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Physiological and histopathological changes in the liver of male rats exposed to paracetamol and diazinon

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ABSTRACT

Objective: The present study was conducted to evaluate the adverse effect of exposure to Diazinon (DIA) and Paracetamol (PARA) and their combination on male rats. **Methods:** Rats were orally administered PARA at a dose of 66.66 mg a.i. kg^{-1} body weight (maximum administration dose) and DIA at a dose 12.50 mg a.i. kg^{-1} b.wt. (1/100 LD50) for 28 consecutive days. **Results:** Significantly, decreased of body weights were observed in all treated groups, while significant increase in relative liver weight were recorded in DIA and DIA+PARA-treated groups compared to control rats. Liver dysfunction enzymes (e.g., aspartate aminotransferase, AST; alanine aminotransferase, ALT; alkaline phosphatase, ALP and lactate dehydrogenase, LDH) and Lipid Peroxidation Level (LPO) were increased in DIA, PARA and DIA+PARA-treated groups. Treatment of DIA and DIA+PARA caused significant decrease in the activity of serum Cholinesterase (ChE). PARA, DIA and PARA+DIA treatments caused histopathological changes and decreases in DNA content in liver cells of rats. The severities of such observations were more pronounced in their combined exposure. **Conclusions:** We can conclude that both paracetamol at maximum administration dose and diazinon caused biochemical and histopathological alteration in the liver of male rats. The severities of such observations were more pronounced in their combined exposure. The data throw light on the problem of simultaneous exposure to OPIs and commonly used drugs especially among agriculture sector workers in developing countries, where the handling of drugs (e.g., PARA) is mainly without medical prescription. Further studies, applied to pregnant women, newborns and childhood may be of great significance.

1. Introduction

Every day, people are exposed simultaneously to a mixture of environmental and occupational stressors as a routine part of their existence. Up until recently, about 95% of all chemical toxicity studies were performed on individual chemicals [1, 2]. The biological activity of a chemical may be modified through prior or simultaneous exposure of a test organism to another chemical agent and such interactions might result in a potentiation, summation, or reduction of the ultimate effect of the chemical [3].

Pesticides are occasionally used indiscriminately in large amounts causing environmental pollution and therefore, are a cause of concern. Organophosphorus insecticides (OPIs) are a major component of many pesticides with widespread use in both agricultural and domestic situations. However, approximately 85%-90% of applied agricultural pesticides never reach target organisms, but disperse through the air, soil, and water [4]. Diazinon (DIA) (0,0-diethyl-0-[2-isopropyl-6-methyl-4-pyrimidinyl] phosphorothioate) is a contact OPIs with a broad range of insecticidal activity and widely used throughout the world with applications in agriculture and horticulture [5]. Various reports have been published with respect to DIA and its effects on biochemical and hematological parameters of rats,[6] rabbits,[7] and mice [8].

In fact, the interaction of chemicals with the biological system is a complex phenomenon and is ultimately an expression of the interplay between the environment, the host and chemical substance. Drugs or pharmaceutical products, which are used to cure diseases, are also xenobiotics with both therapeutic/toxic potentials [9]. It is evident from the literature, which is very limited, that drug/insecticide interactions can result in altered response/toxicity, which is of clinical relevance [10].

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Paracetamol (4′-hydroxyacetanilide, N-acetyl p-aminophenol, acetaminophen, PARA) is a commonly used analgesic/antipyretic that causes hepatotoxicity, which can in severe cases lead to liver failure in experimental animals and humans when taken in overdose [11]. The initial step in toxicity is cytochrome P450 metabolism of acetaminophen to the reactive metabolite N-acetylp-benzoquinone imine (NAPQI), which reacts rapidly with glutathione (GSH)[12,13] and efficiently detoxified at therapeutic doses [13]. Thus, PARA metabolism causes dramatic depletion of cellular glutathione levels in the liver [14]. At large doses, the formation of the reactive metabolite exceeds the capacity of hepatocellular glutathione and NAPQI covalently binds to cellular proteins [15,16]. However, in developing countries, the purchase of large quantities of commonly used drugs (e.g. PARA) does not require a medical prescription. Additionally, in recent years it was reported that even borderline high doses are hepatotoxic for some infants [17,18]. Therefore, the purpose of the present study is to throw light into some physiological and histological changes in the liver of male rats exposed to repeated doses of either individual or combined treatments of paracetamol and diazinon.

2. Materials and methods

2.1. Chemicals

Diazinon (Nasr-Cidol® 60% EC) was obtained from El-Nasr Mediate Chemical Co., Egypt. Paracetamol® tablets (The Arab Drug Co., Egypt), each tablet contain 500 mg paracetamol, was purchased from local pharmacies. All other chemicals were of reagent grades and obtained from the local scientific distributors in Egypt.

2.2. Animals

Male Wistar rats (weighting 150-160 g) were purchased from Animal Breeding House of the National Research Centre (NRC), Dokki, Cairo, Egypt. Animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals." The experimental protocols and procedures were approved by the Local Ethics Committee at the National Research Centre (NRC), Dokki, Cairo, Egypt. Animals were housed in clean plastic cages with free access to food (standard pellet diet) and tap water *ad-libitum*, under standardized housing conditions (12h light/dark cycle, the temperature was 22±1 ℃ and a minimum relative humidity of 40%) in the laboratory animal room.

2.3. Experimental protocols

After 1 week of acclimatization to laboratory conditions, the animals were randomly assigned to four groups, each consisting of eight rats as follows: First group (control), the second group (DIA), the third group (PARA) and the fourth group (DIA + PARA). Dosages of DIA and PAR were freshly prepared in distilled water, given via oral route for 28 consecutive days and adjusted weekly for body weight changes. DIA was administered at a dose 12.50 mg a.i. kg^{-1} b.wt. (1/100 LD_{50}) based on published LD_{50} (1250 mg a.i. kg^{-1} b.wt). [19] The selective dose of paracetamol (4 g/personal/day (66.66 mg a.i. kg^{-1} b.wt) = maximum administration dose) based on the manufacture pamphlet and given daily. The control group received an equivalent volume of distilled water (0.5 ml /rat).

At the end of the experimental period, blood was drawn from the reteroorbital venous plexus in glass tubes. Within 20 min of blood collection, the sera samples were drawn from the blood after centrifugation at 3500 rpm (600g) for 10 min at 4℃ using Hereaeus Labofuge 400R, Kendro Laboratory Products GmbH, Germany. The sera was kept in a deep freezer (-20℃) until analyzed.

2.4. Body weights and relative liver weights

After blood collection, the rats were sacrificed by cervical dislocation. Liver of rats was quickly removed and weighted individually. Then, the organ/ body weight ratios were calculated.

2.5. Serum liver biomarkers and lipid peroxidation

All serum biomarkers were determined using a commercial kit in accordance with manufacturers' instructions using a spectrophotometer (Shimadzu UV-VIS Recording 2401 PC, Japan). Lactate dehydrogenase (LDH) was determined using kit obtained from Spinreact (Santa Coloma, Spain), alanine aminotransferase (ALT), albumin and lipid peroxidation from Biodignostic (Egypt). Cholinesterase (ChE) was determined using kit obtained from Lab-Care Diagnostics, India, total protein from Stanbio Laboratory, aspartate aminotransferase (AST) from ELITech Group Co., SEPPIM S.A.S. Zone industrially 61500 SEES France and alkaline phosphatase (ALP) from Analyticon® Biotechnologies AG. Am Mühlenberg 10, 35104 Lichtenfels / Germany.

2.6. Histopathological study

Small pieces of liver tissues in each group were collected in 10% neutral buffered formalin. These tissues were processed and embedded in paraffin wax. Sections of 5 μm thickness were cut and stained with hematoxylin and eosin (H&E). The sections were examined microscopically for the evaluation of histopathological changes. Other sections of liver were stained with Feulgen stain for DNA content. [20] For each rat two slides were prepared, each slid content two sections. Ten field areas for each section were selected and examined for histopathological changes (64×) under light microscope. The liver fields were scored as follows: nil (normal appearance) = 0%, + = mild (cellular disruption in less than 20% of field area), ++ = moderate (cellular disruption of 20% to less than 40% of field area), and +++ = sever (cell disruption of 40 to less than 70% of field area).

2.7. Image analysis of DNA content

Further histochemical evaluation of total DNA content was done using Qwin Leica image processing and analysis system

(Cambridge, UK). Total DNA optical density measurement was done by measuring the mean gray of the Feulgen stain reaction. Average measurements from each tissue section (15 random fields per section) were pooled to determine means.

2.8. Statistical analysis

The data were analyzed by using SPSS (version 14.0) for Windows and expressed as means±S.D. Paired samples t-test was used to compare between the data of the control and those of treatments.

3. Results

3.1. Body weights and relative liver weights

During the course of present investigations, it was observed that the body weights of the control animals increased progressively throughout the study and recorded a net weekly body weight gain of 13.35% (Fig. 1). However, the net weekly body weight gains of the animals intoxicated with DIA and PARA was a markedly significant decrease ($P \leqslant 0.01$) and was 7.58%, & 8.41%, respectively. In the contrast, significant decrease ($P \leqslant 0.05$) of weekly body weight gains was recorded in combined exposure to DIA and PARA (DIA + PARA group) and accounted 10.54% reduction weekly body weight gains. As shown in Fig. 2, the relative liver weights were significantly increased ($P \leqslant 0.01$) in DIA and DIA+PARA-treated groups. The relative liver weight was accounted for 2.79% in control and increase to 3.64% and 3.95% in DIA and DIA+PARA-treated groups.

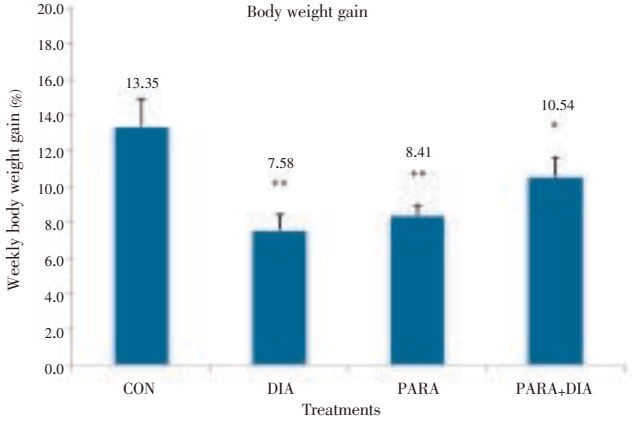

Figure (1): Weekly body weight gains of rats exposed to paracetamol, diazinon, and their combination. Control (Con), paracetamol (PARA) and diazinon (DIA). Each value is a mean of 8 rats ± SD; Statistical difference from the control: * significant at $P< 0.05$& ** highly significant at $P< 0.01$.

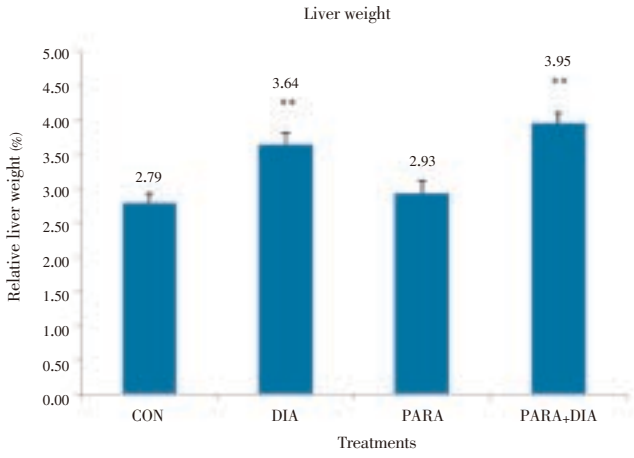

Figure (2): Relative liver weight of rats exposed to paracetamol, diazinon, and their combination. Control (Con), paracetamol (PARA) and diazinon (DIA). Each value is a mean of 8 rats ± SD; Statistical difference from the control: * significant at $P< 0.05$& ** highly significant at $P< 0.01$.

3.2. Serum liver biomarkers and lipid peroxidation

Data in Table (1) shows the activities of AST, ALT, ALP and LDH enzymes in the sera of rats exposed to diazinon and paracetamol for 28 consecutive days. Results showed that the DIA, PARA and DIA+PARA induced significant increase in the activities of AST, ALP and LDH as compared to control group. As shown in Table (2), total protein and globulin were significantly increased ($P \leqslant 0.05$) in the DIA-treated group (7.25±0.46 g/dl & 5.05±0.48 g/dl) compared to untreated control (6.37±0.15 & 4.10±0.22 g/dl). Activity of serum ChE of control and treated groups is shown in Figure 3. The activity of ChE was significantly decreased ($P \leqslant 0.01$) in DIA (1.57 U/ml) and DIA+PARA (1.86U/ml) treated groups when compared to control group (2.14 U/ml). No significant changes in ChE activity were observed in PARA-treated group. Male rats treated with DIA, PARA and DIA+PARA showed highly significant increases ($P \leqslant 0.01$) in serum lipid peroxidation as measured by the amount of MDA formed (Fig. 4). LPO was accounted for 1.70 nmol/ml in control and increase to 2.36 nmol/ml, 2.18 nmol/ml and 2.10 nmol/ml in PARA, DIA and DIA+PARA-treated groups.

Table 1

The activities of AST, ALT, ALP and LDH enzymes in the sera of rats treated with diazinon and paracetamol.

Treatments	AST(U/l)	ALT(U/l)	ALP(U/l)	LDH(U/l)
Control	48.9±5.58	31.0±3.39	106.5±5.0	217.7±9.4
Diazinon (DIA)	58.5±2.71**	45.5±3.39**	165.4±19.3**	255.4±16.7**
Paracetamol (PARA)	64.5±4.91**	34.8±3.08	128.6±12.5*	257.5±18.3*
DIA + PARA	63.31±6.31*	32.7±1.29	181.3±27.1**	281.7±29.3*

Each value is a mean of 8 rats ± SD; Statistical difference from the control: * significant at $P< 0.05$& ** highly significant at $P< 0.01$.

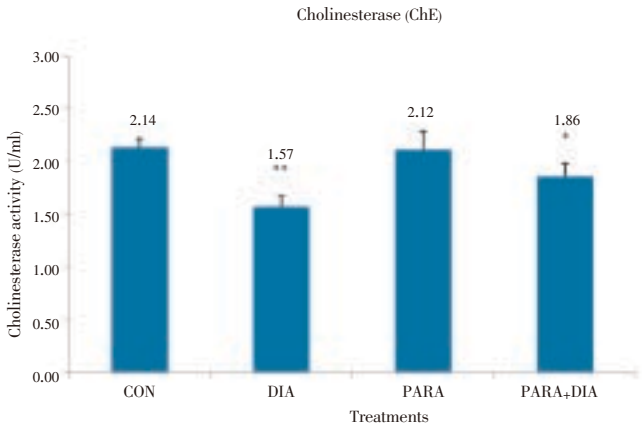

Figure (3): Cholinesterase activity in serum of rats exposed to paracetamol, diazinon, and their combination. Control (Con), paracetamol (PARA) and diazinon (DIA). Each value is a mean of 8 rats ± SD; Statistical difference from the control: * significant at $P< 0.05$& ** highly significant at $P< 0.01$.

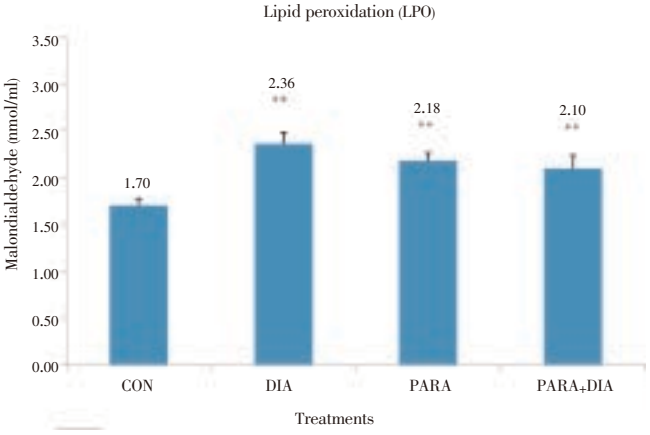

Figure (4): Lipid peroxidation level in serum of rats exposed to paracetamol, diazinon, and their combination. Control (Con), paracetamol (PARA) and diazinon (DIA). Each value is a mean of 8 rats ± SD; Statistical difference from the control: * significant at $P< 0.05$& ** highly significant at $P< 0.01$.

3.3. Histopathological studies

As shown in Figure (5-A), liver sections from control rats showed a normal lobular architecture and normal hepatic cells with a well preserved cytoplasm and well defined nucleus and nucleoli. In PARA-treated group, the histoarchitecture of liver sections of rat showed disarrangement of normal hepatic cells, degeneration of hepatocytes with lymphocyte infiltration, necrosis of cells congestion of portal tract, sinusoidal dilation, pyknotic and binucleate nucleus (Fig. 5-B). There were cellular infiltrations around the central vein and portal tract; degenerative changes of hepatic cells with cell necrosis, pyknotic and binucleate nuclei were recorded in liver sections of DIA-treated rats (Fig. 5-C). In contrast, treatment of DIA+PARA induced marked histopathological lesion which was characterized by liver cells loosing the radial arrangement, diffuse vascular degeneration with lymphocytic infiltration, dilated and congested central vein (Fig. 5-D). The DIA+PARA - treatment showed multiple areas of necrosis, haemorrhagic patches, dilated congested sinusoids, pyknotic, binucleate nuclei and Kupffer cell proliferation. In light microscopic examinations, histopathological changes were observed in the liver of all exposed groups compared to control (Table 3). These changes were more frequent in DIA+PARA-treated group.

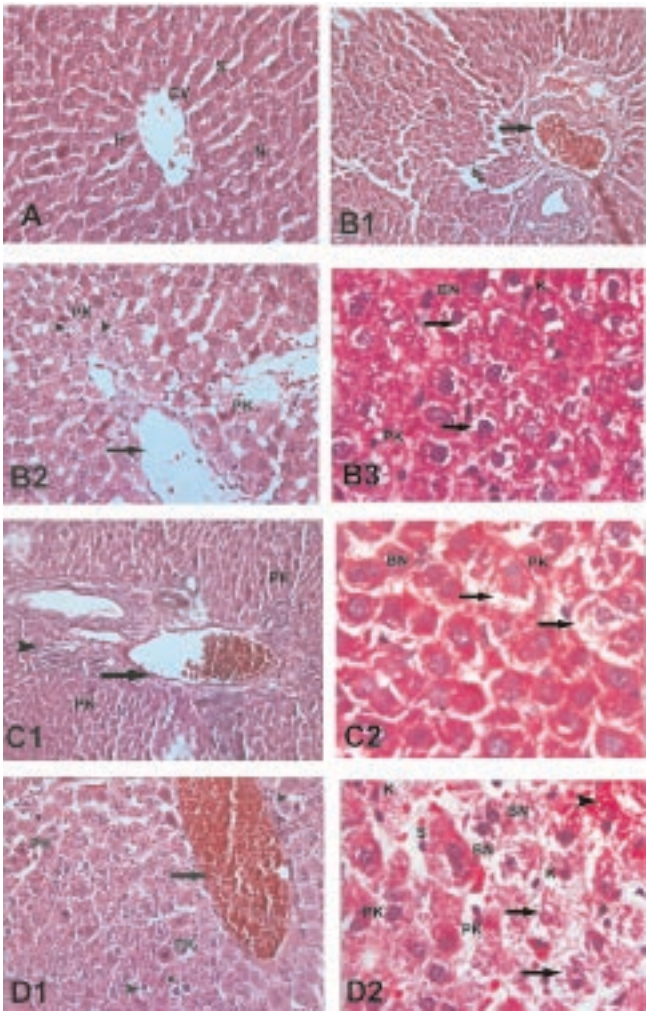

Figure 5: Photographs of sections of the liver of rat show A): control rat showing normal histological structure of hepatocytes (H), central vein (CV), and blood sinusoid (S), nucleus (N). (H&E X400). B1): Liver of rats treated with paracetamol showing dilated and congested portal tract. Notice, the periportal necrosis of the hepatocytes that surrounded the portal area that associated with inflammatory infiltration (arrow), (H&E X400). B2): paracetamol group shows dilated central vein (arrow), and many pyknotic nuclei (PK) of hepatocytes (H&E X400). B3): higher magnification show degeneration (arrow), with many pyknotic nuclei (PK) and binucleated cells of hepatocytes (BN) (H & E X1000). C1): diazinon group show dilated and congested portal tract (arrow) with inflammatory infiltration around the portal area (arrow head) and pyknotic nuclei (PK) of hepatocytes pyknotic nuclei (PK). (H&E X400). C2): higher magnification show vacuolar degeneration (arrow), with pyknotic nuclei (PK) and binucleated cells of hepatocytes (BN) (H&E X1000). D1): Liver of rats treated with diazinon and paracetamol show dilated and congested central vein (arrow), vacuolar degeneration (arrow head), and many pyknotic nuclei (PK) of hepatocytes (H&E X400). D2): higher magnification show vacuolar degeneration (arrow), many pyknotic nuclei (PK) and binucleated cells of hepatocytes (BN), hemorragh (arrow head) (H&E X1000).

3.4. Histochemical studies and DNA content

The histochemical investigation of liver control rat showed that the normal distribution of DNA content and chromatin substances of the cells was marked by Feulgen stain (Fig. 6-A). In PARA and DIA-treated groups mild decrease were found in DNA content in liver cells as compared to control (Fig. 6-B&-C). Treatment of DIA+PARA cause a highly decrease in DNA content in liver cells as compared to control. The nuclei appeared irregular in shape, unlike the control group, with very little peripheral condensed chromatin. (Fig. 6-D). Parallel, the optical densities of total DNA contents were performed using an image analysis system (Figure 7). Results revealed that the histochemical investigation of DNA reaction in liver of rats exposed to PARA and DIA showed a significant decrease ($P \leqslant 0.05$) in total DNA content. In contrast, DIA+PARA cause highly decrease ($P \leqslant 0.01$) in the total DNA content in rat liver.

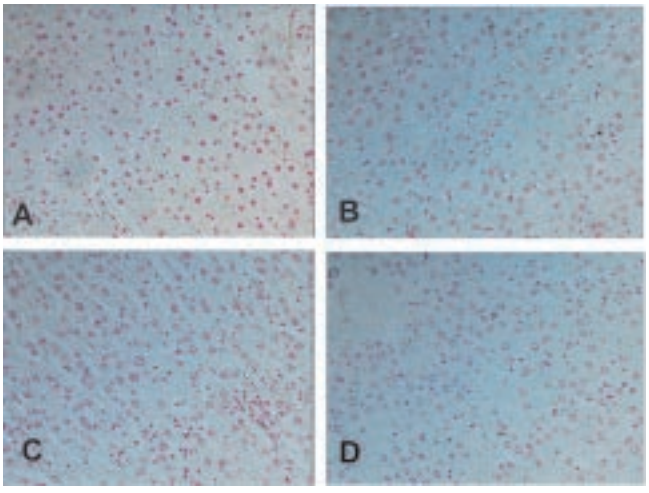

Figure 6: A): Light micrograph of a section in the liver of control group showing normal distribution of DNA content in the nucleus. B): The group that received paracetamol showing a decrease in the DNA content. C): The group that received diazinon showing a decrease in the DNA content. D): The group received paracetamol and diazinon showing a marked decrease in the DNA content in the form of faintly stained nuclei. (Feulgen Stain x400).

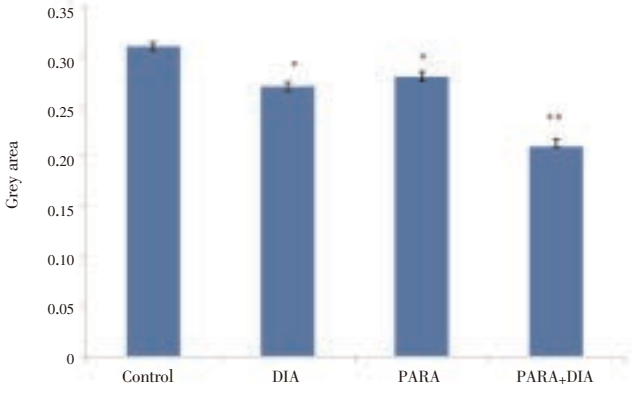

Figure 7: Effect of exposure to paracetamol, diazinon, and their combination on DNA content in rat liver. Control, paracetamol (PARA) and diazinon (DIA). Each value is the mean ± SD; Statistical difference from the control: * significant at $P< 0.05$& ** highly significant at $P< 0.01$.

4. Discussion

In the present study, body weight gains were decreased significantly in rats exposed to diazinon, paracetamol and their mixture. Also, significant ($P \leqslant 0.05$) increase in the relative liver weights were recorded in treating groups. We thought that this decrease in body weight gain may be due to reduced food consumption of exposed-rats (un-tabulated data) and may be due to the overall increased degradation of lipids and proteins as a result of the direct effects of organophosphate compound.[21-23] Stromborg [24] found that dietary levels of diazinon above 50 mg/kg were associated with reduced food consumption, weight loss, and reduction in egg production in northern bobwhites. In addition, others OPIs cause reduction of body weight in rats [23, 25, 26] and mice.[27]

The liver is the primary organ involved in xenobiotics metabolism and is a major target organ for chemicals and drugs. Hepatotoxicity is therefore an important endpoint in the evaluation of the effect of particular xenobiotics. Clinical chemistry and histopathological evaluations are commonly used methods for detecting organ-specific effects related to chemical exposure. Our results showed that the DIA, PARA and DIA+PARA induced significant increase in the activities of AST, ALP and LDH as compared to control group. ALP, ALT, AST and LDH are important indicators of liver damage in clinical findings. These enzymes were secreted into the blood in hepatocellular injury and their levels increase. Changes in these enzyme levels might differ depending on exposure time and dose. The noticed increase in the levels of aminotransferase (ALT and AST) and the level of ALP as well as the decrease in the levels of total protein and albumin in the serum, are the major diagnostic symptoms of liver diseases. [28] Previous studies [23,29-31] showed that OPIs (e.g., diazinon) caused an increase in activities of ALP, ALT and AST enzymes

In the present study, examination of liver function was correlated with the histopathological changes from photomicroscopy observation. These observations indicated marked changes in the overall histoarchitecture of liver in response to PARA, DIA or their combinations, which could be due to its toxic effects primarily by the generation of ROS, causing damage to the various membrane components of the cell. The necrotic conditions observed in the liver of PARA and DIA-treated animals are in corroboration with the observed biochemical changes, wherein an increased level of lipid peroxidation was noticed. Previous studies showed that PARA cause mild focal hepatitis in the lobules and portal areas,[32,33] necrosis in rats [34] and heamorrhagic necrosis in humans mostly characcterised by pyknosis and eosinophilic cytoplasm.[34,35] PARA overdose can cause liver function failure, and death in human as well as experimental animals.[36,37] This could be due to the formation of highly reactive radicals because of oxidative threat caused by paracetamol.[38] LPO is known to disturb

Table 2

The concentrations of protein, albumin and globulin in the sera of male rats treated with diazinon and paracetamol.

Treatments	Total protein(g/dl)	Albumin (A)(g/dl)	Globulin(G)(g/dl)	A/Gratio
Control	6.37±0.15	2.27±0.07	4.10±0.22	0.55
Diazinon (DIA)	7.24±0.46*	2.19±0.04	5.05±0.48*	0.44
Paracetamol (PARA)	6.46±0.25	2.17±0.12	4.29±0.06	0.51
DIA + PARA	6.24±0.15	2.07±0.08*	3.28±0.20	0.48

Each value is a mean of 8 rats ± SD; Statistical difference from the control: * significant at $P< 0.05$& ** highly significant at $P< 0.01$.

Table 3

Histopathological changes in the liver of male rats treated with diazinon and paracetamol, based on scoring severity of injury@ .

Treatment	Hepatic injury	
	Observation	Severity
Control	Normal	−(normal appearance)(0%)
Diazinon (DIA)	Cellular infiltrations around the central vein and portal tract Degenerative changes of hepatic cells with cell necrosis Pyknotic and binucleate nuclei	+ (cellular disruption in less than 20% of field area)
Paracetamol (PARA)	Disarrangement of normal hepatic cells Degeneration of hepatocytes with Lymphocyte infiltration Necrosis of cells congestion of portal tract Sinusoidal dilation Pyknotic and binucleate nucleus	+ (cellular disruption in less than 20% of field area)
DIA + PARA	Liver cells loosing the radial arrangement diffuse vascular degeneration with lymphocytic infiltration Dilated and congested central vein Necrosis Haemorrhagic patches Dilated congested sinusoids Pyknotic, binucleate nuclei Kupffer cell proliferation	++ (cellular disruption of 20% to less than 40% of field area)

@ Scores in terms of numerical values are mentioned in histopathological studies section.

the integrity of cellular membranes, leading to the leakage of cytoplasmic enzymes.[39] Therefore, the increased activities of liver enzymes in the present study could be due to severe histopathological damages and hepatotoxicity of DIA.[8,23] Significant damage in the hepatic cells and glucose metabolism in liver was observed as the result of DIA administration.[40] Previous studies showed that DIA cause significant changes in biochemical and hematological parameters of rats,[6] rabbits,[7] and mice.[8]

In fact, oxidative stress plays an important role in the toxicity of various xenobiotics, including organochlorine and organophosphate pesticides. [23,41] Oxidative stress is a balance between free radical production and antioxidant activity, and the raised LPO may be due to decreased antioxidant activity.[42,43] Increased levels of MDA, an end product of LPO, were observed in the sera of rats treated with PARA, DIA and PARA+DIA. The elevated level of LPO could be due to the increased peroxidation of lipid membranes.[23, 44]

One consequence of oxidative stress and lipid peroxidation is the formation of DNA adducts. Since DNA is believed to be the target molecule for carcinogens, endogenous DNA adducts derived from oxidative stress, lipid peroxidation, and other sources have been proposed to contribute to the etiology of human cancers.[45,46] The histochemical changes of PARA, DIA and PARA+DIA showed declines of DNA staining probably reflect a decline metabolism and oxidative damage of DNA during DIA and PARA+DIA exposure. However, besides being a potent source of ROS, OP compounds also show alkylating properties and alkylating agents are known to cause DNA damage. Alkylation of bases either directly or indirectly via protein alkylation is probably involved in DNA damage.[39] Ansari and Kumar[47] reported that exposure of zebra fish to the DIA for up to 168 hours cause significant reduction in DNA, RNA and the total protein in the liver.

Our previous study indicates that insecticides in both in vivo and in vitro tests alter the enzyme activities associated with antioxidant defense mechanisms. [23, 42-44, 48] In addition, oxidative stress has been implicated in pesticide-induced neurotoxicity based on its role in the cascade of biochemical changes that lead to dopaminergic neuronal cell death.[49] Thomas[11] showed that PARA could in severe cases lead to liver failure in experimental animals and humans when taken in overdose. The mechanisms of PARA hepatotoxicity have been extensively studied.[14,50] The initial step in toxicity is cytochrome P450 metabolism of acetaminophen to the reactive metabolite N-acetyl p-benzoquinone imine (NAPQI). NAPQI is an electrophilic intermediate, which is oxidized by cytochrome P450 and converted to a highly reactive and toxic metabolite as in cases of APAP overdose.[51,52] NAPQI can rapidly react with glutathione (GSH) and lead to a 90% total hepatic GSH depletion in cells and mitochondria, which can result in hepatocellular death and mitochondrial dysfunction.[14]

NAPQI can also induce DNA strand breaks and promote apoptosis and hepatic necrosis [14,53-55]

5. Conclusion

We can conclude that both paracetamol at maximum administration dose (66.66 mg a.i. kg-1 b.wt) and diazinon at 12.50 mg a.i. kg-1 b.wt. caused biochemical and histopathological alteration in the liver of male rats. The severities of such observations were more pronounced in their combined exposure. The data of this study throw light on the problem resulting from simultaneous exposure to OPIs and some commonly used drugs especially among agriculture sector workers in developing countries, where the handling of such drugs (e.g. PARA) is mainly without medical prescription. Further studies, applied to pregnant women, newborns and childhood may be of great significance.

Conflict of interest statement

We declare that we have no conflict of interest.

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