Iranian Journal of Toxicology
ARAK UNIVERSITY OF MEDICAL SCIENCES
July 2022, Volume 16, Number 3

Research Paper

Hepatic Oxidative Damages and Glucose Tolerance in Diabetic Rats Exposed to Repeated Oral Doses of Diazinon

Niloufar Sinaei[1], Elham Jafari[2], Amir Najafi[1], Somayyeh Karami-Mohajeri[1,3*]

1. Pharmaceutical Research Center, Neuroscience Research Center, Kerman University of Medical Sciences, Kerman, Iran.
2. Pathology and Stem Cell Research Center, Department of Pathology, Kerman University of Medical Science, Kerman, Iran.
3. Department of Toxicology and Pharmacology, Faculty of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran.

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How to cite this paper Sinaei N, Jafari E, Najafi A, Karami-Mohajeri S. Hepatic Oxidative Damages and Glucose Tolerance in Diabetic Rats Exposed to Repeated Oral Doses of Diazinon. Iranian Journal of Toxicology. 2022; 16(3):221-228. http://dx.doi.org/10.32598/IJT.16.3.950.1

http://dx.doi.org/10.32598/IJT.16.3.950.1

Article info:
Received: 20 Jan 2022
Accepted: 06 Apr 2022
Online Published: 01 Jul 2022

*** Corresponding author:**
Somayyeh Karami-Mohajeri, PhD.
Address: *Department of Toxicology and Pharmacology, Faculty of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran.*
E-mail: *s_karami@kmu.ac.ir, somayyehkarami@gmail.com*

ABSTRACT

Background: Environmental pollutants including organophosphate insecticides impair glucose metabolism by altering hepatic oxidation and play an important role in the development of diabetes and its complications. The aim of this study was to assess the impacts of repeated oral doses of Diazinon, an organophosphate insecticide, which is known to impair the glucose metabolism and its tolerance through oxidative stress in the rat liver.

Methods: Diabetes was induced in rats by a single dose of freshly prepared Streptozotocin at 60 mg/kg. Both normal and diabetic rats were exposed to daily oral Diazinon at 20 mg/kg for 21 days. Subsequently, the effects on the rats' liver were assessed by glucose tolerance test, histopathology examinations and antioxidant capacity measurement.

Results: The glucose tolerance tests showed impairment in the non-diabetic rats exposed to Diazinon, while the difference in glucose tolerance between the diabetic rats treated with or without Diazinon was not significant. Diazinon in diabetic rats caused greater histopathological changes along with significant elevations in the lipid peroxidation and antioxidant enzymes, including superoxide dismutase and catalase in the liver tissue.

Conclusion: Subacute exposure to Diazinon exacerbated hepatotoxicity by inducing oxidative stress in diabetic rats. The superoxide dismutase and catalase activities increased due to the oxidative damages in rats' liver caused by Diazinon.

Keywords: Diazinon, Diabetes mellitus, Organophosphate, Organophosphorus, Oxidative stress, Pathology, Streptozotocin

Introduction

Organophosphate Pesticides (OPs) accumulate acetylcholine at the cholinergic synapses by the inhibition of Cholinesterase (ChE) activity and consequently over-activation of the nicotinic and muscarinic receptors [1]. Organophosphates induce hyperglycemia and impaired glucose homeostasis in the liver by altering the metabolism of carbohydrates. They also cause oxidative stress, pancreatitis, insulin resistance, disruption of tryptophan metabolism, and stimulation of the adrenal glands [2]. Numerous clinical studies in humans have reported the relationship between exposure to OPs and incidence of type-2 diabetes mellitus [3]. However, the role of environmental pollutants, such as OPs in the induction of insulin resistance is not fully understood [4].

Acute and chronic exposures to OPs induce hepatotoxicity by Oxidative Stress (OS), alterations in hepatic biotransformation, and mitochondrial dysfunctions [5]. The hepatotoxicity of OPs could contribute to the generation of Reactive Oxygen Species (ROS) and the production of reactive metabolites through hepatic processes [6]. Throughout the oxidation and metabolic processes of food substrates in the liver, oxygen is consumed and ROS are produced [5]. The imbalance between the levels of oxidants and antioxidants results in damages to the liver cells and the tissues. Free radicals cause lipid peroxidation, protein carbonylation and damages to DNA through the oxidation of fatty acids, carbohydrates, proteins, and genetic material in the physiological systems [7].

The oxidant byproducts generated by the induction of oxidative stress are also increased in patients with obesity, metabolic syndrome and type-2 diabetes [8]. Studies have shown that augmented OS is linked to the pathogenesis of insulin resistance in target organs by the inhibition of insulin signals and impaired lipid regulation [2, 9]. Insulin resistance and the consequence of its reduced secretion are known to be the signs of type-2 diabetes pathogenesis [10]. Insulin resistance has also been significantly associated with increased lipid synthesis and flow of fatty acids into the liver [2]. Late complications of diabetes due to OS have been implicated in the progression of the disease [11].

Diazinon, a broad-spectrum organophosphate, is widely used in agriculture and households for insect control. As a result of its widespread use, Diazinon enters the environment and accumulates in the food chain. Exposure to Diazinon is known to produce a variety of biochemical and histopathological alterations in the liver through the induction of OS [12]. It has been found that Diazinon leads to hyperglycemia by disruption in the glucose homeostasis [2, 13]. On the other hand, the toxicity of Diazinon may worsen in diabetic rats due to its increased hepatic metabolism [14]. Exposure to Diazinon in diabetic rats has also resulted in the deterioration of glucose tolerance [15]. Therefore, the present study was carried out to investigate the impact of Diazinon on the histopathology, biomarkers of OS in the liver, and glucose tolerance in experimentally-induced diabetic rats following repeated exposures to the oral administration of Diazinon.

Materials and Methods

Test chemicals and reagents: Diazinon at 90% purity was purchased from Shimi-Keshavarz Pesticides Production Company (Tehran, Iran). All other chemicals were obtained at high purity from Sigma-Aldrich (St. Louis, MO, USA).

Experimental animals: Forty adult male Wistar rats, weighing 200–250 g each, were obtained from the animal house of Kerman Neuroscience Research Center (Kerman, Iran). They were exposed to alternating 12hr of light-dark cycles at a room temperature (22–25°C).

Induction of diabetes: Experimental diabetes was induced by the intraperitoneal injection of a single dose of Streptozotocin (60 mg/kg) solubilized in 0.1 M trisodium citrate buffer at pH 4.5, based on the method described previously by Furman [16]. To ensure that the hyperglycemia was established, the fasting blood glucose was measured, which remained at 250 mg/dL on the first, third and the last days of the study. Polyuria and polydipsia were also monitored by the observation of the amount of consumed water and the frequency of bedding exchange.

Experimental design: The study was carried out on four groups of ten rats each (n=40, total) for 21 days as follows. Control group (normal rats) received corn oil orally every day. Diazinon group were non-diabetic rats that received Diazinon at 20 mg/kg/day, which was dissolved in corn oil. Diabetic rats received corn oil orally and the same Diazinon solution at 20 mg/kg/day every day.

Sample preparation: At the end of the experiments, blood samples were collected from each rat under anesthesia with ketamine/xylazine and via cardiac puncture. A portion of the liver tissue from each rat was homogenized in phosphate buffer (0.1 M, pH 7.4) to evaluate the biomarkers of oxidative stress. Another portion of the liver from each animal was preserved in 10% formalin for histological analyses.

Glucose tolerance test: To conduct the Glucose Tolerance Test (GTT), glucose was dissolved in distilled water at 2% (w/v) and administered to the rats by gavage. The blood glucose level was determined every 30 minutes, using the corresponding kit (Pars Azmoon Co., Tehran, Iran). The results were determined by calculating the area under the curve from zero to 120 minutes ($AUC_{0\text{-}120}$) by the trapezoidal method [17].

Histopathologic analyses: Liver tissue specimens, fixed in formalin, were embedded in molten paraffin and sections were made at 5µm thickness, and stained with Hematoxylin and Eosin (H&E). A light microscope (CX41, Tokyo, Japan) was used to examine the histopathological changes by a pathologist in a blinded

manner. The histologic alterations were subsequently recorded, scored and photomicrographs were taken.

Lipid peroxidation assay: The level of Malondialdehyde (MDA), as the end-product of Lipid Peroxidation (LPO), was quantified by the Thiobarbituric Acid (TBA) reactive substances method [18]. Briefly, the supernatants of the liver homogenates were mixed with two volumes of TBA reagent (15% trichloroacetic acid, 0.8% TBA, 0.25N HCl), and heated at 95°C for 15 minutes. Finally, the absorbance of MDA-TBA complex was read for each supernatant at 532nm after centrifugation of the samples for 10 minutes at 4000 rpm and 4°C. The MDA volume was counted, using the standard curve of MDA and the values expressed as nmol/mg of the tissue protein (nmol/mg protein).

Superoxide dismutase assay: The evaluation of Superoxide Dismutase (SOD) activity was performed by the pyrogallol autoxidation procedure [19]. The autoxidation rate of 2 mM pyrogallol in Tris-HCl buffer at pH 8.2 was read kinetically at 420 nm alone and after the addition of 50µl of the tissue samples. One unit of SOD was considered as the amount of enzyme required to inhibit pyrogallol autoxidation up to 50% units per mg of the tissue protein (U/mg protein).

Catalase activity assay: The Catalase (CAT) activity was determined by the procedure based on the amount of decomposition of hydrogen peroxide (H_2O_2) [20]. Briefly, one ml of 30mM H_2O_2 and 50µl of the sample were added to 1.95 mL of phosphate buffer (50 mM; pH 7.0). The absorbance of the solution was read at 240nm in the kinetic mode. The concentration of H_2O_2 was calculated using the following equation: H_2O_2 (mM) = (absorbance×1000)/molar extinction coefficient (43.6 M^{-1} cm^{-1}). The extent of CAT required to breakdown 1µM of H_2O_2 in one minute under standard conditions was considered as being one unit of CAT activity and expressed as U/mg of the tissue protein.

Glutathione assay: Elman's method with minor modifications was used to estimate the Glutathione (GSH) level [21]. For this purpose, 0.25 mL of the reagent, i.e., 10 mM 5, 5′-dithiobis-2-nitrobenzoic acid (DTNB) in 0.1 M phosphate buffer, at pH 8.0 was added to 0.5 mL of the sample and incubated at 20-25°C for 15 minutes in the dark. The samples were read at 412nm and the amount of GSH was calculated, using the GSH standard curve at 412 nm, and the obtained values were expressed as nmol/mg protein.

Statistical analyses: The data were analyzed, using GraphPad Prism software and one-way Analysis of Variance (ANOVA) followed by Tukey's multiple comparison tests. The results were presented as the Mean±SD and the $P \leq 0.05$ were considered as being statistically significant.

Results

Glucose tolerance test: The diabetic rats showed significantly higher blood glucose levels than those of the controls (201.6±6.6 mg/dL/h; $P<0.001$). The exposure of normal rats to Diazinon caused a significant elevation in the AUC_{0-120} values on the glucose tolerance test curve (291.3±13.8 mg/dL/h, $P=0.048$) compared to those in the control group. High AUC_{0-120} (1040±69.6 mg/dL/h, $P<0.001$) was also obtained in diabetic rats exposed to Diazinon compared to those of the diabetic rats that did not receive Diazinon (862.1±147.0 mg/dL/h) (Figure 1).

Liver histopathological findings: As shown in Figures 2A-2D and reflected in Table 1, liver histology in the controls showed normal morphology of the tissue with well-preserved hepatocytes (Figure 2A). Both the diabetic and healthy rats that received Diazinon showed abnormal liver tissue morphologies as characterized by damages in the tissue structures along with hydropic degeneration and increased binucleated hepatocytes, and cytoplasmic vacuolization (fatty changes) in the centrilobular areas (zone 3) (Figures 2B & 2C). The diabetic rats that received Diazinon showed mild lobular necrosis and marked hydropic degeneration, cytoplasmic vacuolization in the hepatocytes (fatty changes) in zone 3, and Kupffer cell proliferation (Figure 2D).

Liver oxidative stress biomarkers: As shown in Figure 3A, there were significantly higher levels of lipid peroxidation in the liver tissue samples of the healthy rats that received Diazinon (71.7±16.4 nmol/mg protein, $P<0.05$) and the diabetic rats that received Diazinon (121.3±43.0 nmol/mg protein, $P<0.001$) than the control rats. The lipid peroxidation levels in the diabetic rats that received Diazinon was higher than the diabetic rats without a Diazinon treatment (57.9±26.8 nmol/mg protein, $P=0.0305$). The superoxide dismutase activities (9.2±3.0 U/mg proteins, $P=0.0305$) in the Diazinon-treated diabetic rats were higher than those in the control (5.1±2.0 U/mg proteins). The activity of superoxide dismutase in the diabetic rats (3.6±2.1 U/mg proteins) was also significantly increased after exposure to Diazinon (6.3±2.8 U/mg proteins, $P=0.0019$) (Figure 3B). Figure 3C indicates that the catalase activities (0.54±0.3 U/mg protein) in the Diazinon-treated diabetic rats were higher than those in

Table 1. Summary of histopathology findings in studied groups

Histopathologic Finding	Control	DM	DZ	DM+DZ
Hydropic degeneration	-	+	+	+++
Fatty change in centrilobular areas	-	+	+	+
Mononuclear cells infiltration	-	-	±	-
Lobular necrosis	-	-	-	+
Kupffer cells hyperplasia	-	-	+	+
Sinusoidal congestion	-	+	++	+
Central vein congestion	±	±	++	++
Portal vein congestion	±	+	+	+
Lobular inflammation	-	-	+	±

Key: -= Not observed, ±= Occasional, += Mild to moderate, ++= Severe, +++= Most severe

Diabetic group (DM), Healthy rats received Diazinon (DZ), Diabetic group received Diazinon (DM+DZ)

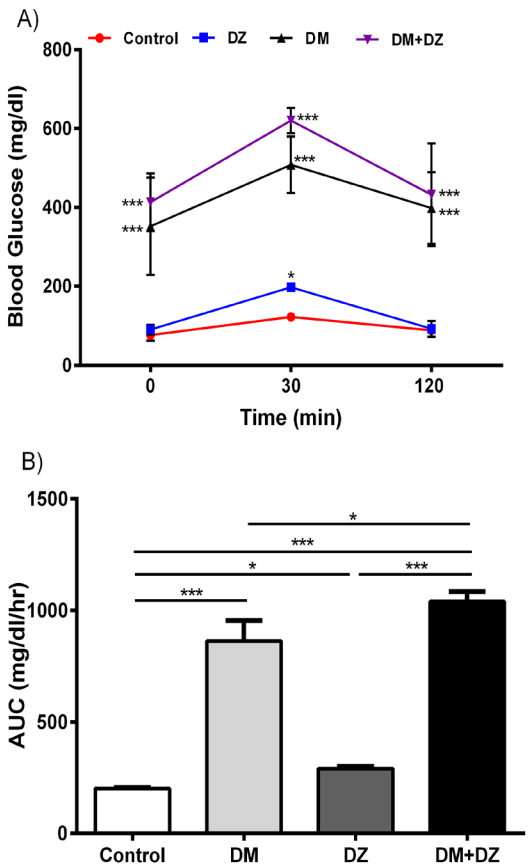

Figure 1. A) Glucose Tolerance Test (GTT) and, B) area under the curve ($AUC_{0\text{-}120}$) of Glucose tolerance test in control group (Control), diabetic group (DM), Diazinon group (DZ), and diabetic group received Diazinon (DM+DZ). Data are expressed as Mean±SD. The significance level set at *P<0.01 and ***P<0.001 for all groups compared to the control group.

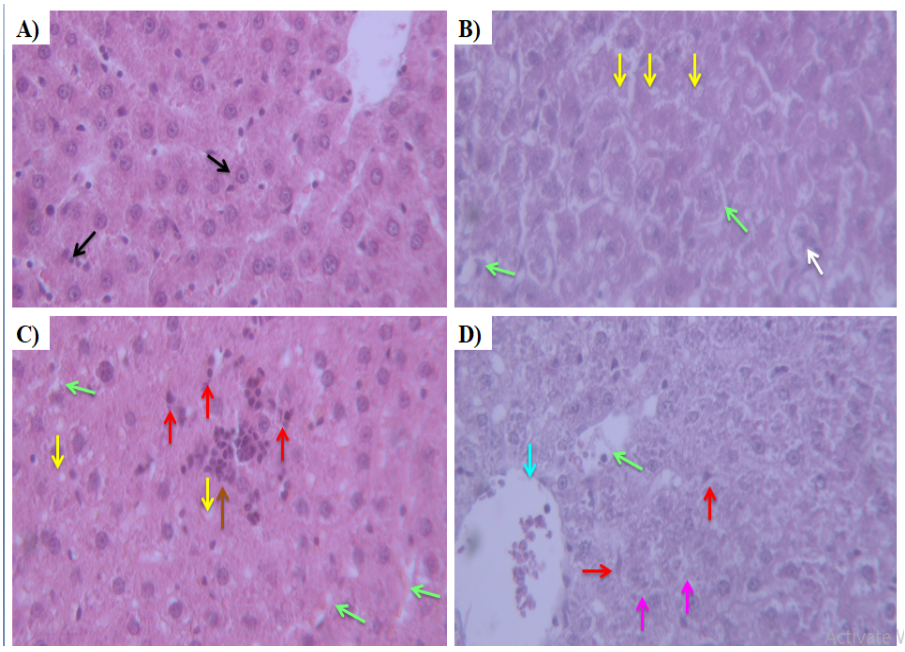

Figure 2. Photomicrographs of liver sections stained with Hematoxylin and Eosin (×400).

A) Control group: preserved architecture with normal Kupffer cells (Black arrows); B) diabetic group: hydropic degeneration of hepatocytes, fatty change in centrilobular areas (yellow arrow), increased binucleated hepatocytes (white arrow) and sinusoidal congestion (green arrows); C) healthy rats received Diazinon: hydropic degeneration of hepatocytes, fatty change in centrilobular areas (yellow arrows), sinusoidal congestion (green arrows), mononuclear cells infiltration (brown arrow), Kupffer cells hyperplasia (red arrows); and D) diabetic group received Diazinon: marked hydropic degeneration of hepatocytes, central vein congestion (blue arrow), necrosis (pink arrows), sinusoidal congestion (green arrows) and Kupffer cells hyperplasia (red arrows)

the control group (0.27±0.20 U/mg protein, P=0.0401), Diazinon group (0.32±0.21 U/mg protein, P=0.0338), and diabetic rats untreated with Diazinon (0.33±0.22 U/mg protein, P=0.0412). The glutathione levels in all of the groups were not significantly different from each other (Figure 3D).

Discussion

Based on our findings, exposure of diabetic rats to repeated low doses of Diazinon elevated the fasting blood glucose in non-diabetic rats but impaired the glucose tolerance in the diabetic rats. These observations agree well with other studies that demonstrated the administration of Diazinon caused significant hyperglycemia [15]. The elevation of blood glucose levels is likely to be a compensatory mechanism in response to the physiological stress caused by OPs and their metabolites due to the depletion of energy reserves. Organophosphates inhibit the secretion or synthesis of insulin [22, 23], and impair the glycogenolysis and gluconeogenesis pathways [24]. Also, the activity of glutamate dehydrogenase is impaired in the pancreatic Langerhans islets [25], which interrupts the normal glucose hemostasis. Another mechanism for the induced hyperglycemia by OPs is the elevation of the muscular glycogenolysis and glycolysis [26].

Hyperglycemia due to OPs exposure induces impaired glycogenolysis and gluconeogenesis caused by defective activities of glycogen phosphorylase and phosphoenolpyruvate carboxy-kinase [2].

As stated by Rezg et al., the hyperglycemia is followed by compensatory insulinemia, which may weaken the metabolic control and result in the loss of physiological response to insulin, leading to diabetes type-2 [27]. Pancreas and liver are the two main organs involved in the induction of diabetes secondary to OPs toxicity [2, 9]. Glucose tolerance test is the most important method for monitoring carbohydrate metabolism in the diagnosis of diabetes in patients with moderate elevation in their fasting blood glucose. Minor changes in blood glucose levels were not noticed after the administration of Diazinon in diabetic rats in the current study, while glucose tolerance test showed remarkable changes among the groups. It may be argued that glucose tolerance test might serve as a reasonable indicator for the assessment of impaired glucose metabolism after exposure to Ops such as Diazinon.

The toxic effect of Diazinon on the liver was evident by the histopathological findings, including hydropic degeneration, necrosis, cellular lipid accumulation, and fo-

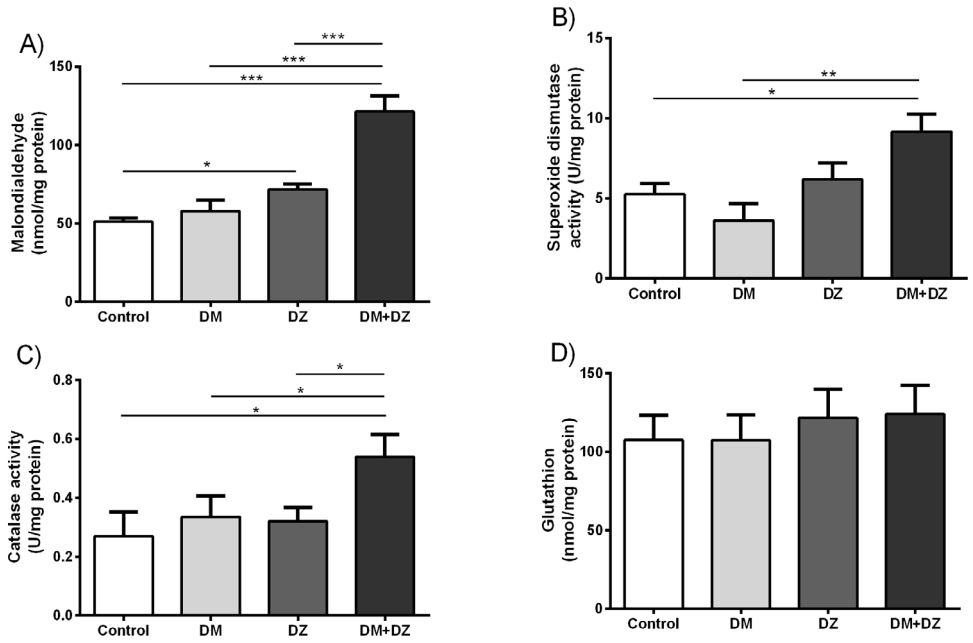

Figure 3. Effect of Diazinon on: A) lipid peroxidation; B) superoxide dismutase activity; C) catalase activity; and D) glutathione level in the liver tissues of control group (Control), diabetic group (DM), Diazinon group (DZ), and diabetic group received Diazinon (DM+DZ). Data are expressed as Mean±SD. The significance level set at *P<0.01 and ***P<0.001 for all groups compared to that of the control group.

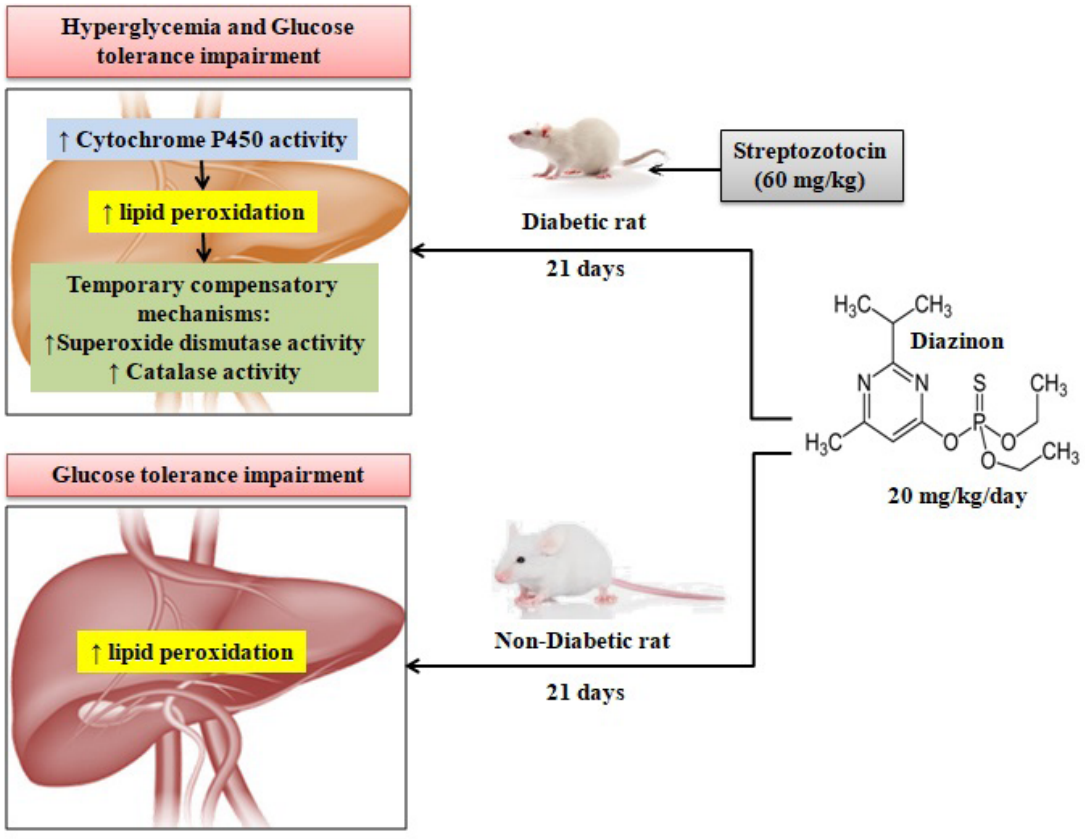

Figure 4. Hepatic oxidative damages and glucose tolerance in diabetic and non-diabetic rats exposed to repeated oral doses of Diazinon.

cal micro-vesicular steatosis [6]. As shown by the results of this study, the destructive effects of Diazinon were clearly demonstrated by the histopathological changes in the rats' liver. After absorption and distribution, Diazinon is known to be metabolized to Diazoxon through oxidative desulfuration by Cytochrome P450 (CYP), leading to the generation of additional reactive metabolites [28]. Organophosphates induce hepatotoxicity through induction of oxidative stress, hepatocyte death, changes in the hepatic metabolism and biotransformation, and mitochondrial disorders [6]. It was also found that Diazinon causes over-production of Diazoxon in diabetic rats by up-regulating the hepatic cytochrome CYP1A2 [14]. As a hepatotoxic agent, Diazinon results in liver damages, including hydropic degeneration, necrosis and focal microvesicular steatosis [29]. In the current study, the exacerbation of the liver histology in diabetic rats that received Diazinon might be due to the synergistic hepatotoxic effect of Diazinon.

In addition to the inhibition of cholinesterase, Diazinon contributes to toxic effects in the liver by the elevation of oxidative stress through over-production of reactive oxygen species and impairment of antioxidant defense system [6]. The imbalance between the generation of reactive oxygen species and the antioxidant defense system causes oxidative damages to the liver, which in turn triggers the compensatory elevations in the activity of antioxidants. In the current study, lipid peroxidation and the activity of antioxidant enzymes were increased, while glutathione, as a radical scavenger of thiol group, did not decrease in the liver samples of all Diazinon-treated groups. This might have contributed to the generation of glutathione through ligation of L-cysteine and glutamate, and the catalytic activity of glutathione synthase. The rise in the enzymatic antioxidant levels might be required to meet the demand for the repair of damaged tissues caused by oxidative stressors.

Conclusions

It may be concluded that exposure to Diazinon, as a widely used Ops, is a risk factor for the induction of diabetes mellitus and aggravation of the diabetic symptoms and side effects. The findings indicate that the induction of oxidative stress and consequent histopathological alterations in liver tissue, as the main organ contributing to carbohydrates metabolism, are the possible mechanisms involved in the exacerbation of Diazinon-induced damages in diabetic rats (Figure 4). The study findings support the role of OPs in the induction of diabetes mellitus and the importance of minimizing OPs applications, thus warrants the search for new and safer pesticides.

Ethical Considerations

Compliance with ethical guidelines

The experiments on animals were approved by the Ethics Committee of Kerman Neuroscience Research Center (Code: IR.KMU.REC.1397.272).

Funding

This research was funded by the Deputy for Research, Kerman University of Medical Sciences.

Authors' contributions

Conceived and designed the research protocol. NS and AN conducted the experiments: Somayyeh Karami-Mohajeri and Niloufar Sinaei; Wrote the initial drafts of the manuscript: Somayyeh Karami-Mohajeri, and Elham Jafari; Analyzed the histopathological data: Elham Jafari. All authors reviewed and approved the final draft of the manuscript.

Conflict of interest

The authors had no conflict of interest.

Acknowledgements

The authors thank Kerman University of Medical Sciences for their support.

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