Cellular Damage After Prolonged Low-Dose Exposure of Neonicotinoid in Rats

Rahat Naseer (rahat.naseer@uvas.edu.pk)
University of Veterinary and Animal Sciences https://orcid.org/0000-0002-8618-2495
Affan Tariq
University of Veterinary and Animal Sciences
Munazza Raza Mirza
Dr Panjwani Center for Molecular Medicine and Drug Research
Muhammad Rashid
Dr Panjwani Center for Molecular Medicine and Drug Research
Syed Qasim Raza
University of Veterinary and Animal Sciences
Sajid Ali Rajput
University of Sindh

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Abstract

Background; Dinotefuran is a new class of neonicotinoids claimed to be harmless to mammals and humans. This claim was daunted by the documented effect of dinotefuran on honeybees and further studies were required.

Aim: The study was designed to assess the capaciousness of damage caused by prolonged exposure of dinotefuran in mammals and probable strategy to neutralize its effect.

Methodology: Ninety-day trial using Wistar rats (n=45) was conducted while dividing them into three groups: untreated control group, insecticide (dinotefuran) treated group, and dinotefuran treated and vitamin E supplemented group. Dinotefuran was administrated orally (LD_{25}). Vitamin E (alpha-tocopherol) supplementation was given in water ad libitum. Blood sampling was done twice a month, and hematological and biochemical data were recorded. After expiry of trial period, the experimental rats were anesthetized and sacrificed. Organs (kidneys, liver, and heart) were isolated from each groups, weighed, and stored at approximately -20°C till further processing, analysis and histopathology were performed.

Results: All the hematological parameters were affected significantly. Histopathology of tissues showed clear necrosis in all the tissues except kidneys. All the biomarkers of oxidative stress and comet assay demonstrated significant cell damage. All the parameters showed improvement after vitamin E supplementation but non-significantly.

Significance: These findings were suggestive that even low dose persistent exposure can lead to mutagenicity and carcinogenicity in mammals and other non-target species hence revised policy guidelines and more intelligent use of these chemicals is required.

Introduction

Pesticides are extensively used in the agricultural field to enhance the productivity of the crops. Pesticides residues can trace their steps from soil to water, food, and environment and the living organisms being the ultimate victims (Pan Wang et al., 2018). There are an estimated three million cases of pesticide poisoning deaths worldwide, whereas, half of the deaths are reported in developing countries. The unintelligent use of any pesticide poses a serious risk to the exposed population (Francisco Prieto et al., 2012). Neonicotinoids are comparatively the latest formulation, which replaced older ones like carbamates (Undeger and Basaran, 2004).

Neonicotinoids are synthetic insecticides, extensively under practice to control unwanted insects. (Bonmatin et al, 2005; Natalia and Robert. 2016). Its mechanism of action involves the binding of neonicotinoid molecule with ligand gated acetylcholine receptors of insect's central nervous system. This binding blocks the neural transmission, resulting paralysis leading to death (Yamamoto and Casida, 1999). Neonicotinoids as an insecticide are frequently applied to most corn and half of soybeans, and

they are potentially used in great quantities on other vegetation, most likely fruit and vegetables (Cimino et al, 2017). Neonicotinoids from last three decades are in use for the crops of vital food values. This group of insecticides were thought to be safe due to its effect on rodents, amphibian and mammals (Tomizawa and Casida, 2005). In 2015, some studies on honeybees were reported indicating its deleterious impact on non-target species. Neonicotinoid contaminated nectar were found to be a source of impaired olfactory learning, disrupting honey bee's life cycle and in some cases death (Wright et al., 2015). Kessler et al. (2015). Gibbons in 2015 also reported neurological and behavior disorders with significant Genotoxicity and Cytotoxic effects (Gibbons et al., 2015).

Dinotefuran is the latest Neonicotinoid developed in 2002 and claimed to have less toxicity to birds, fish, and mammals (Motohiro and Casida, 2005). Dinotefuran (1-methyl-2-nitro-3- guanidine) has a high LD_{50} and a half-life of 3.64 to 15.2h, which contributed to its worldwide acceptance (Alfred Albert et al., 2008; Kiriyama and Nishimura, 2002; Cheng et al., 2000). Dinotefuran is a potent nerve blocker in insects as well as in mammals but with less potency. Neurotoxicity generally works by disturbing pro and anti-oxidant balance, which are producing the reactive oxygen species (ROS) and free radicals. The damage to the cellular function, which leads to mutagenicity and carcinogenicity generally caused by the oxidative stress. Although over the period many adverse effects of dinotefuran have been reported yet little research has been conducted on its effects on mammals or non-target species (Yoneda et al., 2018). Therefore, current project was intended to investigate whether low-dose prolonged exposure to dinotefuran has negative effects on Wistar rats. At the same time, vitamin E supplementation effects were also evaluated on the dinotefuran treated rats.

Oxidative stress is the fundamental indicator correlate to aging, disease production leading to cell necrosis. Macromolecules like DNA and proteins can be effected by cell's microenvironment. This hostile environment of cell with elevated level of oxidative stress take part in impairing respective function important molecules resulting in cell damage. The oxidative stress biomarkers like (catalase (CAT), protein carbonyl content, malondialdehyde (MDA), nitric oxide (NO) and reactive oxygen species (ROS) levels were also determined in this study which is then correlated with Genotoxicity by using comet assay, micronucleus assay, and chromosomal aberration test. This study will improve our understanding about the potential toxic mechanisms of dinotefuran and will provide baseline data to assess its potential health risks to humans.

Materials And Methods

Chemicals and reagents

Pesticide residue, the Dinotefuran with 98% purity, was provided by the *Four Brothers Group* of Industries, Lahore, Pakistan; H_2O_2, HCl, lysis buffer, cytokinesis blocker (cytochalasin B), methanol (case no. RTECS#PC1400000), histopaque, Carnoy's fixative, and Griess reagent were purchased from Sigma Aldrich; ammonium molybdate (case no. Ks542582), NaOH (case no. B102262), and KCl (case no. TA952135) were purchased from MERCK; Trichloroacetic acid (case no. BDH-9310), and DNPH (2,4-

dinitrophenyl hydrazine) (case no. 102573A) were purchased from VWR; Guanidine hydrochloride (case no. 4073-4405) was purchased from DAEJUNG; Phosphate buffer saline (PBS) (case no. 2810305) was purchased from MP Biomedicals; Agarose (case no. CSL-AG500) was purchased from Cleaver; Na-EDTA (case no. S061101) and glacial acetic acid (case no. 27225) were purchased from Riedel-de Haen; Ethidium bromide (case no. Art-Nr-2218-1) was purchased from ROTH; Giemsa solution (case no. 25668) was purchased from SimpSons; and (DCFH-DA)Dichloro-dihydro-fluorescein-diacetate (HDCFDA) was purchased from Invitrogen.

Animal experiments

Wistar rats of mixed sexes and aged between 8–10 weeks were purchased from the local market. All the experimental procedures were carried out in conformity with and approved by the Ethical Committee for Animal Trials, University of Veterinary and Animal Sciences, Lahore. As per institutional guidelines all animals were cared for and treated humanely. Experimental work was performed at the Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences (UVAS) Lahore, Pakistan

All the rats were housed at ambient conditions and 7 days were given to accommodate the new environment. Seemingly healthy rats were choose for the study. The rats were weighed which was (80 ± 20 grams) and randomly divided among various groups in a manner that all groups had approximately equal average body weights. Rats were tagged with distinctive identification number and cage tag cards were placed for the identification of animal with number, there group, dose level etc. Rats were accommodate separately in stainless steel tagged cages and fed according to standard rodent diet and water *ad libitum*. The conditions of cage like temperature and relative humidity were maintained at 22 ± 2°C and 50% -55%, respectively. Light and dark period cycle of 12 was sustained by an automatic timer for 90 days. All 45 rats were divided into three groups: the first group was untreated control with no pesticide and vitamin E supplementation; second group was exposed to pesticide (LD_{25}) only, which was given orally (0.016 gm/gm of body weight); while third group was treated with dinotefuran and vitamin E supplementation. Vitamin E (alpha-tocopherol) dose used was 400 mg/350 mL water per day. The dose repeatedly updated with the increase in body weight.

Estimation of hematological and biochemical parameters

Rats of each group were inspect daily for the existence of any toxicity signs and symptoms or mortality. On day 90, all fifteen (15) rats from each group were fortnightly anesthetized with diethyl ether in a vacuum desiccator. The blood samples were collected directly from the heart puncture and were used for hematological, and biochemical estimations. The blood taken was divided into two parts, one was immediately transferred to EDTA-coated tube for complete blood count (CBC). Hematological tests were performed by using an automatic hematology analyzer MS4SE. The second portion of blood was processed for serum extraction to perform lipid profile, Liver function tests (LFT) and Kidney function tests (KFT). Serum was obtained using standard procedure. The blood samples were kept at room temperature for one hour and allowed to clot, and centrifugation was carried out at 1000g for 10 minutes. The serum samples were collected and immediately frozen at -80^{O}C for further use.

Gross Pathological Evaluation

On day 90 3ml blood was sampled after which five rats were selected randomly from each group and dissected for organ extraction. Liver, kidney and heart tissues were collected from all the experimental groups and fixed immediately in formalin fixative. The visually necrotic sections was cut at 5µm thickness and hematoxylin-eosin (H&E) staining was performed.

Oxidative stress assays

Biomarkers of oxidative stress were evaluated using serum samples initially collected and preserved at -40 ^{0}C.

Catalase, Lipid Peroxidation, and Protein Carbonyl Content Estimation

Catalase (CAT) is responsible for degradation of H_2O_2 and rate of degradation per mg of protein is 1 µmol H_2O_2/min/one unit of catalase. 2 mL of 30% hydrogen peroxide with 10 µL of serum was incubated for 10 minutes at room temperature. Termination of reaction was achieved with the addition of 1 mL of 4% ammonium molybdate. The decrease of H_2O_2 as a function of catalase activity was measured at 410nm Koroliuk et al, (1988).

The amount of malondialdehyde (MDA) act as a biomarker for range of lipid peroxidation (LPO) and was estimated as described by Kamyshnikov et al. (2004). Equal volumes of serum and 20% thiobarbituric acid (TBA) were taken (pH 7.2). The clear solution was obtained and 20 % trichloroacetic acid (1 ml) was added and boiled in a water bath for 10 minutes. After cooling, centrifugation was carried out at 3000g for 10 minutes. The Malondialdehyde (MDA) level was measured at 540nm and was showed in µmol MDA per mg protein, while molar extinction coefficient used was by using 1.56×10^5 mM^{-1} cm^{-1}. Hydrochloric(HCl) buffer acted as a blank.

The protein oxidation was computed from carbonyl derivatives formed. Serum and 2, 4- dinitrophenyl hydrazine (10 mM in 2.5M HCl) in the ratio of 1:1 incubated for 30 minutes at ambient temperature after that 1ml of 20% w/v Trichloroacetic acid (TCA) was added, again incubated(1 hour at 37C) and.pellet obtained after centrifugation washed thrice with ethanol. Pellet was re-suspended in 2.5 mL of 6M guanidine hydrochloride. Another incubation at 90 ^{0}C and centrifugation at 3000g was conducted. The carbonyl content was evaluate by measured absorbance at 370 nm and an absorption coefficient of 22000 M^{-1} cm^1. Georgiou et al. (2018).

Genotoxicity Estimation: DNA Damage

After 90 days study period, blood was collected from the heart of experimental rats, and transferred to the EDTA-coated vials and stored at 4 ^{0}C. The genotoxic effects of dinotefuran on the experimental rats were estimated with the help of comet assay, micronucleus test, and chromosomal aberration test.

Comet Assay

Performed the alkaline comet assay as per the method developed by Undeger and Basaran (2004). The blood sample was diluted by adding phosphate saline buffer (0.5 mL of blood + 1 mL of saline buffered with phosphate). The diluted blood sample was mixed with agarose of low melting point (LMP) in the ratio of 1:2 on pre-coated (1% agarose) microscopic slides and was allowed to solidify. This step was repeated and two more layers of agarose were placed on the sample and solidified. As the gel solidified, slides were dipped in the solution which containing freshly prepared cold lysis buffer (2.5 M NaCl, 10 mM Tris (pH 10), 100 mM Na-EDTA, 10% dimethyl sulfoxide (DMSO) and 1% Triton X-100) after removing cover slips and kept at 4°C for 1 hr. After lysis, the slides were set side by side on a horizontal electrophoresis tank having electrophoresis solution of (1 mM Na/EDTA, 100 mM NaOH and pH 13). The tank was completely filled with freshly prepared electrophoretic solution to a level of 0.25 cm above the slides. Electrophoresis was performed and slides were then neutralized with (0.4 M Tris buffer) and after neutralization stained with ethidium bromide (20µg/mL). Fluorescence microscopy at 100X was performed and 100 cells from each group were scored. Comet score was calculated by the method of Cigerci et al, (2015). The DNA damage was assessed by the visual classification of cells into four categories: comets analogous to the tail length were classified as undamaged (0) to maximum damage (4).

Micronucleus Assay

In the micronucleus assay, the frequency of micro-nucleated cells was evaluated based on the technique described by Karabay and Oguz (2005). Cytokinesis blocker (name) was added in diluted blood and incubated for 72 hrs, followed by centrifugation. The pellet was incubated for 20 minutes at 37°C while merged in 0.075 M KCl, centrifuged for 5 minutes at 1500 g. After centrifugation, the cells pellet was re-suspended in fixative composed of three parts methanol and one part glacial acetic acid.5 mL fixative. Smear was prepared, air dried. 1000 cells were scored from each slide as described by (Anamika Basu, 2004)

Chromosomal aberration

Chromosomal aberrations in lymphocytes were analyzed by a modified method described by Guimaraes et al. (2014). Lymphocytes were isolated by histopaque after centrifugation at 2100 rpm for 20 min. Extracted lymphocytes were washed with 5 mL 0.075M KCl and the cells were pipetted out. The cell suspension was incubated for 20 min at 37°C, followed by centrifugation at 550g for 5 min, and after that supernatant was removed. The cells pellet thus obtained, re-suspended in 5 mL of *Carnoy's* fixative. Centrifugation and fixation were repeated thrice with the intervals of 20 min. Cells were poured onto the microscopic slides and stained for 10 min in 3 mL of 5% buffered Giemsa solution (pH 6.8). One hundred cells from each group were investigated to detect the chromosomal aberrations.

Determination of Reactive Oxygen Species

Dichloro-dihydro-fluorescein-diacetate (DCFH-DA) assay was utilized for determine of intracellular ROS production in serum as described by Keller et al. (2004). The principle of the reaction is the formation of highly fluorescent compound 2,7-dichlorofluorescein (DCF) in the presence of ROS from non-fluorescent Dichloro-dihydro-fluorescein-diacetate (DCFH-DA). Fluorescence intensity was recorded at 485nm for excitation and 530nm for emission.

Determination of Nitric Oxide

Nitric oxide was estimated spectrophotometricaly as described by Greiss reagent (Ridnour et al., 2000). Equal volumes of serum and Greiss reagent (N-(1-naphthyl) ethylenediamine dihydrochloride and 1% sulfanilamide) were mixed and incubated for 5 min. A calibration curve was constructed using sodium nitrite was used. Absorbance was taken at 540nm.Indirect quantification of NO was done by measuring the levels of stable anions- nitrites and nitrates.

Hemotoxylin-Eosin Staining of Tissues

After completion of the study period, the selected experimental rats were given anesthesia, dissected, and organs (liver, kidneys and heart) were dissected out, washed with water and stored in 10% formalin solution until further analysis. A cubical sample 0.5 cm^3 was excised from tissues and immersed in 10% formalin solution. Each fixed tissue sample was desiccated in gradient ethanol solution of (70%, 80%, 85%, 90%, 95%). Later, tissue sample was embedded in paraffin at 58-60 ^oC in order to prepare block and then sliced up by using microtome. Thin section of film placed on a glass slide and dyed by hemotoxylin and Eosin staining as described (Cui et al, 2009). Slides were observed under light microscope (Olympus Model CX31RTSF) and photographs were taken.

Statistical analysis

Statistical data was presented as means ± SD One-way ANOVA was applied to appreciate difference between biological parameters of different experimental groups while Tukey's test was applied for oxidative stress assays. Throughout the notified results, *$p < 0.05$ and ***$p < 0.0001$ were considered statistically significant and highly significant, respectively. Software SPSS version 22.0 (SPSS, Chicago, IL, USA) was used for analysis.

Results

Effect of dinotefuran, and Vitamin E supplementation on body weight and serum biochemical parameters

The maximum weight gains after 90-days trial is 200.03g followed by 193.53g and 174.76g in untreated control, dinotefuran treated, and dinotefuran + vitamin E supplemented groups, respectively (Fig. 1) (Bhardwaj et al 2010).

Biochemical Studies:

The blood is the first indicator of any abnormality. At the end of study period of 90 Days, the hematological parameters of all three groups were studies (Table 1), the percentage of red blood cells and hemoglobin was decreased significantly in treatment groups, and improved non significantly in vitamin E supplementation group. The WBCs count (especially lymphocytes) were increased, showing resistance to the toxicity initially, although, ultimately following the same trend as that of RBCs. The rest of the parameters like mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were effected but non-significant across the groups (Fig. 2) Saravanan, et al 2011).

Table 1
Hematological Parameters of control, dinotefuran treated, and dinotefuran with vitamin E supplemented groups.

Parameters	Control	Dinotefuran treated	Dinotefuran + Vitamin E treated
RBC (10^6/µL)	5.86 ± 1.2	3.83 ± 0.9**	4.50 ± 1.0**
Hb (g/dL)	15.4 ± 0.78	14.1 ± 0.3**	15.6 ± 0.5**
WBC (10^3/µL)	4.70 ± 2.7	7.20 ± 1.0**	6.46 ± 1. **
PLT (10^3/µL)	1253 ± 12.3	869 ± 7.8***	1092 ± 9.2**
Lym (10^3/µL)	5.74 ± 1.4	8.63 ± 2.7*	6.94 ± 2.1*
MCV (fl/RBC)	47 ± 4.1	39 ± 2.8**	41 ± 3.7**
MCH (Pg/RBC)	36.8 ± 3.9	28.4 ± 1.6**	31.7 ±2.4**
MCHC (g/dL)	81.9 ± 4.0	77.2 ± 3.2*	79.8 ± 5.7*

Data was represented as mean ± SD. *p = or < 0.005, **p= or < 0.001, ***p= or < 0.0001. RBC, red blood cells, HB, Hemoglobin; WBC white blood cells; PLT, platelets; Lym, Lymphocytes; MCV, mean corpuscular volume; MCH, mean corpuscular hemoghlobin; MCHC, mean corpuscular hemoglobin concentration

Tissues Specific Toxicity: Liver

Nicotine is a highly toxic alkaloid found in plants. 90-days of dinotefuran exposure to Wistar rats showed sign of toxicity like lacrimation, salivation and mortality. Decrease in the liver weights across the groups was found to be non-significant (p = 0.40).

The markers studied were Aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) and Bilirubin (Fig. 3). All the biomarkers were found to be significantly altered (p< 0.0001) in dinotefuran treated group, the impact of vitamin E supplementation on liver markers indicated significant improvement (p<0.0001) in all the liver biomarkers except bilirubin (Table 2).

Liver histopathology was also carried out by taking tissues from all three groups. The tissue extracted from the liver of the control group showed normal cell line and vascular arrangements. There was no cellular swelling and necrosis observed (Fig. 4-A). However, dinotefrurn exposed rats showed harmful effects on hepatic cells. There was severe necrosis found with moderate degenerative changes, cellular swelling, degenerative hepatic cord, and fatty change (Fig. 4-B). In vitamin E supplementation group, there were mild and reduced degeneration of hepatic cord, RBCs congestion, leucocyte infiltration, and necrosis as compared to the dinotefuran treated rats (Fig. 4-C). Though, some fatty changes were also observed in the liver.

Table 2
Liver weights and parameters of Liver Function Tests (LFT) of control, dinotefuran exposed, and dinotefuran + E vitamin supplemented groups

LFT Parameters	Control	Dinotefruan treated	Dinotefuran + Vitamin E treated
Liver weight	7.2 ± 1.8	6.4 ± 0.9*	6.9 ± 1.2a*
ALT (fL/RBC)	64 ± 3.6	98 ± 4.9***	76 ± 2.4***
AST (pg/RBC)	140 ± 4.2	315 ± 5.2***	218 ± 4.7***
ALP (g/dL)	39 ± 2.9	148 ± 6.8***	91 ± 5.3***
Total Bilirubin (mg/dL)	0.4 ± 0.2	1.7 ± 0.8**	0.8 ± 0.5**

Displayed values are mean ± SD. *p= or < 0.05, ** p= or < 0.001, *** p= or < 0.0001. ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase.

Tissues Specific Toxicity: Kidney

After 90 days' study period, the kidneys from all three groups were also assessed for their function, and histological changes. Creatinine excretion rate is a good indicator of kidney health. In dinotefuran treated rats the blood urea nitrogen (BUN), Creatinine levels were significantly altered ($p<0.0001$). Whereas, vitamin E supplementation showed a non-significant improvement ($p<0.026$) in both BUN and creatinine levels (Table 3). Renal cells of untreated rats showed normal cell line and vascular arrangements (Fig. 5A). There were no cellular swelling and necrosis observed. Dinotefuran treated rats showed minor histological changes, glomerulus atrophy, and necrosis was found with moderate degenerative change in the tubules and glomerulus leading to cellular swelling of rat's kidneys (Fig. 5B). The vitamin E supplementation group only registered very minor changes (Fig. 5C).

Table 3
Renal function tests (RFT) of control, dinotefuran expose, and dinotefuran + E vitamin supplemented groups

RFT Parameters	Control	Dinotefruan treated	Dinotefuran + Vitamin E treated
BUN(mg/dL)	16±4.3	35±3.8***	33±12**
Creatinine(mg/dL)	0.6±0.2	1.4±0.4b**	1.2±30**

Displayed values are mean ± SD. *p= or < 0.05, ** p= or < 0.001, *** p= or < 0.0001. BUN, body urea nitrogen.

Tissues Specific Toxicity: Heart

All the parameters of lipid profile were affected significantly. Cholesterol, triglycerides and LDL levels were found to be much higher in dinotefuran treated rats. However, HDL was found to be decreased in insecticide treated group (Table 4). Histopathology of heart muscles from dinotefuran treated rats showed disorganization, degeneration of myocardial fibers, cytoplasmic vacuolization, and degenerative changes in cardiac muscle cells when compare to the normal vascular arrangement in the heart muscles of untreated rats (Fig. 6A-C).

Table 4
Lipid profile of untreated control, dinotefuran exposed, and vitamin E supplemented groups

Lipid Profile	Control	Dinotefruan treated	Dinotefuran + Vitamin E treated
Cholesterol (mg/dL)	59±3.9	108±8.108***	98±80**
TGC (mg/dL)	63±7.9	167±12.5***	152±13**
LDL (mg/dL)	50±10.5	93.7±6.21**	88±7.1**
HDL (mg/dL)	58±2.9	45±4.1**	49±5.0**

Data are represented as mean ± SD. *p= or < 0.05, ** p= or < 0.001, *** p= or < 0.0001. TGC, triglycerides; LDL, low density lipoproteins; HDL, high density lipoproteins.

Oxidative Stress:

The indicators of oxidative stress, malodialdehydes (MDA), protein carbonyl contents, and catalase (Fig. 7A-C) in the dinotefuran exposed group was found to be significantly higher (Table 5). The current study also showed a significant improvement in oxidative stress markers in vitamin E supplemented rats.

Table 5
Cytotoxic biomarkers level in untreated control, dinotefuran exposed, and vitamin E supplemented groups.

Cytotoxic Biomarkers	Control	Dinotefruan treated	Dinotefuran + Vitamin E treated
Amount of H_2O_2 (µ moles)	0.975+0.540	2.680+ 0.741**	1.057+0.350*
Protein Carbonyle Content (mmoles/mg)	0.806+ 0.43	2.93+0.79**	1.124+ 0.52*
Malondialdehyde (moles/mg protein)	0.620+ 0.56	3.107+0.64***	0.789+ 0.48*
Micronuclei	12 ± 3	36 ± 9***	34±5***
Bi-nuclei	9 ± 2	32 ± 6***	30±8***
Data was represented as mean ± SD. *p = or < 0.005, **p= or < 0.001, ***p= or < 0.0001			

Genotoxicity Studies:

Comet assay was performed using whole liver homogenate from each group. DNA damage was assessed by the frequency of fragmented segments in the dinotefuran exposed group, the score was significantly higher in the insecticide treated rats as compare to the untreated control, which was reflective of a high damage index Vitamin E supplementation also showed non-significant reduction as compared to the control group (Fig. 8A-C).

The micronuclei test showed that 90-day exposure of dinotefuran significantly increased micronuclei (36±9), and bi-nuclei (32±6) in insecticide treated rats (Fig. 9A-C)..

Chromosomal aberration test showed that 90-days exposure of dinotefuran increased chromatid fragments (18.5±0.6), and ring chromosomes (10.5±2.0) in insecticide treated rats (Fig. 10A-C). This significant increase in ring chromosomes and presence of lobulated nuclei reinforced the findings of micronuclei and comet assay. However, we did not found sticky chromosomes, and hence no double stranded break.

Determination of Reactive Oxygen Species (ROS) levels

At the end of 90-days dinotefuran treatment, the level of ROS was significantly higher as compared to the untreated rats. Whereas, vitamin E supplementation significantly reduced ROS levels (Fig. 11).

Determination of Nitric Oxide (NO)

. The NO level was significantly higher dinotefuran treated rats as compared to untreated or control group rats Whereas, Vitamin E supplementation significantly decreased in NO levels as compare to dinotefuran treated rats (Fig. 12).

Discussion

Residual contamination in the environment always has long-time serious consequences for the planet earth. Present study showed the effect of one of the widely used herbicide (Dinotefuran) on mammals when exposed for prolonged period. A comprehensive analysis was conducted.

The first and the most obvious parameter of well-being is weight gain. In developmental studies a strong link was reported between xenobiotic exposure and birth weights. We found a decrease in weight gain, due to probable disruption of hormonal signaling which can ultimately act as a risk factor for metabolic disorders. Dietary interventions improve the situation but to a lesser extent. The duration of the said trial was 90 days so we are not in a position to comment on catch up response of vitamin beyond three months (Svingen T. Ranhoj et al 2018)

Hematological parameters provide better understanding of time dependent side effects of pesticides exposure. Overall reduction in the blood cells (BC) indicate a reduced erythropoiesis or BC destruction. Only white blood cells count increase can point a finger towards a possibility of positive correlation between pesticides toxicity and hematopoietic cancers (Ruifa Hu 2015).

This leukocytosis is also considered as an adaptive measure under stressed condition. Abnormal ESR, can be attributed due to the alteration of plasma proteins. Hematological changes have been reported but conflicting evidence is also present. Regardless of conflicting data abnormality, whether it is in the form of increase or decrease in the blood parameters has been reported persistently (J. L. Del. Prado. Lu 2007). These findings can be due to the disruption of erythropoietic tissue or defensive adaptation, because of less viable circulating cells due to high level of oxidative stress.

Although organophosphates exposure is persistently associated with abnormal low levels of different blood parameters but similar outcomes by Dinotefuran is alarming and require policy revision.

Changes in liver chemistry is always being the first to detect, due to its detoxification role. Liver enzymes are important biomarkers for the assessment of liver function. Any increase or decrease in the activities of these enzymes are indicative of hepato-cellular injury. Abnormal liver function is reflected by the raised values of liver enzymes ALT, AST, ALP as reported by (C. R. Garcia et al 2016), although one conflicting result was reported by (A. A. Malekiand 2013). Liver is the detoxification hub of the body. The changes in the liver biomarkers are well explainable due to liver function of detoxification regarding xenobiotics. It detoxifies xenobiotics by inactivating, removing and transforming them so they can be excreted by intestine or the kidney. Due to persistent or acute heavy exposure, body organs may get overwhelmed, which may lead to the impairment of basic regulatory mechanisms, termed regulatory freeze. This regulatory freeze might induce necrosis and abnormal enzymatic activity. The improvement with the addition of vitamin E can be understand by the notion that ROS-induced oxidative stress was minimized with Vitamin E supplementation that is an antioxidant and maintains redox balance of the body (Miyazawa et al, 2019).

our study is in argument with *Garcia et al 2016* except for the values of ALP, abnormal value of ALP along with other parameters suggest a possibility of hepatocellular necrosis which is afterward confirmed by the histopathology carried out in the same study.

Elevated ratio of BUN Creatinine, Cholesterol, and triglycerides are well explained by the fact that oxidative stress has the capacity to impair the vital metabolism of lipids, carbohydrates, and proteins. So oxidative stress linked inflammatory cytokines, along with the adoptive strategies of insulin sensitive tissue like adipose tissues or muscles, collectively create a crisis, which elevate all the parameters. These findings are well supported by the organophosphate's toxicity studies. However, one lab study conducted by (S. H. Abd 2014) reported low level of cholesterol by exposure to difenoconazole + diclofop methyl in albino rats. Nitrogen containing species, commonly termed as reactive nitrogenous effect (RNS) include NO. Its level is also one of the biomarker of oxidative stress; although less reactive than ROS, but their co-presence with ROS can produce extremely reactive derivatives

From the above findings and discussion, we found that oxidative stress is a plausible explanation for the leading changes in the basic parameters of mammalian system. To elucidate this idea present quantify the oxidative stress by studying cell microenvironment. Vitamin E is a known antioxidant that is thought to prevent cellular damage induced by oxidative stress, the mechanism of reduction in cellular damage is attributed to the potential of vitamin E to inhibit the formation of liquid radicals by preventing liquid peroxidation (LPO) in cell membrane These findings showed that vitamin E treatment had the potential to minimize the deleterious effects of Dinotefuran.

Relationship between environmental exposure of organo-phosphate and oxidative stress is established over the year of research. Pesticides molecule are notably capable of either generating oxidative molecules or can inhibit antioxidant molecules. In the present study as we found elevated levels of cytotoxic biomarkers like H2O, PC, MDA suggested that Dinitofuran is also capable of producing free radicals that can disturb the skin scavenging complex result in lipid peroxidation. The phenomena is supported by the studies conducted by using other organophosphate molecules (Garcia et al, 2016). This interference of antioxidant molecule is supported by the values of vitamin E supplementation group where dietary intake of established antioxidant molecules, show significant improvement in the values of different biomarkers. Increased number of micronuclei has been related to reduce vitality and genome stability. Due to increased number of micronuclei, a genomic chaos is created, as a consequence, genomes tend to reorganize hence become prone to eliminate gene or portion of it ROS are important signaling molecules and contribute significantly as second messengers. Abnormal levels of ROS can cause cell damage even cell death or cell proliferation. Despite their short span of life, ROS can react with biomolecules like lipids proteins, and nucleic acids, resulting in harmful species like lipid adducts and damaged proteins. As a result, these damaged proteins reduce the functionality of enzymes, leading to mutagenicity and carcinogenicity, affecting the tumor micro environment (Kapoor et al 2019). All these findings is verified through histopathology of various tissues, which are in direct correlation with other hematological and cytotoxic parameters.

We can conclude from the present study that like notorious organophosphate, Neonicotinoids has capacity to damage living cells and tissues, if the exposure is persistent even concentration is low. Although further studies with specific reference of Neonicotinoid is required, particularly regarding long term exposure on a population, we recommend policy revision regarding the unintelligent use of herbicides in the developing countries.

Conclusion

The current study was performed to ascertain the influence of low-dose dinotefuran in Wistar rats, and possible reduction in dinotefuran-induced adverse effects through vitamin E supplementation in animal model. After 90-days study period, hematological parameters were affected significantly. Histopathology of tissues showed clear necrosis in all the tissues except kidneys. Biomarkers for oxidative stress and comet assay demonstrated significant cell damage in dinotefuran-treated rats. Most of the parameters used in this study showed improvement after vitamin E supplementation, however, these changes were statistically non-significant. These findings suggests that low-dose but persistent exposure of dinotefuran insecticide can lead to mutagenicity and carcinogenicity in mammals and other non-target species. The policy guidelines to use this insecticide should be revised and more intelligent use of these chemicals is required.

Declarations

Conflicts of Interest

The authors declare no potential conflict of interest.

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Author contributions

Conceptualization: Rahat Naseer

Data curation: Affan Tariq, Munaza Raza Mirza

Formal analysis: Rahat Naseer, Qasim Raza, Munazza Raza Mirza

Methodology: Rahat Naseer, Munaza Raza Mirza

Validation: Rahat Naseer, Muhammad Rashid

Investigation: Rahat Naseer, Munaza Raza Mirza, Sajid Ali Rajput

Writing - original draft: Rahat Naseer, Munazza Raza Mirza, Muhammad Rashid

Writing - review & editing: Munaza Raza Mirza, Sajid Ali Rajput

Ethics approval

Ethical approval was obtained from the Ethical committee for animal trials University of Veterinary and Animal Sciences Lahore

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Figures

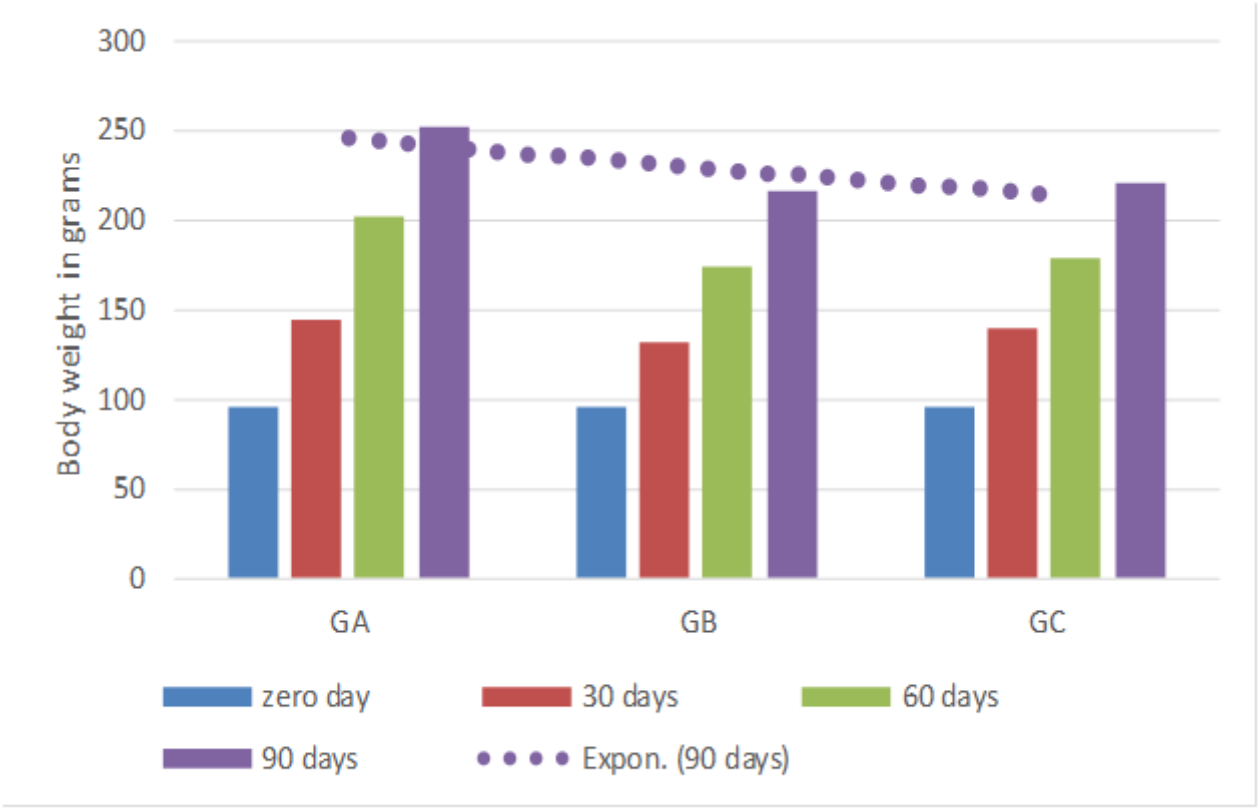

Figure 1

Bodyweight of Experimental Rats: Bodyweight of untreated control group, dinotefuran-treated, and dinotefuran + vitamin E supplemented group form zero-day to day-90.

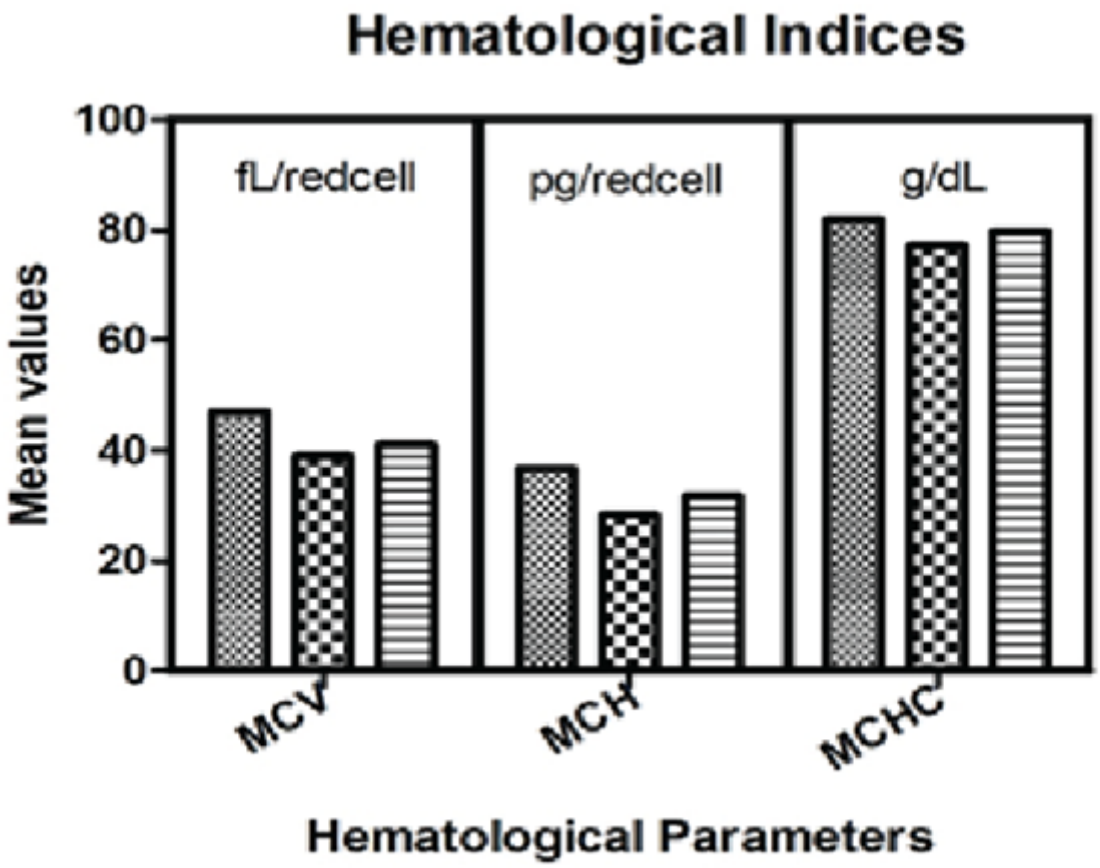

Figure 2

Hematological Parameters of Experimental Rats: Hematological parameters of untreated control group, dinotefuran-treated, and dinotefuran + vitamin E supplemented group on day-90.

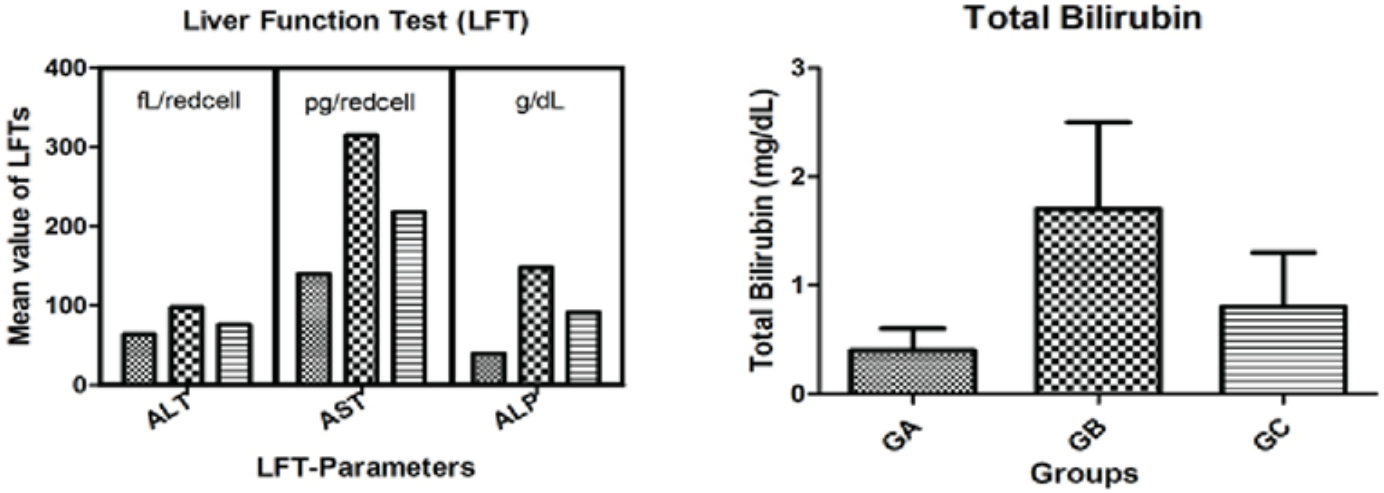

Figure 3

Liver Function Tests of Experimental Rats: Liver function tests of untreated control group, dinotefuran-treated, and dinotefuran + vitamin E supplemented group form zero day to day-90.

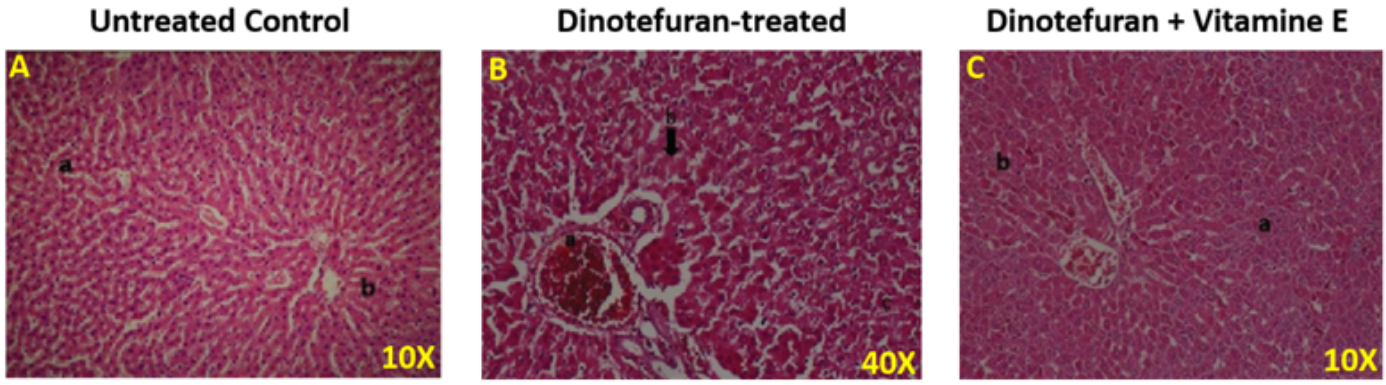

Figure 4

Histopathological Changes in Hepatocytes. Untreated control rat shows normal cell architecture with no lesion (A). Whereas, treatment with dinotefuran has affected the cellular structure, and showing loss of demarcation, aggregation of inflammatory cells, severe necrosis, degenerated hepatic cord, and fatty changes with congestion within the liver tissues (B). However, treatment with vitamin E has lessen the tissue damage and shows some signs of regeneration. There was mild cellular necrosis also present (C).

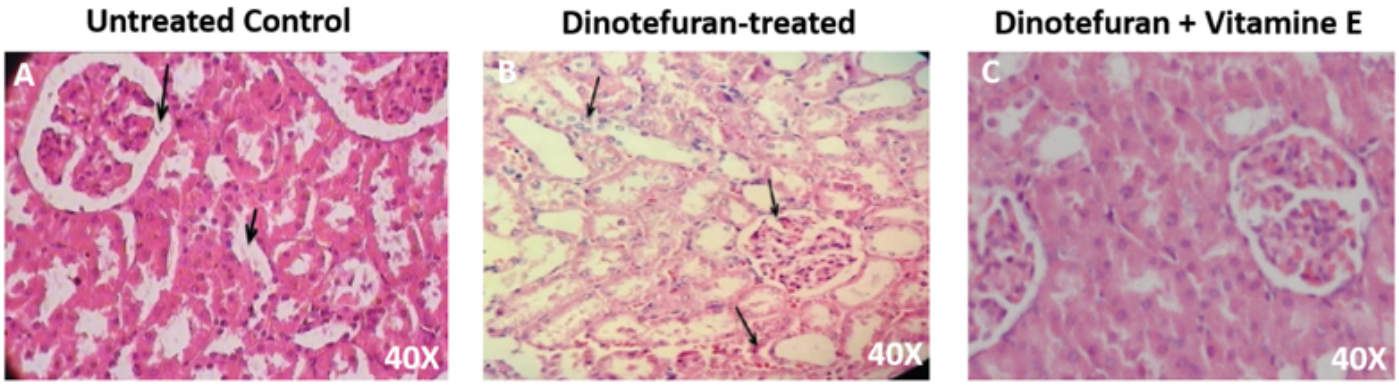

Figure 5

Histopathological Changes in Renal Tissues. Untreated control rat showing normal kidney architecture (A). Whereas, treatment with dinotefuran exposed rat showing diminished and degenerative glomeruli and dilated tubules in kidney tissues (B). However, vitamin E supplementation, the binucleated cells showing regeneration and less damaged (C).

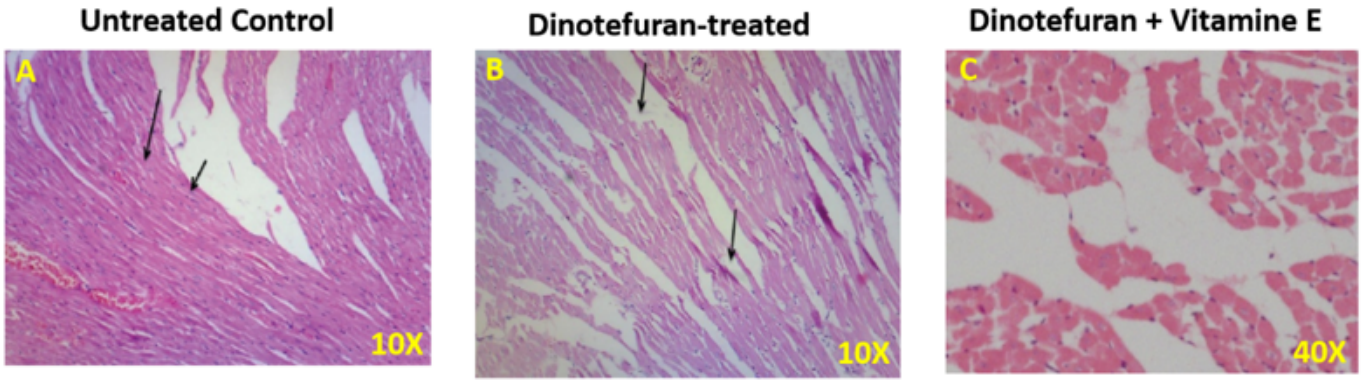

Figure 6

Histopathological Changes in Cardiac Muscle Cells. Control showing normal striated cardiac muscle cells (A). Dinotefuran treated group showing disorganization and degeneration of myocardial fibers and damage to total heart area ratio increased (B). Vitamin E supplement showing no significant signs of improvement (C).

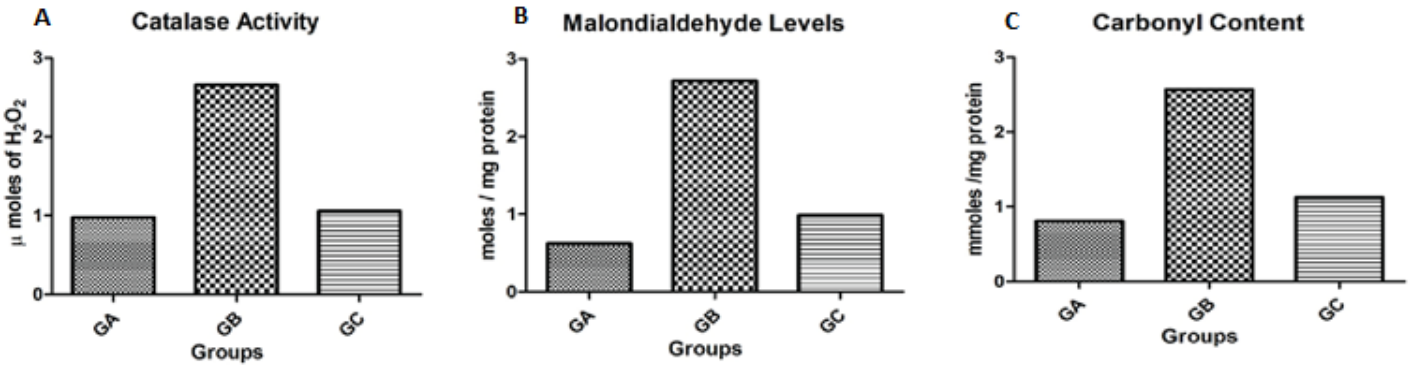

Figure 7

Oxidative Stress Markers in Experimental Rats. The indicators of oxidative stress including catalase (A), malondialdehyde (B), and protein carbonyl content (C) were measured in untreated control rats, dinotefuran treated, and dinotefuran + vitamin E supplemented rat groups.

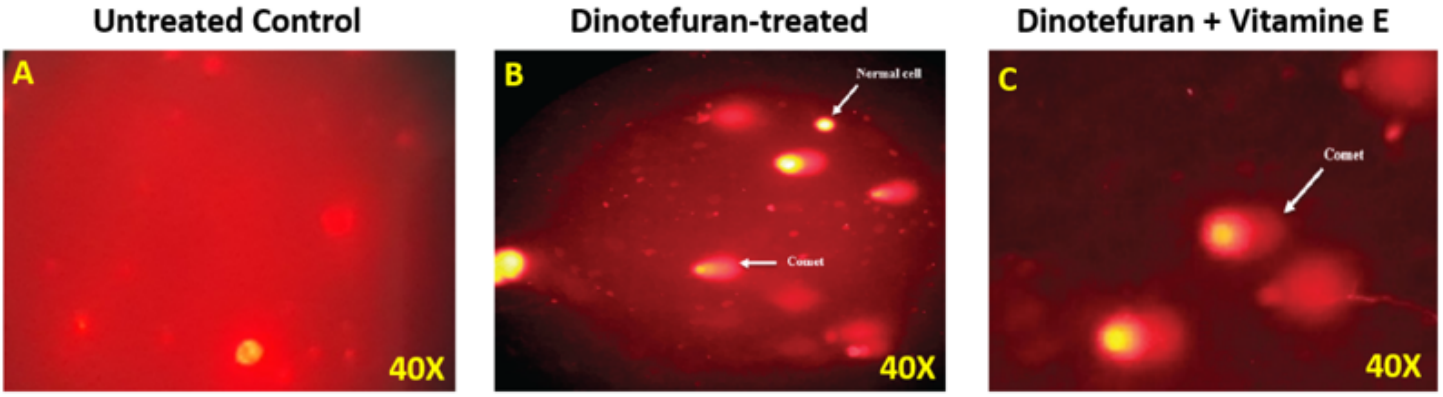

Figure 8

Comet Assay in Different Experimental Groups. Untreated control rat showing intact DNA with no tail (A). Dinotefuran treated group showing comet tails to number of cells (B). In vitamin E supplemented group, comet tails are visible with no significant improvement (C).

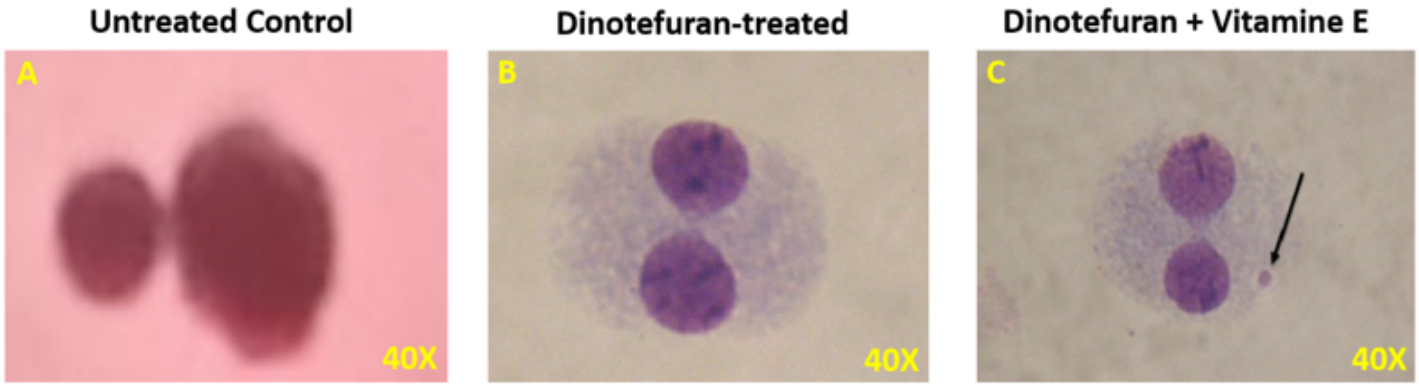

Figure 9

Multinucleated and Binucleated Cells in Different Experimental Groups: Untreated control showing normal cells (A). In dinotefuran treated group, multinucleated cells are visible (B). In Vitamin E supplemented group, binucleated and micronuclear cells are visible (C).

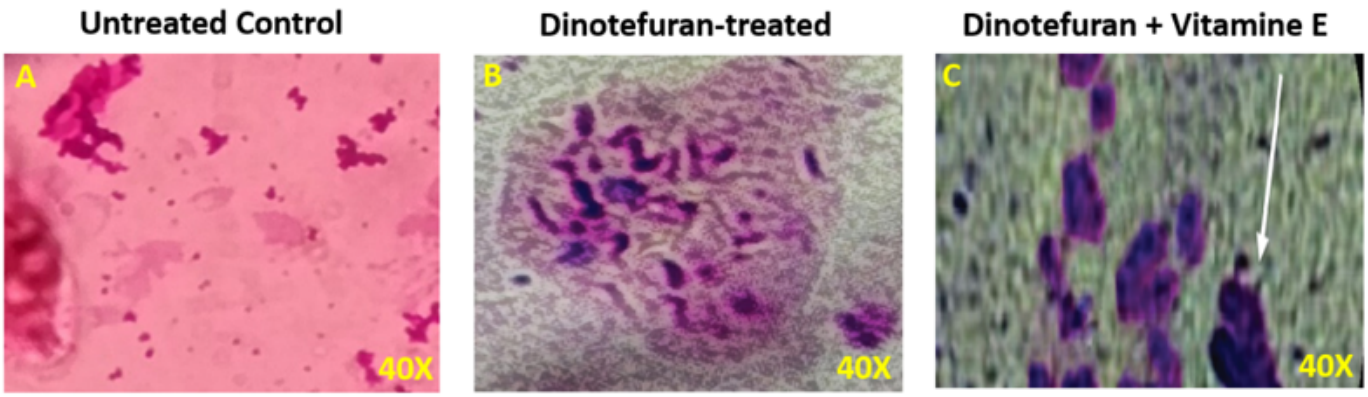

Figure 10

Chromosomal Aberrations in Different Experimental Groups. Untreated control showing no chromosomal aberration (A). In dinotefuran treated group, fragmented chromosomes are visible (B). In Vitamin E supplement group, fragmented (but with less damage) chromosomes are visible (C).

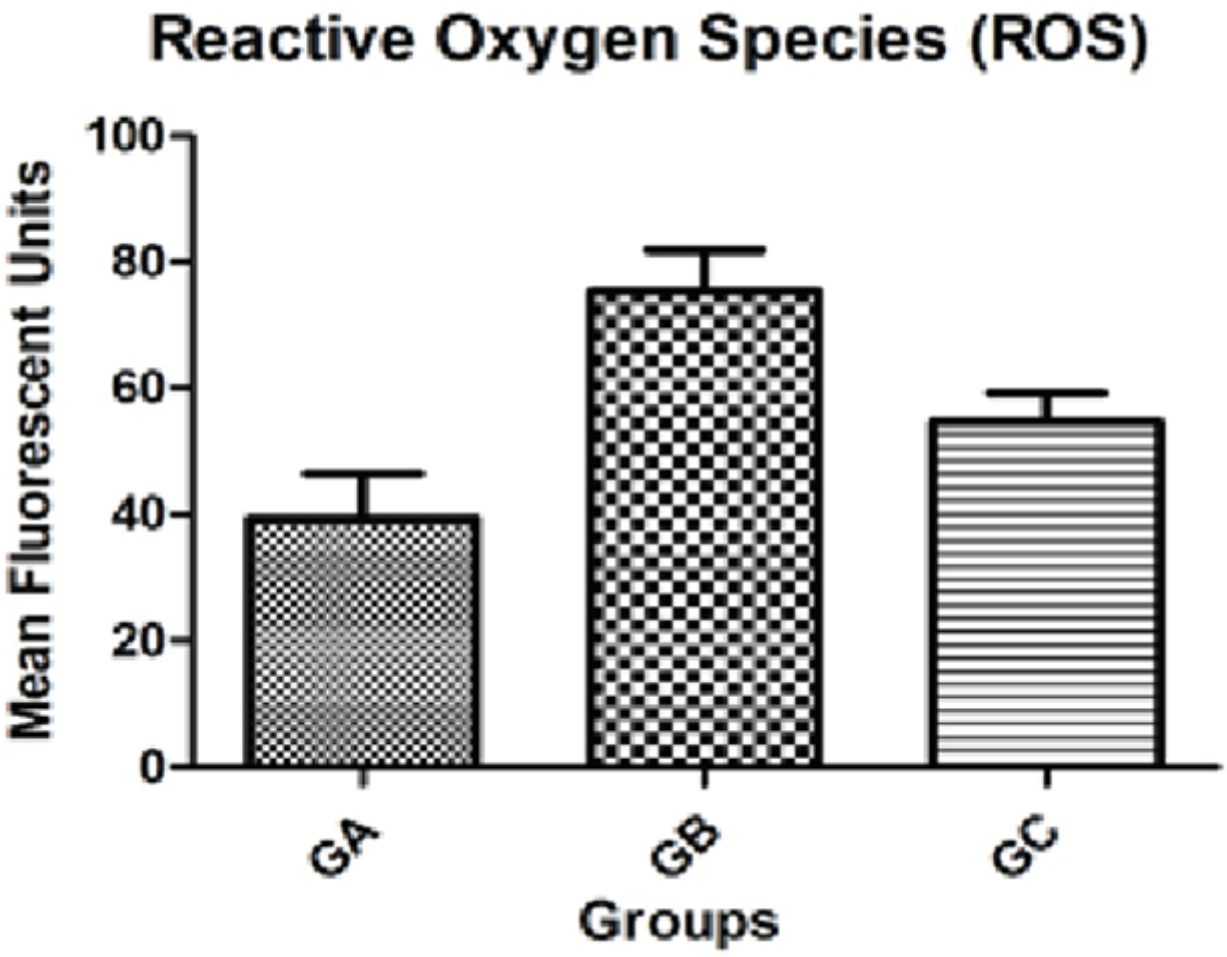

Figure 11

Reactive Oxygen Species Levels of Experimental Rats: Reactive oxygen species (ROS) levels of untreated control group, dinotefuran-treated, and dinotefuran + vitamin E supplemented group on day-90.

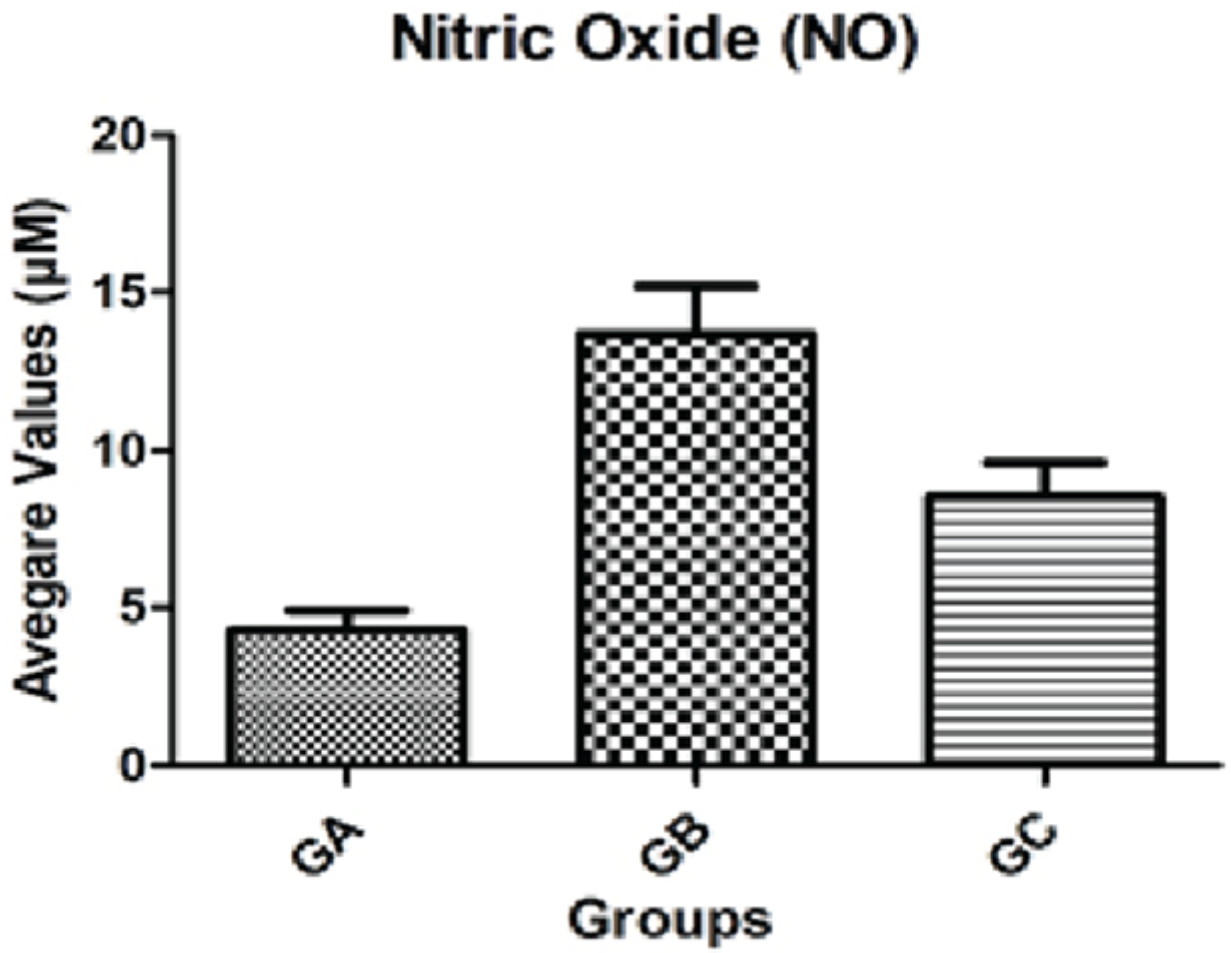

Figure 12

Nitric Oxide Level of Experimental Rats: Nitric oxide (NO) levels of untreated control group, dinotefuran-treated, and dinotefuran + vitamin E supplemented group on day-90.