 | 9.0* | 164 | 91.0* | 1.5±0.07* |
| 50 | 15 | 138 | 9.2 ± 1.4 | 18 | 13.1** | 120 | 86.9** | 1.5±0.03 |

Statistical analysis with Chi-square test: * = $P < 0.05$; ** = $P < 0.001$

The effects of diazinon on total number of implants, resorptions, live fetuses and fetal body weights are summarized in Table II. There was no significant effect on total number of implants at two dose levels tested. The incidence of resorption was dependent on dosage (2.8 % for the control vs 9 % at 25 mg/Kg, and 13.1 % at 50 mg/Kg). Significant fetal body weight depression occurred in all doses.

Diazinon treatment induced significant incidences of some gross and skeletal malformations in the rat fetuses. The most frequent anomalies observed were umbilical hernia, haematoma, and limb anomalies (Table III). Skeletal defects were observed in almost all of the fetuses of rats treated (6.7 % for the control; 100 % at 25 and 50 mg/Kg). The insecticide induced anomalies of the skull, cervical archs, vertebral column, and absence of ossification centres in fore limbs, hind limbs, and sternebrae (Tables IV and V).

Several OP pesticides are now recognized as being teratogenic, in particular to avian embryos. Khera (1966) found that injection of diazinon at any stage of embryonic development caused alterations in the formation of cartilaginous and osseous skeleton, eye cataracts, ascites, and hepatic degeneration in both ducks and chicks. Skeletal defects observed were dwarfism, micromelia, ectrosyndactyly, stunted growht of cervical vertebrae, and irregular beak growth.

Studies with malathion and diazinon in the Wistar rat revealed that diazinon (63.8 mg/Kg) administered on day 9 of the gestation period produced morphological malformations manifested primarily as hydronephrosis, hydroureter,high incidence of fetal resorptions and a reduction in weight of the fetus and placenta (Dobbins 1967). However, the malformations were mild, inconsistent, and apparently not dose related.

Our results indicates that diazinon is embryotoxic and teratogenic in the rat when injected in a single dose (25 or 50 mg/Kg) on gestation days 9 and 11. The presence of resorptions, decrease in fetal body weight, increase in gross malformations and skeletal anomalies confirm this conclusion.

TABLE III. TYPES AND FREQUENCY OF GROSS ANOMALIES IN FETUSES OF RATS EXPOSED i.p. TO DIAZINON ON DAY 9 AND 11 OF GESTATION

| | *TREATMENT (mg/Kg)* | | |
|---|---|---|---|
| *OBSERVATIONS* | *0* | *25* | *50* |
| TOTAL OF FETUSES EXAMINED | 105 | 164 | 120 |
| (%) | | | |
| UMBILICAL HERNIA | 0.0 | 1.8* | 4.9** |
| HAEMATOMAS | 1.0 | 2.5* | 10.7** |
| OLIGODACTYLY | 1.0 | 0.0 | 0.9 |
| CLEF PALATE | 0.0 | 0.0 | 0.9 |
| LIMB ANOMALIES | 0.0 | 1.8* | 0.0 |

Statistical analysis with Chi-square test: * = $P < 0.05$; ** = $P < 0.001$

TABLE IV. INCIDENCE OF SKELETAL VARIATIONS IN FETUSES OF RATS TREATED i.p. WITH DIAZINON ON DAY 9 AND 11 OF GESTATION

| | *TREATMENT (mg/Kg)* | | |
|---|---|---|---|
| *OBSERVATIONS* | *0* | *25* | *50* |
| TOTAL OF FETUSES EXAMINED | 68 | 110 | 83 |
| % OF FETUSES WITH ANY SKELETAL ABNOMALITIES | 6.7 | 100.0* | 100.0* |
| % OF FETUSES WITH REDUCED OSSIFICATION** | | | |
| SKUL BONES | 6.7 | 100.0* | 97.5* |
| CERVICAL ARCHS | 1.8 | 100.0* | 100.0* |
| VERTEBRAL COLUMN | 1.8 | 100.0* | 100.0* |

Statistical analysis with Chi-square test: * = $P < 0.001$, ** = Reduced ossification = combination of incompletely ossified and poorly ossified categories

TABLE V. ALTERATIONS IN THE NUMBER OF OSSIFICATION POINTS PRESENT IN FETUSES OF RATS TREATED i.p. WITH DIAZINON ON DAY 9 AND 11 OF GESTATION

| | *TREATMENT (mg/Kg)* | | |
|---|---|---|---|
| *OBSERVATIONS* | *0* | *25* | *50* |
| TOTAL OF FETUSES EXAMINED | 68 | 110 | 83 |
| $\bar{X}$ ± S.E. OF OSSIFICATION POINTS PRESENT IN: | | | |
| FORE LIMBS phalanges | 5.5 ± 0.14 | 0.0* | 0.4 ± 0.16* |
| HINDLIMBS metatarsals | 7.1 ± 0.16 | 0.0* | 0.0* |
| STERNEBRAE | 3.5 ± 0.28 | 0.0* | 0.0* |

Statistical analysis with Student "t" test: * =P < 0.001

Kushaba-Rugaaju and Kitos (1985) found that application of diazinon by the intravitelline route to chicken eggs at day 3 of incubation, decreased the amounts of the purine and pyrimidine ribonucleotides, and the levels of the tryptophan and histidine were also decreased, while the levels of threonine and aspartic acid were increased.

Tryptophan, the least abundant of all the amino acids, is unique in its ability to protect against the OP insecticide-induced malformations. It plays as least two major roles in the developing embryo: its essential raw material for protein synthesis and pyrimidine nucleotide synthesis (Kushaba-Rugaaju and Kitos 1985). It is possible that these effect *in vivo*, may be responsible for the diazinon-induced teratogenesis and decreased fetal body weight.

Etiological similarities of carcinogenesis, mutagenesis, and teratogenesis can be substainted only to a limited extent. Except for a few agents which are known to be active in mammals in three areas, the common causation agent cannot easily be supported (Fishbein 1976).

## REFERENCES

Beek. B. and Obe G. (1979). Sister chromatid exchanges in human leukocyte chromosomes: spontaneous and induced frequencies in early- and late-proliferating cells *in vitro*. Human Genet. *49*, 51-61.

Branham B. E. and Wehner D. J. (1985). The fate of diazinon applied to thatched turf. Agron. J. *77*, 101-104.

Chen H. H., Hsueh J. L., Sirianni S. R. and Huang C. C. (1981). Induction of sister-chromatid exchanges and cell cycle delay in cultured mammalian cells treated with eight organophosphorus pesticides. Mutat. Res. *88*, 307-316.

Craig-Holmes A. P. and Shaw M. W. (1977). Effects of six carcinogens on SCE frequency and cell kinetics in cultured human lymphocytes. Mutat. Res. *46*, 375-384.

Dobbins P. K. (1967). Organic phosphate insecticides as teratogens in the rat. J. Fla. Med. Assoc. *54*, 452-456.

Fishbein L. (1976). Teratogenic, mutagenic, and carcinogenic effects of insecticides. In: *Insecticides biochemistry and physiology*. (C. F. Wilkinson, Ed.). Plenum Press, New York, pp. 555-603.

Grant W. F. (1982). Cytogenetic studies of agricultural chemicals in plants. In: *Genetic toxicology: an agricultural perspective* (R. A. Fleck and A. Hollaender, Eds.). Plenum Press, New York, pp. 353-378.

Ivett J. L. and Tice R. R. (1982). Average generation time: a new method of analysis and quantitation of cellular proliferating kinetics. Environ. Mutagen. *4*, 358.

Kaur P. and Grover I. S. (1985a). Cytological effects of some organophosphorus pesticides. I. Mitotic effects. Cytologia *50*, 187-197.

Kaur P. and Grover I. S. (1985b). Cytological effects of some organophosphorus pesticides. II. Meiotic effects. Cytologia *50*, 199-211.

Khera K. S. (1966). Toxic and teratogenic effects of insecticides in duck and chick embryos. Toxicol. Appl. Pharmacol. *8*, 345.

Kushaba-Rugaaju S. and Kitos P. A. (1985). Effects of diazinon on nucleotide and amino acid contents of chick embryos. Biochem. Pharmacol. *34*, 1937-1943.

Latt S. A., Allen J., Bloom S. E., Carrano A., Falke E., Kram D., Schneider E., Schreck R., Tice R., Whitfield B. and Wolff S. (1981). Sister-chromatid exchanges: a report of the gene-tox program. Mutat Res. *87*, 17-62.

Lawley P. D. and Brooks P. (1963). Further studies on the alkylation of nucleic acids and their constituent nucleotides. Biochem J. *89*, 137-138.

Leidy R. B., Wright C. G. and Dupree H. E. Jr. (1982). Concentration and movement of diazinon in air. J. Environ. Sci. Health *B17*, 311-319.

Leidy R. B., Wrigt C. G., MacLeod K. E. and Dupree H. E. Jr. (1984). Concentration and movement of diazinon in air. II. Vertical distribution in rooms. J. Environ. Sci. Health *B19*, 747-757.

López D., Alcixandre C., Merchan M. and Carrascal E. (1986). *In vitro* induction of alterations in peripheral blood lymphocytes by different doses of diazinon. Bull. Environ. Contam. Toxicol. *37*, 517-522.

Matsouka A. and Hayahi M. Jr. (1979). Chromosomal aberration test on 29 chemical combined with S9 mix *in vitro*. Mutat. Res. *66*, 277-290.

Meiniel R., Lutz-Ostertag Y. and Lutz H. (1970). Effets teratogenes du parathion sur le squelette embryonnarie de la cailler Japonaise (*Coturnix japonica*). Arch. Anat. Microsc. Morphol. Exp. *59*, 167-183.

Menn J. J., DeBraun J. R. and McBarn J. B. (1976). Recent advances in the metabolism of organophosphorus insecticides. Fed. Proc. *35*, 2598-2602.

Morales-Ramírez P. (1980). Analysis *in vivo* of sister chromatid exchange in mouse bone marrow and salivary gland cells. Mutat. Res. *74*, 61-69.

Mucke W., Alt K. O. and Esser H. O. (1970). Degradation of $^{14}$C-labeled diazinon in the rat. J. Agr. Food Chem. *18*, 208-212.

Severn D. J. (1982). Exposure assesment for agricultural chemicals. In: *Genetic toxicology: an agricultural perspective* (R. A. Fleck and A. Hollaender, Eds.). Plenum Press, New York, pp. 235-242.

Nishio A. and Uyeki E. M. (1981). Induction of sister chromatid exchanges in Chinese hamster ovary cells by organophosphate insecticides and their oxygen-analoges. J. Toxicol. Environ. Health *8*, 939-946.

Tice R. R., Schneider E. L. and Rary J. M. (1976). The utilization of bromodeoxyuridine incorporation into DNA for the analysis of cellular kinetics. Exp. Cell Res. *102*, 232-236.

Tsoneba-Maneya M. E., Kaloyanova E. and Georgieva V. (1969). Mutagenic effect of diazinon on human peripheral blood lymphocytes *in vitro*. Exp. Med. Morphol. *8*, 133-150.

Waters M.. D., Sandhu S. S., Simmon V. F., Mortelmans K. E., Mitchell A. D., Jorgenson T. A., Jones D. C. L., Valencia R. and Garret N. E. (1982). Study of pesticides genotoxicity. In: *Genetic toxicology: an agricultural perspective* (R. A. Fleck and A. Hollaender, Eds.). Plenum Press, New York, pp. 275-326.

Wild D. (1975). Mutagenicity studies on organophosphorus insecticides. Mutat. Res. *32*, 133-150.

Wilson J. G. (1965). Methods for administering agents and detecting malformations in experimental animals. In: *Teratology-principles and techniques* (J. G. Wilson and J. Warkany, Eds.). Univ. of Chicago Press, pp. 262-277.