Estimation of DNA Fragmentation, Mutagenicity and Biochemical Changes in Mice Exposed to Diazinon and Its Commercial Formulation

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Abstract— *The aim of the present study was to determine the effect of organophosphorus (OP) insecticide diazinon (DZN) and its formulation (DZNF) in bone marrow and germ cells, DNA fragmentation and biochemical changes induced in Swiss albino male mice. The mice were randomly divided into 7 groups (10 mice each), the 1st group served as control, the 2nd, 3rd and 4th groups, as well, 5th, 6th, and 7th groups are treated with 1/10LD50 (6.5 mg/kg bw), 1/20 LD50 (13mg/kg bw) and 1/40 LD50 (26mg/kg bw) of DZNF and DZN i.p with single dose/week for 4 weeks, respectively. At the end of treatment all animals were sacrificed by cervical dislocation after 24 h of the last treatment. Bone marrow and spermatocyte cells were subjected to chromosomal analysis. As well, liver and brain tissues were collected from all animals for DNA fragmentation and biochemical analyses. Cytogenetic analysis revealeda significant increase (more than 2 folds) in structural aberrations (Chromatid and chromosomal gaps, breaks, deletions, centromeric attenuation and end to end)as well as numerical variations in DZNF treated groups than DZN treated groups in a dose dependent manner. A significant increase (about 3 folds) also was found in DZNF treated groups in structural and numerical aberrations of spermatocyte cells than DZN treated mice in a dose dependent manner. Levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), malondialdehyde (MDA) and DNA fragmentation were significantly increased (about 2 fold) in mice exposed to DZNF than DZN exposed groups. In conclusion, our findings demonstrate that DZNF is more genotoxic than DZN as assessed by cytogenetic analysis of both somatic and germ cells of mice and had adverse effects on DNA and biochemical parameters. These results suggested that the effects of DZNF and DZN are dose dependent and the treatment with DZNF is more hazardous and toxic than DZN and it is important to avoid toxicities induced by organophosphate insecticides, take a high level of caution and minimize its agricultural and household uses.*

Keywords— Diazinon, formulation, mutagenicity, DNA fragmentation, biochemical changes, mice.

I. INTRODUCTION

Diazinon (DZN) (*O*,*O*-Diethyl*O*-[4-methyl-6-(propan-2-yl)pyrimidin-2-yl] phosphorothioate) is an organophosphate (OP) insecticide which is the most commonly used to control cockroaches, silverfish, ants, and fleas. It has been extensively used in agriculture (including fruits, vegetables and nut trees) and horticulture for controlling insects in crops all over the world [1]. Commercial diazinon formulations (DZNF)are often more toxic than the pure pesticide compound, as they contain surface active ingredients, dyes, stabilizers, activity enhancers, and organic solvents with unknown or poorly characterized toxicity, which raises concern about the current assessment of genotoxicity induced by this pesticide and calls for a high level of caution in agricultural and household uses [2]. It had been used extensively in home and garden applications, in commercial formulations designed to prevent such pests as crickets or cockroaches from infesting homes or offices, and in pet collars. Residential application methods included aerosol cans, spray equipment, and granular spreaders. Due to the emerging health and ecological risks posed by diazinon, manufacturers agreed to phase out and cancel all residential products, so its use is minimized (USEPA 2006) [3].

It has been reported that DZN insecticide have negative effects on different tissues and organs such as the liver, brain, cardiac, kidney, pancreas, immune system, reproductive system, and vascular walls and can induce liver toxicity, neurotoxicity, cardiotoxicity, genotoxicity or cytotoxicity, and apoptosis. Various biochemical and hematological adverse changes in the body can be induced by OP compounds [4]. Moreover, different studies have shown that DZN could induce oxidative damage by increasing the formation of reactive oxygen species (ROS), depletion of the antioxidant enzymes, protein and lipid peroxidation (LPO) and DNA fragmentation in the cells [4,5].

The clastogenic and aneugenic potential of this pesticide was reported, where a significant percentage of chromosome aberrations in bone-marrow cells of the mouse was found [6,7]. Genotoxic effects are considered among the most serious side effects of diazinon which found to increased significantly ($P \leq 0.05$) the level of chromosomal aberrations (7.5 ± 1.04) in albino male rats including gap chromosomes (10%), break chromosomes (7%), fragment chromosomes (5%) and deletion chromosomes (8%) [8]. DZNF also induced increases in the frequency of micronucleated (MN) cells and DNA damage in human peripheral blood lymphocytes [2].

ALT (alanine aminotransferase) and AST (aspartate aminotransferase) are important indicators of liver damage in clinic finding. These enzymes were secreted to blood in hepatocellular injury and their levels increased [9]. A significant increase in ALT and AST were detected from the 2nd to 4thweek in the DZN - exposed rats [10]. The increased ALT and AST values were also found by Kalender *et al.*, [9], who stated that changes in these enzymes level might differ depending on exposure time and dose in adult male Wistar rats treated orally via gavage for 7 weeks. Organophosphorus insecticides treatment caused an increase in the activities of ALT and AST enzymes in the serum of male and female rats [11, 12]. They concluded that the increase in these enzymes may be due to liver dysfunction and disturbance in the biosynthesis of these enzymes with alteration in the permeability of liver membrane. As well a significant increase of malondialdehyde (MDA) levels in the liver associated with a decrease in antioxidant enzyme was found [11].

Therefore, the present study is designed to assess the effect of DZN and one of its formulations on DNA fragmentation, chromosomal aberrations in both somatic and germ cells and biochemical changes in Swiss albino male mice (*Mus musculus*).

II. MATERIALS AND METHODS

2.1 Chemicals

Diazinon insecticide was obtained from ADWIA 60 EC (Emulsifiable concentrate), Cairo, Egypt. A commercial formulation (DZNF) obtained from (Basudin 60EM®, Syngenta, Basel, Switzerland) containing 630 g of DZN per liter of the product. It was diluted in deionized water for final concentration. Malondialdehyde (MDA); phosphate-buffered saline (PBS), thiobarbituric acid (TBA) were purchased from Sigma chemical company (Sigma, St. Louis, MO, USA). Alanine transaminase (ALT), aspartate transaminase (AST) kits were purchased from Biorexfars, UK.

2.2 Animals

Adult Swiss albino male mice (*Mus musculus*) were purchased from the laboratory animal colony of the National Research Center, Dokki, Giza, Egypt. They were housed in appropriate conditions, and acclimatized for two weeks prior to initiation of the study. All mice were caged, and allowed free access to food and water. The mice were randomly divided into 7 groups (10 mice each) and were treated by oral gavage, once a week for four weeks as follow: 1) control group received corn oil, orally; 2) 1/10 LD_{50} (6.5 mg/kg bw) of DZN-treated group; 3) 1/20 LD_{50} (13 mg/kg bw) of DZN-treated group; 4) 1/4 LD_{50} (26mg/kg bw) of DZN-treated group; 5) 1/10 LD_{50} (6.5 mg/kg bw) of DZNF- treated group; 6) 1/20 LD_{50} (13 mg/kg bw) of DZNF-treated group; 7) 1/40 LD_{50}(26 mg/kg bw) of DZNF-treated group. The LD50 were selected according to Koltzsche [13], and El-Shenawy *et al.* [14]. At the end of treatment, all animals were sacrificed by cervical dislocation after 24 h of the last injection. Bone marrow and testis were subjected to chromosomal analysis (5 animals/each). As well, blood samples and liver and brain tissues were collected from all animals for DNA fragmentation, MDA and biochemical analyses.

2.3 Chromosomal analysis

2.3.1 Chromosomal analysis in somatic cells

Mice were subjected to cytogenetic analysis from bone marrow cells using the method of Preston *et al*. [15]. Briefly, mice were treated intraperitoneally (I/P) with Colchicine (0.05 mg/kg) for two and a half hours before sacrifice. Animals were sacrificed and femoral bone marrow cells were flushed with isotonic solution (0.9% NaCl). Hypotonic solution (0.56% KCl) was added to the cell pellet and incubated at 37°C for 30 minutes the solution was fixed, slides were air dried and stained with 10% Giemsa stain for 20 minutes. 50 metaphases were studied per animal scoring different types of chromosomal aberrations (structural and numerical aberrations).

2.3.2 Chromosomal analysis in germ cells

Spermatocyte cells were prepared according to Russo [16]. Briefly, chromosomes were spread on clean glass slides by the gradual fixation/air-drying method. The preparations were stained with 2% Giemsa (Merck, Darmstadt, Germany) in PBS (pH 6.8) for 10 min for conventional chromosome analysis. Aberrations are scored in metaphase chromosomes of dividing cells. Fifty metaphase spreads per animal were analyzed for studying the chromosome aberrations.

2.4 Blood and tissue sampling

After 24 hr of the last dose of administration, animals were euthanized and their blood was obtained. Serum was separated and used for biochemical experiments. Liver and brain tissues were dissected quickly and washed with cold saline and were homogenized (1:10 w/v) in phosphate buffered saline (PBS) (50 mM sodium phosphate buffer, pH=7.4). Homogenate tissues were centrifuged at 10,000 rpm, for 15 min at 4°C, and supernatants were used for determination of MDA (lipid peroxidation indicator).

2.5 Biochemical assays and analysis

Enzyme activities of ALT, AST were assessed in the blood serum described in section 2.4, using kits and were expressed as international units per liter (IU/L).

2.6 DNA fragmentation analysis

To evaluate genotoxicity induced by DZN and its commercial formulation (DZNF), DNA fragmentation was assessed in combined liver and brain samples using spectrophotometer (Farag *et al.*, 2021) [17]

2.7 Determination of lipid peroxidation (LPO) in tissues

Malondialdehyde (MDA), as the main marker of lipid peroxidation, was measured in the brain and liver tissues. Levels of MDA were measured according to the method of Fernandez *et al.*[18], using the spectrophotometric measurement of color developed by reaction of MDA with thiobarbituric acid (TBA).

2.8 Statistical analysis

All values were expressed as mean±SE. Differences between groups were determined using one-way analysis of variance (ANOVA) and Tukey's as *post hoc* testing was performed for comparisons between groups. Values were regarded as significantly different at $P<0.05$.

III. RESULTS

The novelty of our study are the DZNF effects on DNA fragmentation, chromosomal aberrations and biochemical alterations induced in exposed mice, because there were a few reports on this subject. In the present study, DZNF and DZN insecticide were investigated for their toxicity in exposed Swiss albino male mice by genetic (chromosomal analysis in both bone marrow and spermatocyte cells), DNA damage analysis (DNA fragmentation) and biochemical analysis (AST, ALT and MDA assays). Cytogenetic analysis (Table 1) of bone marrow cells revealed that a significant increase (more than 2 folds) instructural aberrations (Chromatid and chromosomal gaps, breaks, deletions, CA and end to end) and total structural aberrations (33.60 ± 0.87^{g}, 26.40 ± 0.74^{f} , 14.80 ± 0.37^{d} and17.20 ± 0.48^{f} , 11.20 ± 0.20^{c} , 7.80 ± 1.31^{b}, for 1/10 , 1/20 and 1/40 LD_{50} of DZNF and1/10 , 1/20 and 1/40 LD_{50} of DZN, respectively) as well in numerical (N±1) and total numerical variations (17.40 ± 0.67^{g}, 14.60 ± 0.60^{f} , 11.60 ± 0.50^{e} and 8.40 ± 0.67^{d} , 4.80 ± 0.58^{c} , 2.80 ± 0.37^{b} , for 1/10 , 1/20 and 1/40 LD_{50} of DZNF and1/10 , 1/20 and 1/40 LD_{50} of DZN, respectively) in DZNF treated groups than DZN treated groups in a dose dependent manner.

TABLE 1
MEAN FREQUENCY OF CHROMOSOMAL ABERRATIONS IN MICE BONE MARROW CELLS EXPOSED TO DZN AND ITS COMMERCIAL FORMULATION (DZNF)

Treatment	Structural aberrations							Numerical variations		Total numerical variations
	Chromatid gap	Chromosomal gap	break	deletion	CA	End to end	Total structural aberrations	Hypoploidy	Hyperploidy	
Control	0.40±0.24	0.20±0.20	0.00±0.00	0.20±0.20	0.60±0.24	0.00±0.00	1.40±0.74[a]	0.00±0.00	0.20±0.20	0.20±0.20[a]
DZN (1/10 LD_{50})	3.20±0.20	3.20±0.20	3.20±0.37	3.20±0.20	2.60±0.40	1.80±0.20	17.20±0.48[f]	4.40±0.24	4.00±0.44	8.40±0.67[d]
DZN (1/20 LD_{50})	2.00±0.00	2.00±0.00	2.00±0.00	1.80±0.20	1.60±0.24	1.80±0.20	11.20±0.20[c]	2.20±0.37	2.60±0.24	4.80±0.58[c]
DZN (1/40 LD_{50})	0.60±0.24	1.20±0.37	1.20±0.20	1.60±0.24	1.80±0.37	1.40±0.24	7.80±1.31[b]	1.20±0.20	1.60±0.24	2.80±0.37[b]
DZNF (1/10 LD_{50})	3.00±0.00	9.60±0.50	2.60±0.24	6.20±0.37	4.40±0.24	7.80±0.37	33.60±0.87[g]	8.20±0.48	9.20±0.37	17.40±0.67[g]
DZNF (1/20 LD_{50})	4.80±0.20	7.20±0.58	6.60±0.40	4.00±0.31	2.20±0.20	2.20±0.20	26.40±0.74[f]	5.40±0.24	9.20±0.37	14.60±0.60[f]
DZNF (1/40 LD_{50})	1.80±0.20	2.80±2.20	3.60±0.24	3.20±0.20	2.80±0.32	2.60±0.24	14.80±0.37[d]	5.20±0.20	6.40±0.40	11.60±0.50[e]

DZN: diazinon insecticide; DZNF: diazinon commercial formulation; CA: centromeric attenuation .
Values in the same column with different superscript letters are differing significantly (p<0. 05).

TABLE 2
MEAN PERCENTAGE OF CHROMOSOMAL ABERRATIONS IN SPERMATOCYTE CELLS OF MICE EXPOSED TO DZN AND ITS COMMERCIAL FORMULATION (DZNF).

Treatment	Number of examined cells	Structural aberrations								Total structural aberrations		Numerical variations				Total numerical variations	
		Chain		Ring		x-y univalent		Autosomal univalent				N-1		N+1			
		No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%
Control	250	1	0.4	1	0.4	0	0.0	0	0.0	2	0.8	1	0.4	0	0.0	1	0.4
DZN (1/10 LD_{50})	250	8	3.2	10	4	6	2.4	8	3.2	32	12.8	9	3.6	8	3.2	17	6.8
DZN (1/20 LD_{50})	250	7	2.8	6	2.4	3	1.2	3	1.2	19	7.6	6	2.4	5	2	11	4.4
DZN (1/40 LD_{50})	250	3	1.2	5	2.0	2	0.8	1	0.4	11	4.4	4	1.6	3	1.2	7	2.8
DZNF (1/10 LD_{50})	250	28	11.2	35	14.0	10	4.0	11	4.4	84	33.6	19	7.6	16	6.4	35	14.0
DZNF (1/20 LD_{50})	250	25	10.0	28	11.2	4	3.6	10	4.0	72	28.8	16	6.4	13	5.2	29	11.6
DZNF (1/40 LD_{50})	250	16	6.4	18	7.2	8	3.2	9	3.6	51	20.4	11	4.4	10	4	21	8.4

DZN: diazinon insecticide; DZNF: diazinon commercial formulation

Table (2) showed a significant increase (about 3 folds) in DZNF treated groups than DZN treated in structural aberrations (chain, ring, x-y and autosomal univalents), (33.6, 28.8, 20.4% and 12.8, 7.6, 4.4% for 1/10 , 1/20 and 1/40 LD_{50} of DZNF and1/10 , 1/20 and 1/40 LD_{50} of DZN, respectively) and numerical variations (N ±1) (14.0, 11.6, 8.4% and 6.8, 4.4, 2.8 % for 1/10 , 1/20 and 1/40 LD_{50} of DZNF and 1/10 , 1/20 and 1/40 LD_{50} of DZN, respectively) of spermatocyte cells in mice in a dose dependent manner.

In the present study DNA damage assay was evaluated in combined liver and brain samples by DNA fragmentation (Table 3). Administration of DZNF resulted in significant increase in DNA fragmentation (about 2 fold) in mice exposed to DZNF than DZN treated groups in a dose dependent manner, as well, a significant difference were found between all treated groups either by DZN or DZNF and the untreated control. For determination of liver and brain damage induced by DZN and DZNF treatment, the activity of the hepatic enzymes (ALT and AST) and brain damage biomarker MDA (lipid peroxidation indicator) were investigated. As shown in Table (3) regarding the level of MDA as lipid peroxidation indicator, and in Table (4) for serum AST and ALT levels, there was a significant increase (about 2 fold) in mice exposed to DZNF than DZN treated groups for these parameters. There was also a significant increase in ALT, AST and MDA in DZNF treated groups than DZN treated groups in a dose dependent manner.

TABLE 3
DNA FRAGMENTATION AND MDA LEVEL IN MICE TREATED WITH DZN AND ITS COMMERCIAL FORMULATION (DZNF).

Treatment	DNA Fragmentation	MDA	
		Liver	Brain
Control	7.85 ± 0.41^{a}	2.23 ± 0.34^{a}	4.02 ± 0.57^{a}
DZN (1/10 LD_{50})	22.24 ± 0.30^{d}	28.93 ± 0.48^{d}	34.83 ± 0.40^{d}
DZN (1/20 LD_{50})	17.96 ± 0.51^{c}	21.44 ± 0.23^{c}	26.25 ± 0.33^{c}
DZN (1/40 LD_{50})	13.86 ± 0.42^{b}	15.28 ± 0.30^{b}	16.87 ± 0.43^{b}
DZNF (1/10 LD_{50})	38.26 ± 0.41^{g}	54.22 ± 0.35^{g}	67.44 ± 0.22^{g}
DZNF (1/20 LD_{50})	31.92 ± 0.32^{f}	46.87 ± 0.43^{f}	53.90 ± 0.46^{f}
DZNF (1/40 LD_{50})	26.23 ± 0.34^{e}	36.25 ± 0.40^{e}	41.88 ± 0.44^{e}

DZN: diazinon insecticide; DZNF: diazinon commercial formulation; MDA: malondialdhyde. Values in the same column with different superscript letters are differing significantly (p<0. 05). Values represents means ± standard errors. Number of animals/group = 10.

TABLE 4
ALT AND AST LEVELS IN MICE TREATED WITH DZN AND ITS COMMERCIAL FORMULATION (DZNF).

Treatment	ALT (U/L)	AST (U/L)
Control	32.33 ± 0.28^{a}	33.78 ± 0.56^{a}
DZN (1/10 LD_{50})	47.70 ± 0.30^{d}	61.87 ± 0.43^{d}
DZN (1/20 LD_{50})	36.84 ± 0.41^{c}	55.52 ± 0.33^{c}
DZN (1/40 LD_{50})	30.26 ± 0.60^{b}	42.90 ± 0.46^{b}
DZNF (1/10 LD_{50})	84.87 ± 0.43^{g}	98.22 ± 0.35^{g}
DZNF (1/20 LD_{50})	75.22 ± 0.35^{f}	87.47 ± 0.29^{f}
DZNF (1/40 LD_{50})	51.95 ± 0.50^{e}	71.73 ± 0.51^{e}

DZN: diazinon; DZNF: diazinon commercial formulation; ALT: alanine aminotransferase; AST: aspartate aminotransferase. Values represents means ± standard errors. Number of animals/group = 10. Values in the same column with different superscript letters are differing significantly (p<0. 05).

IV. DISCUSSION

Results showed that DZNF had a higher mutagenic effect than DZN itself on both somatic and germ cells of mice. These results supported those of Aboul-Ela [6], Alabi *et al.* [7], Ahmed and Alwan [8], Altamirano-Lozano *et al.* [19], who recorded a significant percentage of chromosome aberrations and sister chromatid exchange in bone-marrow cells of the mice exposed to DZN and also in mammalian spermatogenic cells of mice [20]. DZNF were found more toxic than DZN as compared to control, as they contain surface active ingredients, dyes, stabilizers, activity enhancers, and organic solvents [2]. In addition, studies conducted by the National Institute of Hygienic Sciences in Japan, confirmed that DZN exposure increased the frequency of abnormal chromosomes in hamster [21], and in human blood cell cultures exposed to diazinon [22]. However WHO, found that the effects of DZN on human health and the environment, gave no evidence of a mutagenic potential [23].

The evidence for the genotoxicity of diazinon is strong and appears to operate in humans. Studies in experimental animals in vivo showed either DNA damage (oxidative DNA damage, DNA strand breaks) or chromosomal aberration. In vitro, human cell lines also showed DNA damage (DNA strand breaks) or chromosomal damage (micronucleus formation, sister-chromatid exchange) (IARC, 2017) [24]. The findings presented in this study were consistent with previous studies that associated the exposure to OP pesticides with DNA damage, because DNA is a target for mutagens and carcinogens, which induce changes in DNA structure giving rise to mutations and/ or cell death [25-27]. Free radical generated following insecticide exposure may lead to extensive DNA damage [26]. So, DZN and its formulation are capable of inducing chromosomal aberrations and DNA damage [28]. The increased DNA damage in mice exposed to DZNF and DZN in our study raises concern about the current assessment of the health risk posed by this pesticide and calls for a high level of caution in agricultural and household uses due to the extensive use of OP, in particular, by young workers.

These findings also support earlier reports on DZN-related DNA effects and point to the main mechanism of DZN action on the cell level: genotoxicity, which was established by DNA fragmentation and comet assays [10, 29]; because OP induced ROS (reactive oxygen species) which attack DNA, causing DNA damage and cell death [30]. The evidence that diazinon can induce oxidative stress is strong. Diazinon induced oxidative stress in human and mammalian cells in vitro, and in a variety of tissues in numerous studies in rodents in vivo. Diazinon induces oxidative stress through alteration of antioxidant enzyme activity, depletion of glutathione, and increasing lipid peroxidation (IARC, 2017) [24].

Biomarkers of oxidative tissue damage include AST, ALT in serum and MDA in liver and brain tissues; we found that DZN increases the levels of these biomarkers. In support to our study findings, treatment with DZN increased AST, ALT and MDA levels in male rats compared to control [12]. DZN and DZNF, commonly used as organophosphorus, were found to cause oxidative stress by increasing the level of reactive oxygen species (ROS) in several organs including liver and brain after acute and chronic exposure [5, 31]. Oxidative stress (OS) induced by reactive oxygen species may be a main mechanism, which can be associated with oxidation, membrane damage, sperm DNA damage, apoptosis, and subsequent male infertility, enzyme inactivation and biochemical alteration [12, 32].

V. CONCLUSION

These findings demonstrated that DZNF is more genotoxic than DZN as assessed by cytogenetic analysis of both bone marrow and germ cells in mice and had adverse effects on DNA and biochemical parameters (AST, ALT and MDA levels) and lead to chromosomal aberrations, DNA fragmentation and biochemical alterations. Therefore, the treatment with DZNF is more toxic and harmful than DZN and it is important to avoid toxicities induced by organophosphates, take a high level of caution and minimize its agricultural and household uses.

ABBREVIATIONS

OP: Organophosphorus; DZN: Diazinon; DZNF: Diazinon formulation; DNA: Deoxyribonucleic acid; WHO: The World Health Organization; MDA: Malondialdehyde; PBS: Phosphate-buffered Saline; ALT: Alanine transaminase; AST: Aspartate transaminase; USEPA: US Environmental Protection Agency; NPIC: National Pesticide Information Center; ROS: Reactive oxygen species; LPO: Lipid peroxidation; ANOVA: Analysis of variance; IARC: International Agency for Research on Cancer.

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