GENOTOXICITY OF DIAZINON IN MALE ALBINO RATS FED ON DIET SUPPLEMENT WITH CHITOSAN

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ABSTRACT

In order to determine the genotoxic effects of diazinon and the role of chitosan to neutralize these effects, our study performed in (24) male rats (*Rattus norvegicus*) were divided into four groups and treated for (60) days as following, group (A) treated with normal saline and served as control, group (B) treated with [(1/10LD50) 3.8mg/kg. bw] of diazinon, group (C) treated with [(1/10LD50) 3.8mg/kg. bw] of diazinon and fed on diet supplement containing (1gram/1kg ration) chitosan, group (D) fed on diet supplement containing (1gram/1kg ration) chitosan only. The genotoxic effect of diazinon was evaluated by using the micronucleus assay showed increasing of micronucleated polychromatic erythrocytes were (11.6%) in group B, while (7%) in group C . The chromosomal aberration showed increase of presence of chromosomal aberration in group B was (7.5±1.04), while in the group C showed mild elevation in (3.25±0.8). The polymorphism of GSTM1 and GSTT1 genes showed highly incidence of both genes polymorphism in group B was (66.6%) while group C was (50%) . we concluded that diazinon is genotoxic pesticide and chitosan ameliorate it effects.

INTRODUCTION

Diazinon is a commonly used organophosphorous pesticide (diethox- [(2-isoprophyl-6-methyl-4 pyrimidinyl)oxy] thioxophosphorane). It is a synthetic chemical substance with broad spectrum insecticide activity (1). It was released for experimental evaluation in the early 1950's and today diazinon was used extensively by commercial and home applicators in a variety of formulations to control flies, cockroaches, lice on sheep, insect pests of ornamental plants and food crops, nematodes and soil insects in turf, lawns and croplands (2). Organophosphorous (OPs) pesticide can damage different tissues in human and animals, neurotoxicity, myocardial injury, cytotoxicity, respiratory failure and immune system dysfunction have been reported in OPs poisoning. The main mechanism of OPs intoxication is the inhibition of acetyl cholinesterase and overstimulation of its receptors as a result of accumulation of acetylcholine (3). Water fowl and other wildlife may acquire diazinon by drinking contaminated water, by absorbing it through legs and feet, by consuming treated grass or grain, or by ingestion of pesticide impregnated carrier particles (4). The wide spread of pesticides is connected with serious problems of pollution and health hazards (5). Genotoxic effects are considered among the most serious side effects of pesticides, the effects include heritable genetic diseases, carcinogenesis, reproductive dysfunction and birth defects, also several studies all over the world showed the cancer risk after exposure to insecticides. (6,7). Several studies in population exposed to pesticides and showed that the efficiency of micronucleus assay to detect DNA damage under the effect of pesticides (8). The GSTM1 and GSTT1 genes code for the cytosolic enzymes GSTM-mu and GST-theta, respectively, these enzymes are involved in the conjugation reactions in phase two metabolism of xenobiotics, it is thought that most GST substrates are xenobiotics or products of oxidative stress, including some environmental carcinogens (9). GSTT1 is relevant determinants of susceptibility to chronic pesticide (10).

Chitosan (de-N-acetylated chitin), is a form of a crustacean source and the rare alkaline polysaccharide in nature, has many applications in medicine, agriculture and aquaculture, chitosan has been proved effective in enhancing the immune capacity (11). Chitosan exhibited an inhibitory effect against DNA and protein oxidation, in addition, intracellular glutathione (GSH) level and direct intracellular radical

scavenging effect against cellular oxidative stress (12). This study aimed to determine the genotoxic effect of diazinon in laboratory animals model.

MATERIALS AND METHODS

Before the beginning of experiment, the oral LD50 of diazinon was determined by using 5 male albino rats (*Rattus norvegicus*) according to method of (13) and it was (38 mg/kg bw),then (24) male rats (*Rattus norvegicus*) were used to do this study, their weights were ranged (150-200) grams. they were housed under authority of animal house in college of Veterinary Medicine at University of Baghdad, then divided into four groups in which each group contain (6) animals, these groups included: Group (A): administered normal saline orally for (60) days and served as control ; Group (B): administered [(1/10LD50) 3.8mg/kg. bw] diazinon daily in oral dose for (60) days. Group (C): administered daily [(1/10LD50) 3.8mg/kg.bw] diazinon and fed on diet supplement contained (1gram per 1kg of animals diet) of chitosan for (60) days. Group (D): fed on diet supplement contained (1 gram per 1kg of animals diet) of chitosan for (60) days.

The insecticide diazinon (60%) obtained from local agriculture offices in Basrah, the molecular formula is (C12-H21-N2-03-P-S) and the structure formula is (C2H5O)2-P(=S)-OC4HN2-(CH3)CH (CH3) 2(C4HN2 pyrimidine ring).

The chitosan was brought from Vitex pharmaceutical company (Australia) under trade name (fat sorb).

Micronucleus (MN) assay developed to assess the induction of chromosome damage which described by (14), after the sacrifice of animals, both femurs were desiccated out, both cartilaginous epiphyses were cut off, the marrow was flushed out with 2ml of thermal inhibited human plasma (AB blood group) into a centrifuge tube, the samples were centrifuged at 1000 rpm for 10 minutes, the supernatant was discarded and the cells resuspended in a drop of plasma, the suspensions were spread on slides and air dried, the slides were fixed by Giemsa stain, and rinsed in distilled water, a thousand of polychromatic erythrocytes (PCE) was scored. The frequency of micronucleated cells was expressed as percent of total polychromatic cells.

Chromosomal aberration study was done according to (15) which the animals injected with colchicine solutions prior to bone marrow sampling in order to accumulate metaphases, then sacrificed after 2 hours, the bone marrow was flushed

from the femur into a neutral medium as physiological solution (0.9%) which put in a tube and the cells were centrifuged for 15 minutes at 2000 rpm. The supernatant was discarded and a hypotonic solution (KCL) was slowly added after centrifugation, the cells were fixed by cold methanol/ glacial acetic acid mixture (3:1) then slide making by dropped the suspension from suitable height and stained by (5%) Giemsa solution (pH 6.8).

The extraction of DNA from the blood was obtained according to (16). The genotypes of DNA samples were determined by polymerase chain reaction (PCR) methods, PCR for the glutathione S-transferase (GSTM1 and GSTT1) was done according to the method describe by (17), the β-globin gene primer was included in the PCR reaction to confirm the presence of amplifiable DNA in the samples (18). The GSTM1 gene primers were (F): 5'ACCATCCCTgAgAAAATgAAgC 3' and (R): 5'CTTgggCTCAAAgATACggT 3'. The GSTT1 gene primers were (F): 5'TCCTTACTggTCCCCACATCT 3' and (R): 5' TCACTggAT CATggTCAg CA 3'. The β-glubin gene primers were (F)5'CAACTTC ATCCACGTTCACC3'and (R) 5'GAGCCAAGGACAGGTAC3'. A total volume of (20µl), containing (5µl) DNA; (5µl) premix master mix; (1µl) of forward and reverse primers of each genes and then added (4µl) of de-ionized double distilled water in order to complete the volume to (20µl), the reaction was then subjected to (35) cycles of amplification, (94°C) for (30) seconds, (59°C) for (30) seconds and (72°C) for (45) seconds, after (35) cycles, (5µl) of PCR product were run on 2% agarose gel in Tris borate EDTA (TBE) buffer and stained with ethidium bromide.

The statistical analysis done by using the (SPSS 14.0), data was given in the form of arithmetical mean values and standard errors in ($P \leq 0.05$) significance. One-way analysis of variance was performed and variant groups were determined by means of the LSD method.

RESULTS AND DISCUSSION

In the present study, the DNA assay damage was evaluated by micronucleus test, chromosomal aberrations and GST gene polymorphism. The number of micronuclei was evaluated and compared between group was (1000) cells were examined per rat which illustrated in table 1 and shown in figures (1,2,3,4).

The statistical analysis of (group B) showed (19.3±0.71) of micronucleated polychromatic erythrocytes which represent (11.6%). While (group C) showed (11.6±0.42) micronucleated polychromatic erythrocytes in (7%). In addition there was no significant ($P \geq 0.05$) differences between (group A) and (group D). The obtained results showed that diazinon group (group B) highly increased in the frequency of micronucleated polychromatic erythrocytes while slight increased in the number of micronuclei in (group C). The micronucleus test has been used as an in vivo cytogenetic test to estimate the clastogenic potential of chemicals. Micronuclei (MN) are a centric chromosome fragments or whole chromosomes left behind during mitotic cellular division and appear in the cytoplasm of interphase cells as small additional nuclei, the micronucleus assay has shown to be a reliable and sensitive biomarker (19).

Our results being as similar as the findings were reported by (20) on the effect of two pesticides : Alpha-cypermethrin and diazinon on rat bone-marrow cells, also (21) mentioned the effect of 2, 4- Dichlorophenoxy acetic acid (2, 4-D) herbicide on both whole blood and isolated lymphocytes; and (22) reported the effect of alpha-cypermethrin on rat bone marrow cells and (23) on the effect of diazinon on male rats, who reported the ability of organophosphorus to induce a significant increase in the frequency of micronucleated erythrocytes.

The statistical analysis of chromosomal aberrations of all groups as in table 2 showed highly significant ($P \leq 0.05$) increased of level of chromosomal aberrations (7.5±1.04) in (group B) which included gap chromosomes (10%), break chromosomes (7%), fragment chromosomes (5%) and deletion chromosomes (8%). While in the (group C) showed reduce level of chromosomal aberrations (3.25±0.8) which included gap chromosomes (3%), break chromosomes (5%), fragment chromosomes (4%) and deletion chromosomes (1%). In addition there was no significant ($P \geq 0.05$) differences between (group A) and (group D). These effects are thought to occur because of diazinon's ability to inhibit the synthesis of pyridine nucleotides, and possibly also the amino acid tryptophan (24). Studies have shown an increased risk of non-Hodgkin's lymphoma with exposure to diazinon (25). Also some workers found that diazinon induced sister chromatid exchanges in human lymphoid cells (26). The statistical analysis results of polymorphisms of both GSTT1

and GSTM1 as in table 3 showed normal positive genotype in (group A) and (group D). The polymorphism in (group B) showed losing of GSTM1 gene in (66.6%) and losing of GSTT1 gene in (83.3%) while losing of both gene occurred in (66.6%). While the polymorphism in (group C) showed losing of GSTM1 gene in (50%) and losing of GSTT1 gene in (66%) in addition to (50%) of losing of both gene.

GST enzyme activity is known to be involved in pesticides detoxification , also GST- mediated glutathione conjugation is known to play a role in the detoxification of several groups of pesticides (27). Some researchers showed when blood samples obtained during and one month after the end of intensive pesticide treatments were analyzed to cover a period of high and low exposure, respectively, but no effect of pesticide exposure was detected. Each donor was genotyped for polymorphisms in the GSTMI, GSTT1 and NAT2 genes, involved in xenobiotic metabolism, but no association was observed between MN frequency and the genetic polymorphisms analyzed (28). Nevertheless, a subsequent study showed that GSTM1 positive associated to micronucleus increases (29). Finally, a study carried out in Colombia with women working in open fields observed significant increases in micronucleus associated to pesticide exposure (30). Other study performed in pesticide spraying field in Caxias do Sul (Brazil), 108 vineyard workers showed high rates of MN than controls, when the subjects were genotyped for GSTT1, GSTM1, GSTP1, CYP1A1, CYP2E1 and PON, it was shown that genetic polymorphisms in PON modulated the frequency of micronucleus in the exposed group, in addition, some associations between GSTM1, GSTT1 and CYP2E1 polymorphisms were suggested (31).

Table (1): Micronuclei percentages (%) in 100 polychromatic erythrocytes in bone marrow.

Groups	Mean ± SE	Percentage
Group A	3.8 ± 0.40 a	2.3%
Group B	19.3 ± 0.71 b	11.6%
Group C	11.6 ± 0.42 c	7%
Group D	4.1 ± 0.47 a	2.5%

P≤0.05 highly significant when treated group compared to control group.

Table (2): Statistical analysis of chromosomal aberration.

Groups	Total no. of examined cells	Chromatid gap	Break chromatid	Fragment chromatid	Deletion chromatid	Total damage		
						No.	%	$\bar{X}$±SE
Group A	100	1	2	0	0	3	3	0.75 ± 0.4 a
Group B	100	10	7	5	8	30	30	7.5 ± 1.04 b
Group C	100	3	5	4	1	13	13	3.25 ± 0.8 c
Group D	100	1	0	0	1	2	2	0.5 ± 0.2 a

P≤0.05 highly significant when treated group compared to control group.

Table (3): Statistical analysis of polymorphisms in genes GSTM1, GSTT1 and both.

	GSTM1 mutation	**GSTT1** mutation	**Both**
Group A	0	0	0
Group B	4 (66.6%)	5 (83.3%)	4 (66.6%)
Group C	3 (50%)	4 (66.6%)	3 (50%)
Group D	0	0	0
Chi square	71.79	79.63	71.79
P value	0.01	0.01	0.01

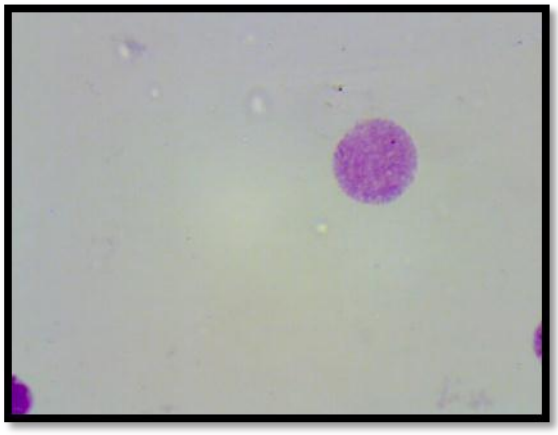

Figure (1): Normal polychromatic erythrocyte in control group (group

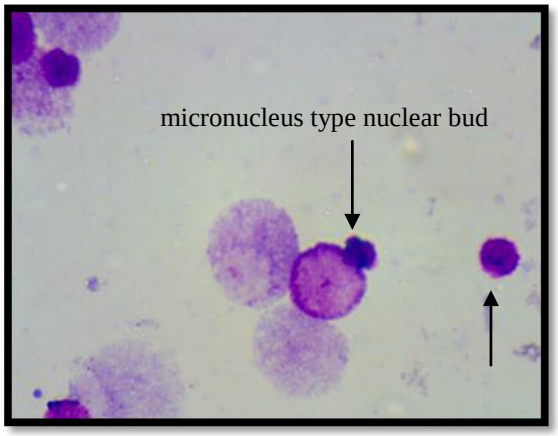

Figure (2): Micronuclei in polychromatic erythrocyte in group B.

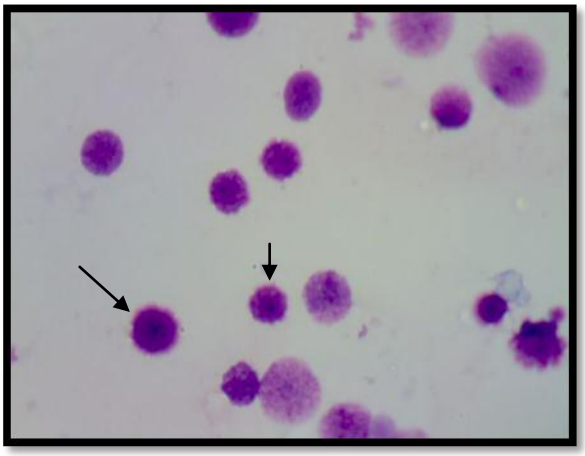

Figure (3): Micronucleus in polychromatic erythrocytes in group C.

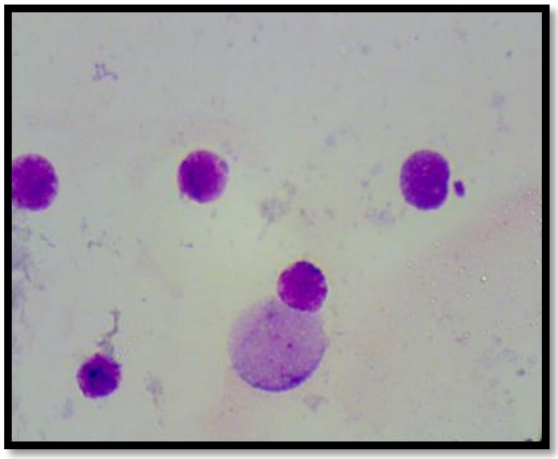

Figure (4): Normal polychromatic erythrocytes in group D

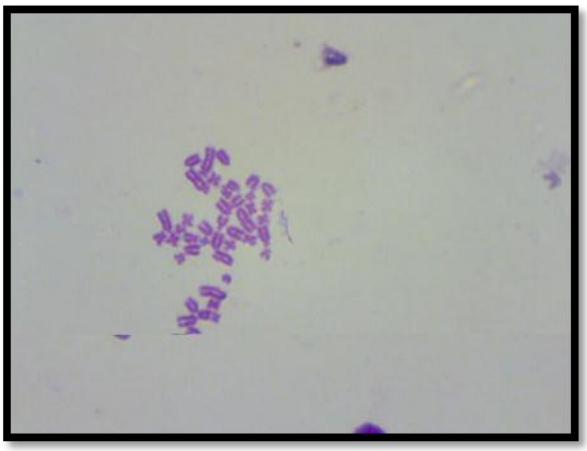

Figure (5): Normal karyotyping of group A.

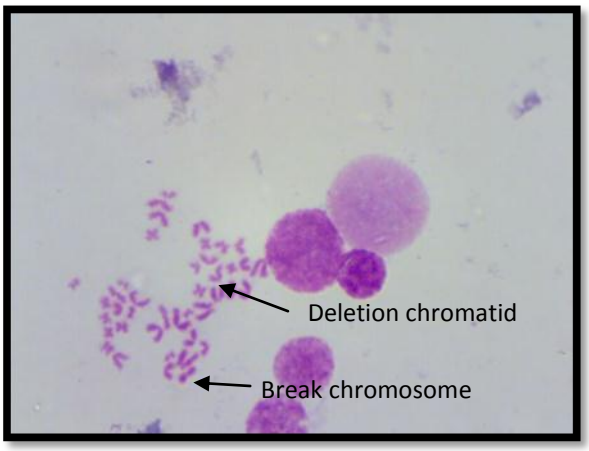

Figure (6): chromosomal aberration in group B.

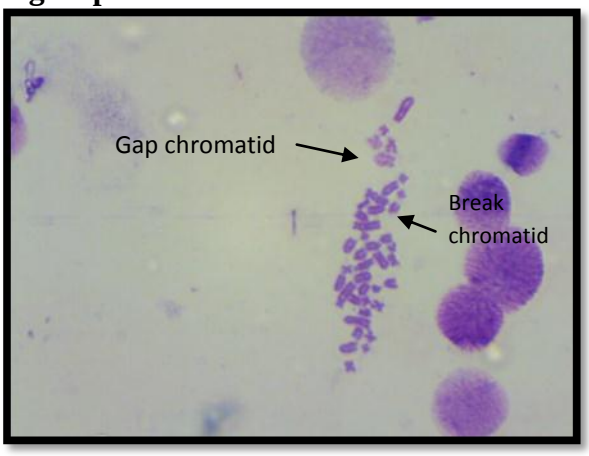

Figure (7): Chromosomal aberration in group C.

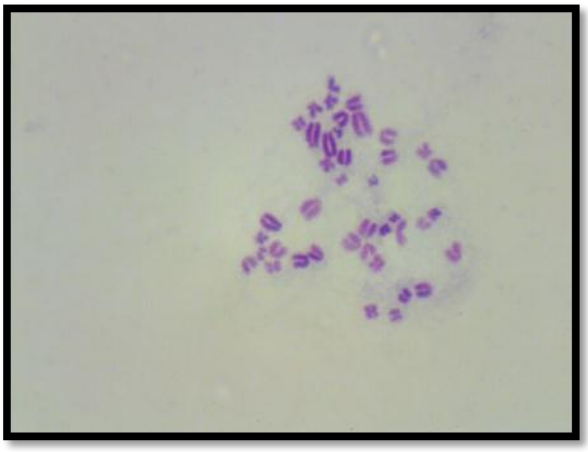

Figure (8): Normal karyotyping of group D.

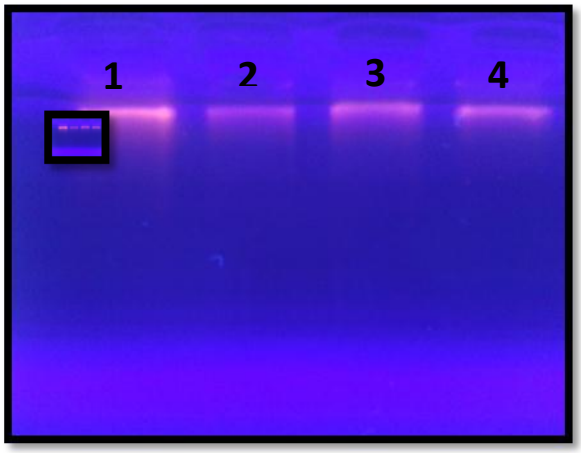

Figure (9): Whole genomic DNA, (lane 1: group A) ; (lane 2: group B) ; (lane 3: group C) ; (lane 4: group D).

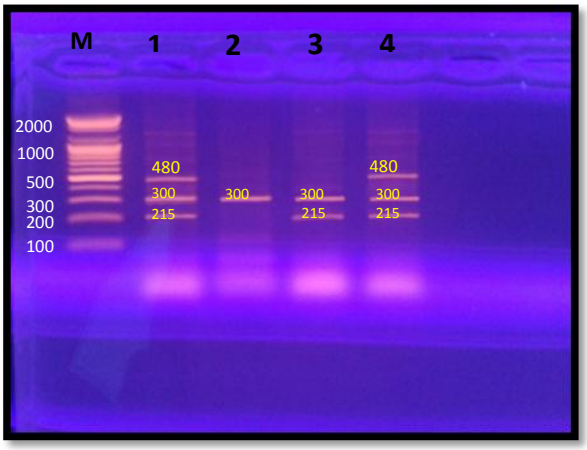

Figure (10): PCR product of GSTM1,GSTT1 and glubin ; M: Marker; Lane 1: normal genotype of GSTT1,GSTM1 and globin genes in group A ; Lane 2: mutation of both GSTT1 and GSTM1 genes in group B ; Lane 3: mutation of GSTT1 in group C, Lane 4: normal genotype of all genes in

CONCLUSION

we concluded that diazinon is genotoxic pesticide and when using chitosan lead to ameliorate it effects.

التاثير السمي الجيني للديازينون في ذكور الجرذان المختبريه المغذيه على عليقه حاويه على الكيتوسان

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الخلاصة

لغرض تحديد التاثيرات السميه الجينيه للديازينون ودور الكيتوسان في معادله تاثيره، اجريت دراستنا على 24 ذكر جرذ مختبري حيث قسمت الى اربع مجاميع وعوملت لمده 60 يوم كما يلي: المجموعه (أ) اعطيت المحلول الملحي الفسلجي فقط واعتبرت مجموعه سيطره، المجموعه (ب) عوملت ب (10/1 من LD50) من الديازينون، المجموعه (ج) عوملت ب (10/1 من LD50) من الديازينون وغذيت على عليقه حاويه على (1غم كيتوسان لكل 1كغم علف)، اما المجموعه (د) فغذيت على عليقه حاويه على (1غم كيتوسان لكل 1كغم علف) فقط.

التاثير السمي الجيني للديازينون قيم بواسطه استخدام طريقه النواه الدقيقه المجهريه، حيث كانت هناك زياده بنسبه (11.6%) بالمجموعه (ب) ، بينما كانت الزياده بنسبه (7%) في المجموعه (د).اما التغيرات الكروموسوميه فلوحظ زياده في وجود تلك التغيرات في المجموعه (ب) بقيمه (1.04±7.5) اما الزياده بالمجموعه (ج) فكانت طفيفه (0.8±3.25). اما التغيرات الجينيه لجيني GSTM1 و GSTT1 فلوحظ زياده

عاليه في التعدد الجيني لكلا الجينين بنسبه (66.6%) بالمجموعه (ب)، اما المجموعه (ج) فكانت نسبه التعدد الجيني (50%). وبذلك يمكن القول بان للديازينون تاثير سمي على الجينات وان الكيتوسان يودي الى تقليل تلك التاثيرات.

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