Glutathione, Antioxidant Enzymes and Oxidative Stress in Acute and Subacute Exposure of Diazinon-Mediated Renal Oxidative Injury in Rats

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Abstract: This study aimed at investigating the possible nephrotoxic effects of the diazinon induced oxidative stress in rats following the acute and subacute administration. Oxidative stress markers in renal tissues, as well as serum biochemical parameters, were evaluated using colorimetric spectrophotometric techniques. Our data showed that diazinon administration to rats induced oxidative stress in kidney, as evidenced by increasing of renal lipid peroxidation level which was accompanied by decreased activities of antioxidant enzymes and depletion in the level of GSH (p<0.05) compared to saline-treated control. The activities of renal γ-glutamyl transpeptidase and quinone reductase were increased (p<0.05) compared to saline-treated control. In addition, diazinon treatment augments renal injuries as evident by the increase in serum creatinine and blood urea nitrogen (p<0.05). Histopathological analysis of renal tissues was in concurrence with the biochemical studies. Overall results suggest that oxidative stress-induced lipid peroxidation and alteration in activities of glutathione and antioxidant enzymes play roles in diazinon-mediated renal injury and toxicity in rats.

Key words: Diazinon, GSH, Oxidative stress, Antioxidant enzymes, Renal dysfunction.

1. Introduction

Glutathione (GSH), a multifunctional tripeptide (gamma-glutamyl-cysteinyl-glycine) is the most abundant low-molecular-weight thiol (0.5–10 mmol/L) which is present in almost all cells in high concentration [1, 2]. It plays an important role in cellular metabolism. These include maintenance of sulphydryl dependent enzymes, preservation of the integrity of biological membranes, protection of cellular components against reactive oxygen species (ROS) such as free radicals and peroxides [3], reaction with various electrophiles, physiological metabolites (e.g., estrogen, melanins, prostataglandins, and leukotrienes), and xenobiotics [4], removal of formaldehyde etc [5]. In addition to this, GSH also conjugates with NO to form an S-nitroso-glutathione adduct, which is cleaved by the thioredoxin system to release GSH and NO (both are important for hepatic action of insulin-sensitizing agents) [4, 6].

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Diazinon (O,O-diethyl-O-[2-isopropyl-6-methyl-4-pyrimidinyl] phosphorothioate), is an organophosphate insecticide with a broad range of activities which inhibit acetylcholinesterase activity [7]. It has been widely and effectively used throughout the worlds with applications in agriculture and horticulture for controlling insects in crops, ornamentals, lawns, fruits, and vegetables and as a pesticide in domestic and agriculture [8]. Diazinon affects mitochondrial membrane transportation in rat liver [9]. Furthermore, it disturbs the cytochrome P450 system in human liver [10, 11]. Exposure of hepatocytes to diazinon accelerated cell killing as well as the loss of cellular ATP [9]. Meanwhile, diazinon causes toxic effects on other organisms [12]. Diazinon is classified as a moderately hazardous class II-organophosphate insecticide. It can be absorbed through the digestive system, the skin, or via the respiratory tract when inhaled. Although it is mainly eliminated by the kidney, microsomal enzymes in the liver oxidize diazinon producing more potent acetylcholinesterase inhibitors, such as diazoxon, and hydroxydiazoxon and hydroxydiazinon [7]. We have shown that chronic diazinon exposure induced renal oxidative stress in rats [13]. As might be predicted that diazinon exposure stimulates ROS production, which may be important in initiating all these effects [9-13]. Because a deficiency of GSH leads to oxidative stress in many tissues [14], we hypothesized that diazinon-mediated depletion of renal GSH might contribute to diazinon-mediated renal injury. In this study, we show that acute and subacute diazinon-mediated exposure deplete renal GSH, causes a decrease in the activities of GSH metabolizing and antioxidant enzymes, and leads to the excessive generation of lipid peroxides, events concomitant with the onset of kidney damage. Our data, therefore, suggest that simultaneous depletion of renal antioxidant enzymes and concomitant generation of oxidants may be important contributors to diazinon-mediated renal oxidative injury.

2. Materials and methods

Chemicals

Diazinon, tris HCl, flavin adenine dinucleotide (FAD), thiobarbituric acid (TBA), oxidized and reduced glutathione, β-nicotinamide adenine dinucleotide phosphate reduced (NADPH), 1-chloro-2,4-dinitrobenzene (CDNB), glutathione reductase, 5,5’-dithio-bis-2-nitrobenzoic acid (DTNB), sulfosalicylic acid (SSA), glycylglycine, bovine serum albumin (BSA), tween 20, hydrogen peroxide (H₂O₂), 2,6-dichloroindophenol, diacetyl monoxime, urea, picric acid, sodium tungstate, ethylenediaminetetraacetic acid (EDTA), creatinine and L-γ-glutamyl-p-nitroaniline were purchased from either Sigma Chemical Company, St Louis, MO, USA or Aldrich, USA. All other solvents and chemicals used were either of analytical grade or the highest purity commercially available.

Animals

Adult Sprague Dawley male rats at 4-8 weeks of age, with an average body weight of 150-200 g were used in the study. They were obtained from Tes Jaya Laboratory Services,
Pulau Pinang, Malaysia. The animals were kept in plastic (polypropylene) cages using paddy husk bedding at room temperature (25 ± 1°C) and 50 ± 5% humidity with water and chow ad libitum. The animals were segregated into four groups (6 rats in each group) and acclimatized to the environment for one week before the commencement of the experiment. All experiments were conducted in accordance with the guidelines for the care and use of laboratory animals of the National Institute of Health [15] and were also approved by the Animal Ethics Committee, Universiti Malaysia Sabah (UMS/IP7.5/M3/4-2012).

Experimental protocol

For acute study, the rats were divided randomly into four groups of six rats per group, Group I (n=6) normal control treated with saline for seven consecutive days; Group II, III, and IV (n=18 having 6 rats per group) were treated with diazinon at a dose level of 10 mg/kg body weight, 15 mg/kg body weight and 30 mg/kg body weight in corn oil via oral (gavage), respectively, for seven consecutive days. Similarly, for subacute study, dose regimen was the same as described for acute study, except the animals were treated daily for a period of two weeks. The selection of dose regimen was based on published data [13, 16, 17]. 24 Hours after the last dose of diazinon or saline, these animals were sacrificed by cervical decapitation to excise the kidney which was washed with ice-cold phosphate-buffered saline and it was stored at a temperature -80°C until further analysis. Blood samples were collected by cardiac puncture into sterile tubes and was centrifuged at 3,000 x g for 15 min at 15°C to obtain serum, which was then used for the determination of blood urea nitrogen and serum creatinine. Portion of the kidney was fixed in 10℅ neutral formalin for histopathological light microscopic analysis and rest of the kidneys was homogenized in chilled phosphate buffer (0.1 M, pH 7.4) containing KCl (1.17% w/v) using homogenizer (Polytron PT 1200E, Switzerland). The homogenate was centrifuged at 2,000 × g for 15 min at 4°C in a refrigerated centrifuge (model Avanti J-E) to separate the nuclear debris. The aliquot so obtained was centrifuged at 10,000 × g for 30 min at 4°C to obtained post-mitochondrial supernatant (PMS) which was subjected to biochemical analysis [18].

Biochemical assays

The level of reduced glutathione (GSH) in the kidney was determined through the measurement of 5-thiol-2-nitrobenzoic acid formation at 412 nm [19]. Lipid peroxidation in renal tissues was determined spectrophotometrically from the reaction of malondialdehyde with thiobarbituric acid conjugate formation at 535 nm [20]. Activity of glutathione peroxidase was estimated spectrophotometrically at 340 nm by measuring the rate of oxidation of nicotinamide adenine dinucleotide [18]. Glutathione reductase activity was determined by the reduction of oxidized glutathione using NADPH as a coenzyme at 340 nm [21]. Catalase activity was determined by monitoring the decomposition of hydrogen peroxide spectrophotometrically at 240 nm [22]. Glutathione S-transferase activity was
estimated by the conjugation reaction of 1-chloro 2,4 dinitrobenzene to glutathione reduced by measuring changes in absorbance at 340 nm [23]. Quinone reductase was determined by measuring the disappearance of 2,6-dichlorophenolindophenol at 600 nm [24]. The method of Orlowski and Meister [25] with γ-glutamyl p-nitroanilide as substrate was used for the determination of γ-glutamyl transpeptidase activity. Blood urea nitrogen was estimated by diacetyl monoxime method [26] which condenses with urea to form a pink chromogen measured at 520 nm. Serum creatinine was estimated by the method of Hare [27]. Alkaline picrate, a color compound was produced by the reaction of creatinine with picric acid in an alkaline medium, which was measured spectrophotometrically at 546 nm. Method of Aitken et al. [28] was used for protein determination in each sample using bovine serum albumin as a standard.

**Histopathological assessments**

Sections of kidneys were excised for histopathological studies and fixed in 10% neutral buffered formalin solution for at least 24 h, trimmed, processed, embedded in paraffin, sectioned 5 to 6-μm in thickness and stained with hematoxylin and eosin stain (H&E) for light microscopic examination. Sections were analyzed by pathologist and photomicrographs were taken.

**Statistical analysis**

Statistical analysis was carried out by using SPSS 17.0 windows statistical package (SPSS Inc, Chicago IL). Differences between groups were analyzed using analysis of variance (ANOVA) followed by Dunnet’s multiple comparisons test. All data points are presented as the treatment group means ± standard error of the mean (S.E.). A P values <0.05 were regarded as significant.

**3. Results**

GSH acts as a non-enzymatic antioxidant in the detoxification pathway that reduces the reactive toxic metabolites of diazinon. The acute and subacute effects of diazinon administration on GSH levels in the rat kidney are shown in figure 1. The result revealed that treatment of animals with all doses of a diazinon caused a significant reduction in renal GSH in the treated groups when compared to the saline-treated control group (p<0.05).

Glutathione reductase, catalase, glutathione peroxidase, glutathione S-transferase, and quinone reductase were measured in the kidney as an index of antioxidant status of the tissues. The activities of antioxidative enzymes viz., glutathione reductase, catalase, glutathione peroxidase, and glutathione S-transferase were decreased as compared to saline-treated control group (p<0.05) after acute and subacute diazinon treatment (Table 1). This showed that the renal tissues were undergoing oxidative stress where enzymatic antioxidants were
consumed largely. On the contrary, the activity of quinone reductase was increased after diazinon administration at all doses and time studied (Table 1).

Oxidative stress is the formation of oxidized macromolecules such as lipid peroxidation. Therefore, lipid peroxidation used as a marker of oxidative stress and measured in all groups in term of MDA formation. As shown in figure 2, MDA formation in the diazinon-treated group was significantly elevated compared to saline-treated control group (p<0.05), reflecting the formation of activated species, indicating diazinon induced oxidative renal damage.

The acute and subacute effects of diazinon treatment on renal γ-glutamyl transpeptidase are shown in figure 3. Diazinon administration showed an increase in renal γ-glutamyl transpeptidase. At the higher dose of diazinon 30 mg/kg body weight, the induction was around 109% and 112% of saline-treated control value (p<0.05), whereas at the lower dose of diazinon 10 mg/kg body weight, it reached a value of 103% and 103% that of saline-treated control (p<0.05) (Fig 3).

Blood urea nitrogen and serum creatinine is a useful and inexpensive method for evaluating kidney function. As shown in table 2, one and two weeks after diazinon administration, an elevation of creatinine and blood urea nitrogen in sera was evidence indicating the level of kidney damage caused by diazinon as compared to saline-treated control group (p<0.05).

Renal injuries in rats treated with diazinon at all doses and time was revealed by histopathological studies as shown in figure 4. Histopathological studies of rats treated with diazinon following acute and subacute exposure have shown many abnormalities in kidney glomeruli and convoluted tubules compared with saline-treated control (Fig 4). Histological changes in kidney were more pronounced at a higher dose of diazinon 30 mg/kg body weight as compared to lower diazinon dose of diazinon10 mg/kg body weight. The main findings were the appearance of kidney swelling with obliteration of space in Bowman’s capsule, glomerular sclerosis, interstitial spaces, necrosis of proximal tubules and congested blood vessels etc.

4. Discussion

Glutathione (GSH) is a major cellular antioxidant and is crucial for maintaining the balance between oxidation and antioxidation [3]. It plays important roles in cellular detoxification and protecting the cells against oxidative stress [4-6]. Oxidative stress has been reported to be the chief initiator of most of the kidney disorders. Many studies have demonstrated that diazinon can induce dysfunction and histopathological changes in kidney by generating free radicals [7-14]. Moreover, these free radicals also attack on the antioxidant defense system, leading to loss of antioxidant components [8]. The results of the present study demonstrated that diazinon administration to rats significantly increased MDA formation while it depletes enzymatic and non-enzymatic antioxidant armory in the kidney including catalase, glutathione reductase, glutathione S-transferase, glutathione peroxidase

139
and GSH levels, suggesting impairment in the antioxidant defense system of rats. In addition, it also suppresses the activity of glucose-6-phosphate dehydrogenase. Similar alterations in activities of antioxidant enzymes including the enzyme involved in glutathione metabolism and associated oxidative stress have been reported following diazinon administration to rats [7-14]. The observed elevations in γ-glutamyl transpeptidase may further deplete GSH, leading to oxidative stress. Finally it is well known that diazinon catalyzes the generation of highly reactive oxidants [7-14]. Creatinine and blood urea nitrogen are vital indicators of kidney function [26, 27]. The higher levels of urea and creatinine after acute and chronic administration of diazinon suggest that it may interfere with renal function. Therefore, the diazinon-mediated renal damage may be due to the decrease in tissue levels of GSH, a fall in the activities of antioxidant enzymes including the enzymes involved in glutathione metabolism and simultaneous generation of various oxidants including lipid peroxide and other ROS.

Histopathological analysis of renal tissues was in concurrence with the biochemical studies. In fact, histopathological changes, seen in the kidney of rats treated with diazinon are characterized by a narrowed Bowman’s space, degeneration of tubular epithelial cells and widened tubular lumen. Besides these, extensive necrosis in renal proximal tubules was also observed. Our results confirmed previous findings of others who had found degenerative changes in the kidney of rats exposed to methidathion and methyl parathion (OPs pesticides) [29-32].

In summary, our observations clearly indicate that diazinon treatment eventuates in decreased renal GSH, a fall in the activities of antioxidant enzymes including the enzymes involved in glutathione metabolism and excessive production of oxidants with concomitant renal damage, all of which are involved in the cascade of events leading to diazinon-mediated renal injury.

Acknowledgments

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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FIGURES CAPTIONS 1 to 4

**Fig. 1** Dose-dependent acute and sub acute effects of diazinon administration on renal GSH in rats.
Each value represents mean ± SE of six animals. Saline treated animals served as control. Dose regimen, treatment protocols and other details are described in text. Values marked with asterisks differ significantly from the corresponding values for saline treated control (*p<0.05).

**Fig. 2** Dose-dependent acute and sub acute effects of diazinon administration on renal lipid peroxidation in rats.
Each value represents mean ± SE of six animals. Saline treated animals served as control. Dose regimen, treatment protocols and other details are described in text. Values marked with asterisks differ significantly from the corresponding values for saline treated control (*p<0.05).

**Fig. 3** Dose-dependent acute and sub acute effects of diazinon administration on renal γ-glutamyl transpeptidase in rats.
Each value represents mean ± SE of six animals. Saline treated animals served as control. Dose regimen, treatment protocols and other details are described in text. Values marked with asterisks differ significantly from the corresponding values for saline treated control (*p<0.05).

**Fig. 4** Dose-dependent acute and sub acute effects of diazinon administration on renal histopathological alterations in rats.
Saline treated animals served as control. Dose regimen, treatment protocols and other details are described in text. (a) Saline treated control (b) Corn oil treated (c) Diazinon treated (10 mg/kg body weight) (d) Diazinon treated (15 mg/kg body weight) (e) Diazinon treated (30 mg/kg body weight). Specimens stained with haematoxylin and eosin. (a, b, c, & d) X 20.
<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Glutathione reductase (nmol NADPH oxidized/min/mg protein)</th>
<th>Catalase (nmol H₂O₂ consumed/min/mg protein)</th>
<th>Glutathione peroxidase (nmol NADPH oxidized/min/mg protein)</th>
<th>Glutathione-S-transferase (nmol CDNB conjugate formed/min/mg protein)</th>
<th>Quinone reductase (nmoldichloroindophenol reduced/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control saline</td>
<td>Acute 48.8 ±0.89 Subacute 48.7 ±0.60</td>
<td>Acute 73.7 ±1.01 Subacute 72.5 ± 1.90</td>
<td>Acute 73.5 ±1.09 Subacute 83.2 ± 2.77</td>
<td>Acute 17.1 ± 0.25 Subacute 20.2 ± 0.29</td>
<td>Acute 7.4 ± 0.10 Subacute 4.4 ± 0.16</td>
</tr>
<tr>
<td>Diazinon (10 mg/kg body weight)</td>
<td>Acute 48.0 ±0.54 Subacute 47.4 ±1.33</td>
<td>Acute 71.5 ±0.81 Subacute 70.0 ± 2.89</td>
<td>Acute 72.8 ±0.98 Subacute 81.1 ± 2.96</td>
<td>Acute 16.4 ± 0.20 Subacute 19.3 ± 0.25</td>
<td>Acute 7.7 ± 0.15 Subacute 4.8 ± 0.24</td>
</tr>
<tr>
<td>Diazinon (15 mg/kg body weight)</td>
<td>Acute 47.3 ±0.45 Subacute 46.8 ±0.89</td>
<td>Acute 71.4 ±1.28 Subacute 66.6 ± 2.53</td>
<td>Acute 71.7 ±0.80 Subacute 81.4 ± 1.67</td>
<td>Acute 16.3 ± 0.35 Subacute 19.3 ± 0.20</td>
<td>Acute 7.8 ± 0.16 Subacute 4.8 ± 0.18</td>
</tr>
<tr>
<td>Diazinon (30 mg/kg body weight)</td>
<td>Acute 45.2 ±0.44* Subacute 45.1 ±0.49*</td>
<td>Acute 69.0 ±0.88* Subacute 62.4 ± 5.40*</td>
<td>Acute 70.5 ±0.69 Subacute 77.2 ± 1.62</td>
<td>Acute 16.3 ± 0.54 Subacute 18.6 ± 0.35*</td>
<td>Acute 8.2 ± 0.14* Subacute 5.3 ± 0.11*</td>
</tr>
</tbody>
</table>

Each value represents mean ± SE of six animals. Saline treated animals served as control. Dose regimen, treatment protocols and other details are described in text. Values marked with asterisks differ significantly from the corresponding values for saline treated control (*p<0.05).
**Table 2:** Acute and subacute effects of diazinon on blood urea nitrogen and serum creatinine in rats.

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Blood urea nitrogen (mg/dl)</th>
<th>Serum creatinine (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acute</td>
<td>Subacute</td>
</tr>
<tr>
<td>Control saline</td>
<td>24.6 ± 1.35</td>
<td>24.2 ± 1.78</td>
</tr>
<tr>
<td>Diazinon (10 mg/kg body weight)</td>
<td>25.6 ± 2.18</td>
<td>27.1 ± 2.08</td>
</tr>
<tr>
<td>Diazinon (15 mg/kg body weight)</td>
<td>27.4 ± 1.77</td>
<td>31.5 ± 1.52*</td>
</tr>
<tr>
<td>Diazinon (30 mg/kg body weight)</td>
<td>30.7 ± 1.46*</td>
<td>36.1 ± 2.65*</td>
</tr>
</tbody>
</table>

Each value represents mean ± SE of six animals. Saline treated animals served as control. Dose regimen, treatment protocols and other details are described in text. Values marked with asterisks differ significantly from the corresponding values for saline treated control (*p<0.05).
Fig. 1 Shah et al.
**Acute**

![Graph showing lipid peroxidation levels in Acute phase with different treatments compared to control saline.]

**Subacute**

![Graph showing lipid peroxidation levels in Subacute phase with different treatments compared to control saline.]

**Fig. 2 Shah et al.**
Acute

Subacute

Fig. 3 Shah et al.
Fig. 4 Shah et al.