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Study of the Effects of Diazinon on Fetal Liver in BALB/c Mice

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Abstract

Background: Diazinon is an organophosphate that is broadly used as a pesticide to control insects and environmental pollutions. This toxic material is absorbed via inhalation, contact, or digestion and affects different tissues.
Objectives: This research was a histomorphometric and immunohistochemical study of the fetal liver of mice after exposure to Diazinon.
Materials and Methods: Twenty-five pregnant BALB/c mice (25 - 30 gr) were divided into five equal groups in the animal lab of Baqiyatallah University of Medical Sciences, Tehran, Iran. The normal group was without any intervention, and two sham groups received an emulsifier as 0.52 and 5.2 μL/volume (5000 cc in desiccator) and two experimental groups received Diazinon 1.3 and 13μL/volume from the seventh to eighteenth days of pregnancy every other day via forty minutes of inhalation. The pregnant mice were killed on the eighteenth day of gestation and their fetuses were removed and evaluated for fetal growth and liver development. Five fixed fetuses were dehydrated through a series of graded ethanol, embedded in paraffin wax and their whole bodies were sectioned sagittally and stained via the hematoxylin-eosin method. Quantitative computer-assisted morphometric studies were done on the fetal liver tissues occupied by hepatocytes, blood islands, liver sinusoids, and apoptosis.
Results: The mean crown-rump of the fetuses and their mean weight were increased in the experimental group as compared to the sham and normal groups, but the differences were not significant. The mean percentage of the hepatocyte area significantly increased in the experimental group as compared to the sham and control groups ($P < 0.0001$). However, the mean sinusoid area significantly decreased in the experimental group as compared to the sham and control groups. The mean percentage of the area occupied by apoptotic hepatocytes in the experimental group - 13 μL /volume (8.6143 ± 1.00945) and 1.3 μL /volume (6.1091 ± 0.93093) - significantly increased as compared to the normal and sham groups ($P < 0.0001$).
Conclusions: Our data showed that inhalation of Diazinon during pregnancy increased the hepatocyte area and hepatocyte apoptosis while it decreased the sinusoid area of the fetal liver.

Keywords: Diazinon, Fetal Liver, Apoptosis, Histomorphology

1. Background

Organophosphate (OP) pesticides have been widely used in agriculture as a broad-spectrum pesticide based on its action as an inhibitor of acetylcholine esterase. Several studies have indicated widespread exposure to OP pesticides among some susceptible populations, including pregnant women and children (1, 2). Diazinon is one of the most effective organophosphorus insecticides to control insects and agricultural products (3).

Diazinon is a persistent and dangerous chemical compound. Organophosphates pesticides are commonly used as insecticides and they are generally the most toxic pesticides for the animal species, especially vertebrate animals (4).

Some studies have reported increased exposure to pesticides on women and children and suggested an association between environmental exposure to certain agricultural pesticides, like organophosphorus (OP) compounds, and adverse reproductive outcomes in men and women working on or living near farms (5). Studies reported an association between pesticide exposure and changes in histomorphological parameters and the spermatogenesis of rat testes (6).

The toxicity of OP combinations has been shown in different organs, including the safety and genitourinary systems. It also causes biochemical changes in the blood (7).

During the past few years, a good number of epidemiological studies have been conducted to show the effects of exposure to OPs and other compounds on pregnant

women, but there is a paucity of literature on animal models concerning the effects of OPs during different stages of pregnancy (8). Peiris-John et al. suggested that high OP pesticide level might adversely affect the duration of gestation (5). Some investigators also revealed evidence of the impairment of fetal growth and development brought about by prenatal exposure to OPs. A similar effect was noted with Parathion (9, 10). The deleterious effects due to pre- or post-conception exposure to OP pesticides include menstrual disorders, sterility, fetal toxicity, abortion, stillbirth, and developmental deficits (11, 12).

Eskenazi reported that in utero exposure to OP pesticides may decrease fetal growth, shortening the gestational period (13), and Czeizel also demonstrated an increase in congenital malformations after exposure to OP (14). Kalender reported that Diazinon exposure increased levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP). In addition, exposure of hepatocytes to Diazinon accelerates hepatocyte apoptosis and loss of ATP (15).

2. Objectives

Since data concerning the effects of OPs, such as Diazinon, on fetal hepatic tissue have not been shown, this research was carried out to study the histomorphometric and immunohistochemical evaluation of the fetal liver of mice after maternal exposure to Diazinon.

3. Materials and Methods

3.1. Diazinon and Emulsifier Preparations

This OP has 60% Diazinon and 40% emulsifiers as a solvent of Diazinon preparation (Mahan Co. Ltd, Iran).

3.2. Animals and Diazinon Administration

All procedures were approved by the institutional review board of our medical school. The transportation, treatment and experimentation upon the animals was carried out in strict accordance with the guidelines of the animal care and use ethics committee of Baqiyatallah University of Medical Sciences (Tehran, Iran) for experimental study under Code 33 in 2014. The animals were obtained from the animal breeding unit of Baqiyatallah University and the guiding principles for the care and use of laboratory animals were strictly adhered throughout the study.

The mice, weighing 30 $\pm$ 5 gr, were maintained on a 12-hour light-dark cycle. The animals were housed at 21 $\pm$ 0.5°C with a relative humidity of 50 $\pm$ 10% and were given ad libitum access to food and water. Males were housed individually with estrous females for approximately 16 hours. Mating was confirmed by the presence of a copulatory plug. The day that the plug was detected was considered day 0 of pregnancy. Twenty-five pregnant mice were separated and then randomly divided into five equal groups (five mice for each group). 1, normal group, without any intervention; 2, sham group, receiving emulsifier as solvent of Diazinon of 0.52 and 5.2 μL/volume (5000 cc in desiccator) and then two experimental groups that received 1.3 and 13 μL/volume of Diazinon respectively (Mahan, Tehran chemical Co. Ltd) for 40 minutes every other day by inhalation from the seventh to the eighteenth day of pregnancy.

3.3. Macroscopic Assessments of Fetuses

All the pregnant mice on Day 18 of gestation were sacrificed via an overdose of chloroform (Merk Co.). The uterine horns were cut and the fetuses and their placentas were removed from the uteruses. The fetuses, randomly selected from four to five litters per group, were examined for congenital malformations using a stereomicroscope. Fetal body weights were assessed using laboratory balance equipment (Sartorius, Japan) and crown–rump lengths were measured using a digital vernier caliper (Germany) and recorded.

3.4. Tissue Preparation and Morphometric Studies

Each fetus was fixed overnight in 10% formalin and processed in paraffin for sectioning. The paraffin blocks of the whole bodies of the fetuses were sagittally sectioned at 5 μm thickness and the sections were stained by the hematoxylin and eosin method. The surfaces of the sinusoid and blood island areas were directly measured using a Motic hardware and software system (version 1. 2) with a microscopic magnification of 40$\times$ of the objective lens. For each section, three measurements were randomly taken from non-adjacent points of the fetal liver. Twenty tissue sections were randomly selected at predetermined intervals for each of the fetuses and histomorphometric studies and cell counting were performed in the following manner - the percentage area that was occupied by hepatocytes per unit area (counting frame 23 $\times$ 20 μm equal to 460 μm^2) was counted with the aid of a rectangular calibrated ocular micrometer using a 100 $\times$ objective lens. Only hepatocytes that completely fell within the counting frame were counted. Three visual fields were counted for each section. The percentage of the area occupied by hepatocytes, blood islands, and liver sinusoids was measured by Motic software.

3.5. Detection of Apoptotic Hepatocytes by TUNEL Assay

Apoptotic hepatocytes were detected using the technique of terminal-transferased UTP Nick End Labeling

(TUNEL Apoptag plus peroxidase in situ Apoptosis detection kit, S7101, Chemicon). The liver of the fetuses was collected, fixed in 10% formalin, and then processed and embedded in paraffin. Blocks were sectioned at five microns. Sections were mounted on slides and a proteinase k digestion (20 μg/mL) was carried out for 15 minutes. Endogenous hydrogen peroxidase activity was quenched by 3% hydrogen peroxide. The nucleotides contained in the Reaction Buffer were enzymatically added to the DNA by terminal deoxynucleotidyl transferase (TdT). The incubation was carried out for 60 minutes and the labeled DNA was detected using anti-digoxigenin-peroxidase for 30 minutes. The chromogen, diaminobenzidine tetra hydrochloride (DAB), resulted in a brown reaction product that was evaluated by light microscopy and cells were counted. Positive and negative controls were carried out on slides from the same block. Incubation without TdT served as the negative control.

3.6. Statistical Analysis

Data are presented as mean ± standard deviation (SD). Randomly selected animals (n = 5) were used for data analysis. Statistical analyses were performed using SPSS 13 for windows (SPSS Inc., USA). Data were tested for normal distribution using the Kolmogorov-Smirnov test. For data with normal distribution, one-way ANOVA by Tukey's test was used to compare the groups. The samples were randomly allocated to the study group by a randomized block procedure of size five. $P < 0.05$ are usually reported as statistically significant.

4. Results

4.1. Fetal Body Weights, Crown-to-Rump Lengths

No significant differences in the indicators of embryo toxicity were found between the sham and normal groups. The mean crown-to-rump lengths of the fetuses in the experimental groups were not significantly different as compared to the normal and sham groups ($P < 0.924$). Likewise, the mean body weight of the fetuses increased in the experimental groups as compared to the normal and sham groups. However, the difference was not statistically significant ($P < 0.803$) (Table 1).

4.2. Morphometric Findings

The mean percent-area occupied by sinusoids in the experimental groups [doses 1.3, (20.85 ± 8.72) and 13, (16.86 ± 7.55)] significantly decreased when compared with the normal (37.00 ± 16.79) and sham groups [doses 0.52, (35.38 ± 7.53) and 5.2, (36.12 ± 4.40)] ($P < 0.0001$). On the other hand, the mean percent-area occupied by blood islands increased in the experimental groups [doses 1.3, (25.47 ± 16.19) and 13(27.05 ± 13.02)] as compared to the normal (22.80 ± 19.95) and sham groups [doses 0.52, (25.36 ± 8.32) and 5.2, (22.77 ± 5.88)]. However, the difference was not statistically significant (Figure 1).

The mean percent-area occupied by hepatocytes in the experimental groups at doses of 1.3 (53.69 ± 17.12) and 13 (56.09 ± 14.90) μL/volume of Diazinon significantly increased when compared to the normal (40.20 ± 4.76) and sham groups [doses 0.52, (39.26 ± 3.66) and 5.2, (41.11 ± 1.15)] ($P < 0.0001$) (Figures 1 and 2).

Table 1. Fetal Crown-Rump Lengths (Crl) (Mm) and Fetal Weights (G), Are Shown in Different Groups

Groups	Crown-Rump Lengths Fetuses (CRL)	Weight of Fetuses
Normal	20.76 ± 4.059	1.040 ± 0.458
Sham 0.52	20.602 ± 0.899	1.040 ± 0.105
Sham 5.2	20.65 ± 1.311	1.045 ± 0.080
Exp 1.3	20.49 ± 0.937	1.085 ± 0.075
Exp13	20.97 ± 1.908	1.092 ± 0.177
P-Value (between groups)	0.924	0.803

4.3. Apoptotic Hepatocyte Results

The mean number of apoptotic hepatocytes in the experimental groups at doses of 1.3(6.109 ± 3.087) and 13(8.614 ± 3.777) μL/volume of Diazinon significantly increased when compared to the normal (2.970 ± 2.152) and sham groups [doses 0.52, (3.100 ± 2.612) and 5.2 (3.300 ± 3.103)] ($P < 0.0001$) (Figures 3 and 4).

5. Discussion

Diazinon is a non-systemic insecticide used in agriculture to control soil and foliage insects and pests on a variety of fruit, vegetables, nuts and field crops (16). In addition, Diazinon is one of the most applicable organophosphorus insecticides to control insects and agricultural products (3). Reproductive abnormalities caused by organophosphates (OP) have been observed in many animals (17-19). Jorsaraei showed that Diazinon had a significant effect on the structure of rat testes (6). Diazinon induced lipid peroxidation and increased oxygen free radicals in the reproductive tissues in both genders, so the gonads in male and female rats were vulnerable to oxidative stress and its damages, including infertility (20). Sargazi showed that, considering the effect of oxidative stress in multiple physiological processes, from oocyte maturation

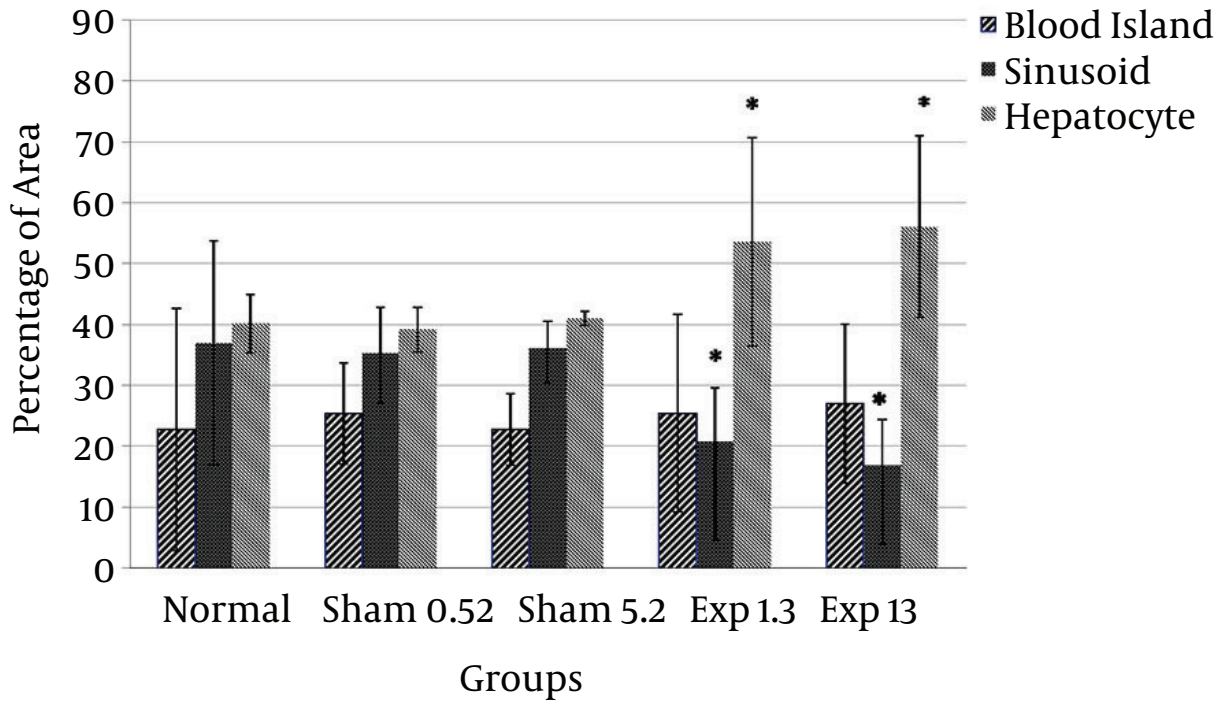
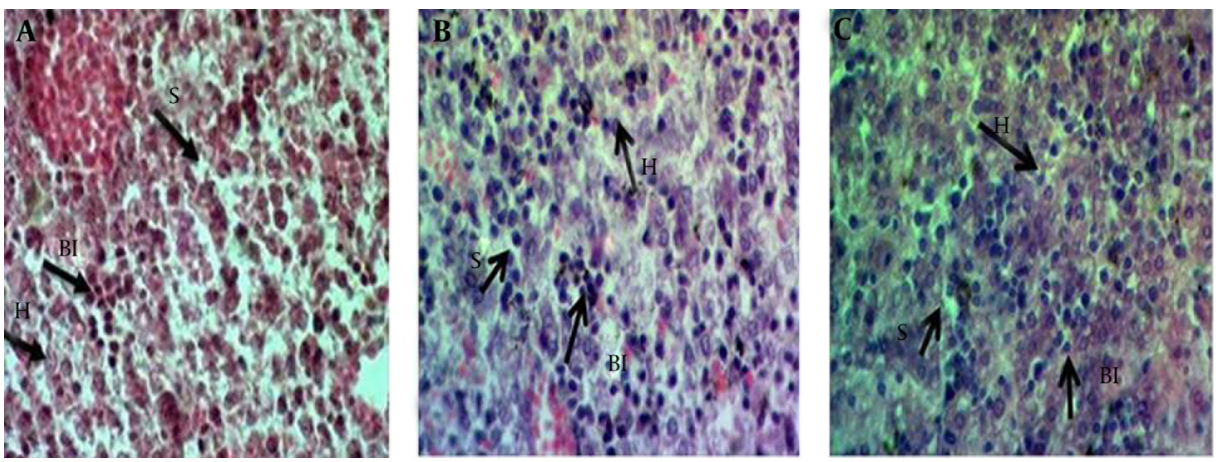


Figure 1. The Mean Percent-Area Occupied by Hepatocytes, Sinusoids, and Blood Islands in 18 Day Fetuses in Different Groups

Figure 2. Photomicrographs of the Livers of 18 Day Fetuses



A, Normal; B: Exp 1.3 (at dose of 1.3 μL/volume of Diazinon); C, Exp 13 (at dose of 13 μL/volume of Diazinon); H, hepatocyte; S, sinusoids; BI, blood islands) (H and E: 400 ×)

to fertilization, embryo development, and the pathophysiology of infertility, it can be concluded that female rats are more vulnerable to oxidative stress and its consequences, including infertility. Therefore, it is necessary to prevent further toxin entry into the body which causes gonadal dysfunction (20).

Our results showed a non-significant increase in fetal weight after maternal exposure to Diazinon. Our findings are in agreement with previously published research examining prenatal developmental toxicity in pregnant rats which were fed Diazinon doses up to 100 mg/kg/day during days six through fifteen of gestation which showed

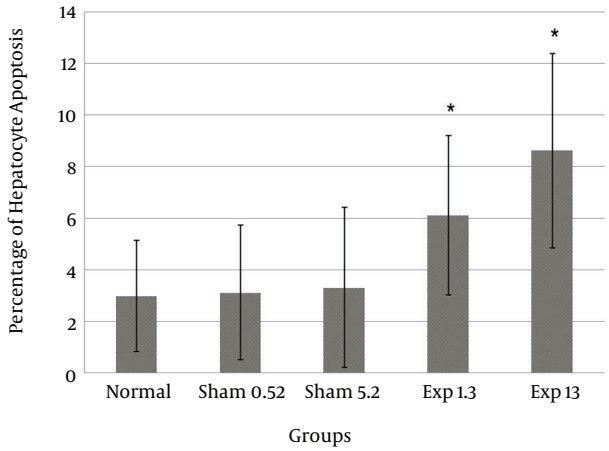


Figure 3. The Mean Number of Apoptotic Hepatocytes in 18 Day Fetuses in Different

fetal weights increasing at the highest dose. However, the number of live fetuses decreased and pre- and post-implantation losses were increased at the highest dose (21). In contrast to our results, some researchers have shown that if rat embryos are exposed to Diazinon in culture, the neonatal weights of the experimental group significantly decreased as compared to the control group (22). It has been shown that the teratogenic effects of a drug or chemical agents directly depend on the amount or timing of drug concentration in maternal plasma, but the most important external factor concerning the impact of the drug on the fetus depends on the toxin concentration and duration of the fetal body (23, 24). In addition, our results demonstrated that Diazinon decreased sinusoidal area while it increased hepatocyte and blood island surface area in the fetal liver. On the other hand, our findings also revealed that the mean number of apoptotic hepatocytes in the experimental groups significantly increased when compared to the normal and sham groups.

Some other studies have shown the evacuation of liver tissues, cell infiltration, irritation, vein enlargement, glomeruli degeneration, glomeruli removal, and compression by Diazinon in the treated group (25). Their results are in agreement with our findings about fetal liver tissue. Since the liver is one of the most complex and vital organs and its primary function is detoxification of absorbed substances from the digestive system before their distribution into the systemic circulatory system (26), Diazinon's in vitro and in vivo degradation rate of metabolites in the liver requires further research (27). The exposure to OPCs during pregnancy is an important factor because it affects two organisms - the mother and the fetus (28-30). Moreover, it has been shown that Diazinon can also act as a substrata for glutathione peroxidase (GST) and GPx enzymes that neutralize poisons (16, 31-33).

In addition, antioxidants are scavengers that detoxify excessive ROS and have an important role in maintaining oxidant/antioxidant balance in the body (34).

On the other hand, Yilmaz reported that oxidative stress contributes to Diazinon-induced brain toxicity and the vitamins E and C in combination may have a protective effect against this toxicity (35). Khan et al. also showed that consumption of cypermetrine and malathion by desert mice decreases liver glutathione (36). Fujita and colleagues (1993) reported that the need for oxygen during hypothermia due to high liver metabolism makes the liver hepatocytes susceptible to the negative effects of hypoxia and anoxy (37). It has been shown that OP combinations cause toxicity in different organs, including the safety and genitourinary systems. It also causes biochemical changes in the blood (7). Since most of the OP compounds in the body are converted to the form of an active metabolite through the hepatic cytochrome P450 system and by oxidative desulfurization (38) and this combination is rapidly absorbed over a few hours through the intestine and converted to metabolized diazoxon in liver (39), it seems the fetal liver in our study was affected by Diazinon and suffered apoptotic changes.

Many of these effects have no relation to the control of the acetyl cholinesterase enzyme, but they are induced by other cell mechanisms (40, 41). One of these mechanisms is the production of free radicals and the disorder of the antioxidant systems. Naturally, there is a balance between the production and removal of free radicals. An imbalance of these processes causes oxidative stress. If this stress continues, it can produce serious cell damage (42). One strength of our study was that exposure of Diazinon to pregnant mice occurred via inhalation. Histomorphometric evaluations and immunohistochemical studies were also some of the advantages of our research. In contrast, one of the limitations of our study was that we could not measure how much of Diazinon was absorbed into the bodies of the pregnant mice. Nor could monitor the function of the liver in newborn mice.

In conclusion, the data suggest that prenatal Diazinon exposure has toxic effects on fetal development, as well as increasing apoptosis in fetal hepatocytes.

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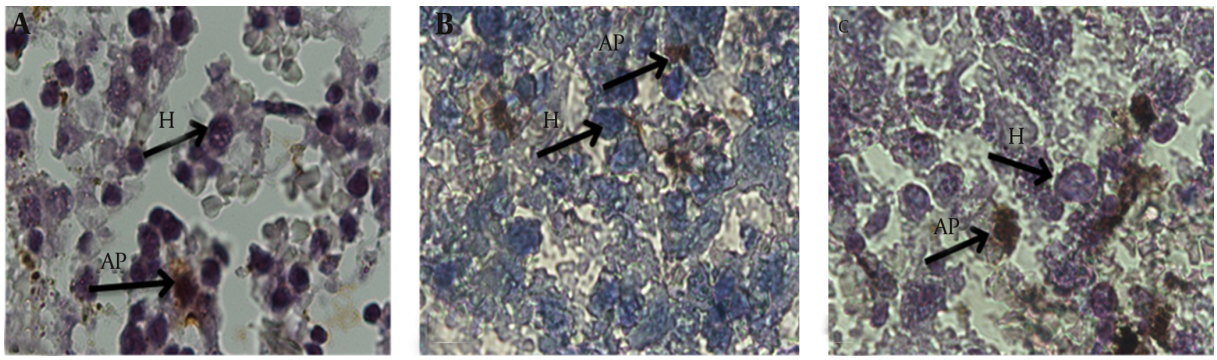
Figure 4. Photomicrographs



A, Normal; B, Exp 1.3 (at dose of 1.3 μL/volume of Diazinon); C, Exp 13 (at dose of 13 μL/volume of Diazinon); H, hepatocyte; AP, apoptosis) (Tunel: 1000 ×).

Footnotes

Authors' Contribution: Designing the method of study; collection, validation, and analysis of the data; drafting the manuscript, and final revision, Fatemeh Saraei; validation and analysis of the data and final revision, Mehrangiz Sadoughi, Gholamreza Kaka, Seyed Homayoon Sadraie and Mohsen Foadoddin.

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