Diazinon-induced ulcerative keratitis in C57bl/6 mice

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Abstract As a well-known organophosphate insecticide, diazinon (DZN) has been used for several decades in agriculture. The major signs of ophthalmic toxicity of DZN have been reported to be cholinergic overstimulation (lacrimation, myosis). Here, we report, for the first time, ulcerative keratitis in C57bl/6 mice secondary to sub-acute exposure to DZN. Four groups of female C57bl/6 mice were administered intraperitoneally either DZN (1, 5, 25 mg/kg/day) or vehicle for 14 consecutive days. Then, histopathological examinations on mice eyes were performed using light microscope and scored for corneal keratitis. Furthermore, blood cholinesterase activity, and hematologic examinations were performed. Data indicated a significant ulcerative keratitis with prompt vision loss in mice exposed to 25 and 5 mg/kg/day ($P < 0.05$) doses. These results suggest that diazinon might induce ulcerative keratitis secondary to its immunosuppressive effects at high doses in C57bl/6 mice.

Keywords Diazinon · Cornea · Keratitis · Immunosuppression

Introduction

Diazinon (DZN) (IUPAC name: O,O-diethyl O-[4-methyl-6-(propan-2-yl)pyrimidin-2-yl] phosphorothioate) has been widely used as a household and agriculture insecticide for decades to control insects and other pests [1]. Its residential uses have been canceled since 2004 by US Environmental Protection Agency but its agricultural application is still very common [2]. It belongs to a class of pesticides called organophosphates (OPs) which could efficiently phosphorylate and inhibit pests' acetylcholine esterase resulting in cholinergic overstimulation [3]. It also could inhibit other esterases which might play a role in its not-fully-understood mechanism of neurotoxicities [4]. Organophosphates have shown a wide range of toxic insults to many organs in human and some other animals, probably through oxidative stress or indirectly through their immunotoxic effects [5–8]. As a medium toxic insecticide, DZN has been used for several decades in agriculture with no reported ocular toxicities (e.g., corneal adverse effect) except for cholinergic overstimulation in the eye [1]. The cholinergic overstimulation signs mainly include lacrimation, myosis, and blurred vision [9]. The current study describes the ulcerative keratitis induced by DZN in C57bl/6 mice which has not been reported in this species so far.

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Materials and methods

Animals and exposure schedules

Female C57bl/6 mice (4–6 weeks old) were purchased from Pasteur Institute (Karaj Breeding Center, Iran) weighting 19–21 g at the beginning of the experiment. After 1 week (for acclimatization), four groups of mice (six to seven mice per group) were treated either by different doses of diazinon (DZN: 1, 5, 25 mg/kg/day, i.p.) or received only vehicle (negative control), for 14 consecutive days (five injections per week). Mice were housed in polystyrene cages (six to seven mice per cage) with free access to food and water with a 12/12 light–darkness cycles and ambient temperature of 20–25 °C.

Chemicals

Diazinon technical grade (Daewoo Chemical Division Ltd; purity >98 %, $d=1.15$ g/ml) was offered by the National Institute of Agriculture Supporting Services (Tehran, Iran) and was diluted in sweet almond oil (SAO) pharmaceutical grade, purchased from a domestic source to prepare appropriate concentrations of 0.2, 1, and 5 mg/ml DZN in SAO. These oily solutions were prepared weekly and stored at 4 °C. The solutions were prewarmed to 37 °C prior to injection.

Hematological examinations

On day 14, mice were anesthetized by injecting intraperitoneal pentobarbital (50 mg/kg) and 0.5 ml of heart blood was collected by a heparinized syringe and hematological examinations (WBC, CBC differential, and hemoglobin concentration) were performed using Coulter-Counter®.

Determination of RBC cholinesterase activity

The activity of acetyl cholinesterase (true cholinesterase) in compact RBCs was measured using colorimetric method previously described by Ellman et al. and reported as millimoles per liter per minute [10]

Ophthalmologic examination of mice vision and cornea

Mice were checked every day for their vision status. Before and during DZN injection, the eyes were observed for any gross changes in the cornea and vision status according to a score table developed originally for keratitis evaluation [11] with some minor modifications (Table 1). In the case of difference in scores of each mouse eyes, the higher score was reported and used for statistical analysis.

Table 1 Keratitis score based on macroscopic observations

Score	Corneal haziness ^a	Vision loss ^b
0	–	–
1	+	+
2	++	++
	+++	+
3	+++	+++

^a Corneal haziness scale: normal cornea (–), mild changes in cornea color and its clarity (+), significant changes in cornea color with partial turbidity (++), gross and complete cornea haziness (+++)

^b Vision loss (blindness scale): normal vision with normal head reflexes to visual stimulus (–), delayed or decreased head reflexes to visual stimulus (+), partial field blindness (++), complete blindness (no reflexes to visual stimulus) (+++)

Eye histopathology

The anesthetized mice were sacrificed by cervical dislocation at the end of the experiment and the whole scalp containing the eyes was collected and fixed in 10 % formalin. After staining the 5 µm section of the eyeballs with hematoxylin and eosin, the cornea was observed and scored with light microscope for ulcerative keratitis according to Table 2.

Statistical analysis

The quantitative data reported as mean±SD and were analyzed using one-way ANOVA followed by Dunnet's post hoc test. Histopathological findings, reported as median in score tables, were analyzed using the nonparametric

Table 2 Ulcerative keratitis score according to the microscopic findings

Score	Microscopic findings
0	Normal cornea with intact epithelium and stromal layer thickness <85 µm
1	Intact epithelium with a mild increase in stromal thickness (85–125 µm) and scanty PMNLs (<50 cells/field) in stromal layer
2	Marked increase in stromal thickness (>125 µm), mild detachment of epithelium, numerous inflammatory cells in stroma (>50 cells/field)
3	Significant increase in stromal thickness (>150 µm), detachment/rupture of corneal epithelium, numerous inflammatory cells in stroma (>50 cells/field)

In the case of different scores for each mouse eyes, the higher score was reported for statistical analysis

PMNLs polymorphonuclear leucocytes cells

Table 3 Hematology results for different doses of DZN-treated mice and control group (sweet almond oil: SAO) at the end of experiment

Hematological assays	DZN (mg/kg)			
	0	1	5	25
RBC ($\times 10^6/\mu\text{lit}$)	8.6 $\pm$ 0.7	8.4 $\pm$ 0.6	8.3 $\pm$ 0.7	8.1 $\pm$ 0.5
Hemoglobin (g/dl)	14.1 $\pm$ 0.6	13.8 $\pm$ 0.5	13.9 $\pm$ 0.6	13.2 $\pm$ 0.7
WBC (cells/ μl)	9,266 $\pm$ 633	9,012 $\pm$ 644	8,425 $\pm$ 576	8,125 $\pm$ 510
Lymphocyte	7,376 $\pm$ 544	6,848 $\pm$ 403	6,536 $\pm$ 400	6,155 $\pm$ 327
Neutrophil	1,163 $\pm$ 105	1,266 $\pm$ 122	1,543 $\pm$ 131*	1,497 $\pm$ 131*
Monocyte	546 $\pm$ 133	425 $\pm$ 114	340 $\pm$ 69*	288 $\pm$ 101*

* $P < 0.05$ compared to the control group

Kruskal–Wallis test. P values < 0.05 were considered as statistically significant.

Results

Hematological examinations

In order to evaluate the hematologic status of the mice exposed to different doses of DZN, the animals' blood samples were analyzed and the corresponding data are shown in Table 3. The results indicated that while there were no significant differences in total WBC or lymphocytes percentage, a significant difference in the neutrophil and monocyte percentages among all DZN-treated groups compared to the control (Table 3) exist. Also, the blood hemoglobin levels showed no significant difference compared to the control group.

Determination of RBC cholinesterase activity

The mice's RBC cholinesterase activity results indicated that DZN at medium (5 mg/kg) and high doses (25 mg/kg, i.p. for 14 days) has a significant anticholinesterase effect ($P < 0.05$) which indicates marked systemic exposure to DZN (Table 4).

Table 4 RBC cholinesterase activity of mice treated with different doses of DZN

	DZN (mg/kg)			
	0	1	5	25
Cholinesterase activity (mmol/lmin)	10.1 $\pm$ 0.3	8.8 $\pm$ 0.5	5.0 $\pm$ 0.7*	2.8 $\pm$ 0.8**

* $P < 0.05$

** $P < 0.01$

Vision and macroscopic cornea examination

Mice vision and macroscopic cornea examination results showed that keratitis (determined by gross changes in the cornea) has occurred during and at the end of exposure to DZN. It was observed in most of the animals treated with high doses of DZN (25 mg/kg) while in a few animals at medium dose (5 mg/kg, i.p. for 14 days; Table 5).

Eye histopathology

The histopathological results of the eyes are shown in Table 6 which indicates partially a dose-dependent relationship with corneal microscopic changes in the DZN-treated groups. The inflammatory cells infiltrated into the cornea and consequent ulcerative keratitis are observable in Figs. 1, 2, and 3.

Discussion

Subchronic exposure to DZN, like other OP insecticides, could inhibit significantly the RBC cholinesterase (true cholinesterase) [9] which have been used as a marker of exposure to OPs [12]. In our study, the inhibition of this enzyme has been monitored as a marker of exposure (Table 4).

Table 5 Gross changes in mice cornea (corneal haziness) for different doses of DZN during the exposure period

DZN doses (mg/kg)	Exposure day			
	0	3	7	14
0	–	–	–	–
1	–	–	–	–
5	–	–	–/+	–/+
25	–	–/+	++	++

Scores were determined according to the table score (Table 1)

Table 6 Light microscopic changes (ulcerative keratitis) observed in mice treated with different doses of DZN

	DZN doses (mg/kg)			
	0	1	5	25
Ulcerative keratitis score ^a	–	–	+	+++

^a Scores were determined according to the table score (see Table 2)

Ocular adverse effects resulting from diazinon exposure has been reported to be the same as the other OPs insecticides, mainly consisting of cholinergic overstimulation such as lacrimation and myosis [9]. However, these signs are mostly observed with acute toxicity and there are no reports on specific ophthalmologic effects with diazinon after chronic/subchronic exposure [13]. According to the obtained results in this study, gross changes in C57bl/6 female mice

eyes could occur after subchronic exposure to DZN (25 mg/kg/day for 14 days) manifested as ulcerative keratitis with corneal haziness. This ocular toxicity showed, to some extent, dose- and time-dependent effect at 1–25 mg/kg/day for 14 days (Tables 5 and 6).

The extensive infiltration of inflammatory cells into the stromal layer (Figs. 2 and 3) might trigger the corneal haziness and subsequent mice field blindness via cytokines release resulting histopathological changes [14]. These changes could not be interpreted in the shade of DZN cholinesterase inhibitory effects. The RBC cholinesterase activity measured at the end of this experiment could only be used as a marker of subchronic exposure to DZN and based on the best of our knowledge, there is no correlation between cholinesterase inhibition and histopathological changes observed in the cornea [13].

The microscopic examinations of the affected corneas could not differentiate between a direct toxicity and fungal/microbial infection, the later cause is more probable. It has been well documented that immunosuppressive agents (i.e., steroids) could facilitate the bacterial proliferation, most commonly *Streptococcus viridans*, on the corneal surface after minor trauma [15]. Subsequent corneal ulcer could disrupt the epithelium layer with a profound polymorphonuclear leucocytes infiltration into the stromal layer [14, 15]. Hematological examination results of DZN-treated C57bl/6 mice (Table 3) are in concordance with previous immunotoxicological findings showing a suppressive effect on both humoral and cellular activities [7]. This might be causative for microbial/fungal infections and secondary keratitis [15]. On the other hand, DZN did not induce any significant changes in the mice blood hemoglobin levels or RBC counts (Table 3). The risk of fungal infection keratitis has been showed to be higher in C57bl/6 mice receiving cyclophosphamide (an immunosuppressive agent) [16]. Nevertheless, the exact mechanism of DZN-induced ocular toxicity after systemic exposure needs further investigations.

Many toxic agents including insecticides induce their chemical insults towards different tissues by oxidative stress [17–20]. Diazinon could directly or indirectly (via its metabolites) produce reactive oxygen species which is assumed to be involved in DZN mechanism of toxicity in other organs (e.g., DZN-induced nephrotoxicity and neurotoxicity) [21, 22]. This oxidative stress might be responsible for the immunotoxic effects of DZN and consequent keratitis induced by infections.

As a conclusion, diazinon could induce ulcerative keratitis secondary to its immunosuppressive effect at high doses in C57bl/6 mice. This keratitis might lead to severe stromal damage resulting to absolute blindness in these animals.

Fig. 1 Normal cornea (vehicle-treated group), with intact corneal epithelium. P.C. posterior chamber, A.C. anterior chamber

Fig. 2 Numerous inflammatory cells (*PMNLs* polymorphonuclear leucocytes), across the cornea observed in DZN treated group at high dose (ulcerative keratitis score: +++). *P.C.* posterior chamber, *A.C.* anterior chamber

Fig. 3 A profound ulcerative keratitis (DZN-treated group) with increase in stromal thickness full of inflammatory cells (*PMNLs* polymorphonuclear leucocytes) and (ulcerative keratitis score: +++)

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