Toxicology Reports 10 (2023) 32–39

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Role of biotransformation in the diazinon-induced toxicity in HepG2 cells and antioxidant protection by tetrahydrocurcumin

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ARTICLE INFO

Handling Editor: Prof. L.H. Lash

Keywords:
HepG2 cell line
Organophosphorus insecticides
Toxicity
RONS
Antioxidants

ABSTRACT

Diazinon (DZN) is an insecticide extensively used to control pests in crops and animals. However, its indiscriminated use may lead to liver damage in animals and humans. This study aimed to evaluate the toxicity of DZN (25–150 μM) on human hepatoblastoma (HepG2) cells after 24 and 48 h of exposure and the role of its biotransformation on the toxicological potential. We also tested the protective effect of tetrahydrocurcumin (THC), an antioxidant agent, in the DZN-induced citotoxicity. DZN caused cytotoxicity in the HepG2 cells, inhibiting cell proliferation and reducing cell viability in a dose- and time-dependent manner. The pre-incubation of HepG2 cells with chemical inducers of cytochrome P450 monooxygenase 3-methylcholanthrene and phenobarbital resulted in a further decrease of cell viability associated with DZN exposure. In addition, the metabolite diazoxon was more toxic than DZN. Our results also revealed that THC alleviated DZN-induced cytotoxicity and reactive oxygen and nitrogen species (RONS) generation in HepG2 cells. In conclusion, our data provide novel insights into the involvement of biotransformation in the mechanisms of DZN-induced cytotoxicity and suggest that amelioration of RONS accumulation might be involved in the protective effect of THC on DZN-induced liver injury.

1. Introduction

Diazinon (DZN) is a non-systemic organophosphorus insecticide used in agriculture to control soil and foliage insects and pests on a variety of fruit, vegetable, nut and field crops. It is also used to control ectoparasites on sheep, pigs, cows, horses, goats and pets [3,10]. DZN inhibits the enzyme acetylcholinesterase (AChE), which catalyzes the hydrolysis of the neurotransmitter acetylcholine (ACh) in cholinergic synapses and neuromuscular junctions. This leads to ACh accumulation in the nervous system, resulting in the insect's paralysis and, eventually, death [9].

In the environment, DZN is moderately persistent, but highly mobile. It can be adsorbed by the soil and degraded by several processes, including hydrolysis, photolysis, and biodegradation [3,73], but it also may moveaway from the application site by leaching or volatilization, being commonly detected in groundwater, drinking water and surface water of several countries ([29,38,39]; Dahmardeh Behrooz, 2021). The concentration of DZN in these water systems is variable. In a Canadian study, water samples taken from raw, treated and distribution systems showed a maximum detected level of 0.43 μg DZN/L [35]. In addition, environmental residues of DZN have been detected in the air, dust, soil and food [20,34,73].

Exposure of laboratory animals to DZN elicits a series of toxic effects such as: structural changes in proteins, changes in liver enzymes, swelling of mitochondria in hepatocytes, production of free radicals, depletion of antioxidants and the induction of oxidative stress, causing dysfunction of the liver, kidney, neurological system, and pancreas [22–24,32,4,72].

DZN is metabolized in the liver through phase I and phase II

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https://doi.org/10.1016/j.toxrep.2022.12.005
Received 5 October 2022; Received in revised form 27 November 2022; Accepted 11 December 2022
Available online 12 December 2022

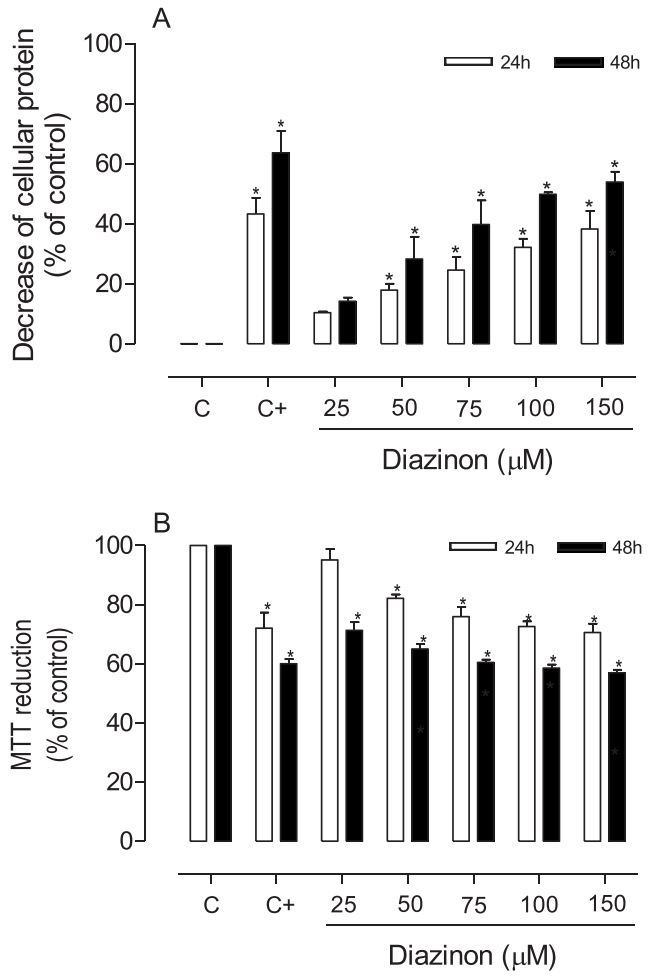

Fig. 1. Effect of diazinon on proliferation (amount of cell protein) (A) and viability (MTT reduction) (B) of the HepG2 cell line after 24 and 48 h of exposure. The graphs represent the mean ± SEM of three independent experiments. *p < 0.05 in relation to the control group. C = control, 0.1% DMSO only; C+ = 100 μM tert-butyl hydroperoxide (A) and 10 μM carbonyl cyanide m-chlorophenyl hydrazone (B).

biotransformation reactions, resulting in its detoxification or bioactivation. The main degradative mechanisms are thiol ester hydrolysis, side chain oxidation, isopropyl group hydroxylation and pyramidyl ring conjugation with reduced glutathione (Matsumura, 1985). Activation products include several DZN monohydroxides [33] and diazoxon (DZO) (Matsumura, 1985), which is a more potent AChE inhibitor than DZN itself [67]. Although studies have been conducted to compare the potential of inhibition of DZN and DZO in AChE activity [16,50,74], no studies were found reporting the effect of cytochrome P450 monooxygenase-mediated biotransformation in the DZN-induced toxicity and the cytotoxicity of DZO in HepG2 cells.

HepG2 is a cell line derived from a human hepatoblastoma that has been commonly used as a tool in the evaluation of xenobiotic metabolism and liver toxicity. Regarding the weak or absent expression of some members of the cytochrome P450 (CYP) superfamily [30], which are involved in phase I xenobiotic oxidation in the liver, HepG2 cell has retained most of the metabolic functions of normal hepatocytes [7]. HepG2 cells also provide a suitable in vitro model to study the involvement of specific endpoints as mitochondrial toxicity and oxidative stress in the hepatotoxicity [31,63].

Tetrahydrocurcumin (THC), a curcumin metabolite, possesses strong antioxidant activity. Both in vitro and in vivo studies have suggested that THC scavenges free radicals such as superoxide, hydroxyl radicals, ferric ions and peroxyl radicals efficiently [48,53]. The hepatoprotective effect of THC has been demonstrated in chloroquine- and arsenic-induced toxicity in rats [45,49].

In vivo administration of DZN causes an increase in the levels of biomarkers of oxidative stress such as lipid peroxidation and protein carbonylation in the liver. It also causes a decrease in the activity of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), and a decrease in antioxidant defenses like reduced glutathione (GSH) [22,43]. The co-administration of vitamin E and curcumin can ameliorate oxidative stress markers [43].

So, in the present study, we conducted experiments to determine: 1) the cytotoxic effects of varying concentrations of DZN on HepG2 cells; 2) the role of biotransformation in the cytotoxic effects of DZN; 4) the comparison between the cytotoxicity of DZN and DZO; 4) the mechanism by which THC protects against DZN-induced citotoxicity. Our results provide new evidence for understanding the mechanisms of DZN-induced liver injury and indicate THC as a promising preventive agent.

2. Materials and methods

2.1. Chemicals

DZN (O,O-Diethyl O-[4-methyl-6-(propan-2-yl)pyrimidin-2-yl] phosphorothioate; purity 100%) and THC (1,7-Bis(4-hydroxy-3-methoxyphenyl)− 3,5-heptanedione; purity ≥ 96%) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and DZO (Diethyl (6-methyl-2-propan-2-ylpyrimidin-4-yl) phosphate; purity 97.6%) was purchased from Chem Service (West Chester, PA, USA). All other reagents were of commercial products of standard chemical grade. Ultrapure water (Direct Q3, Millipore, Bedford, MA, USA) was used to prepare the solutions. The volume of dimethyl sulfoxide (DMSO) used to dissolve DZN, THC and DZO never exceeded 0.1% (v/v) of the total medium and had no effect on the assays.

2.2. Cell culture

The human hepatocellular carcinoma HepG2 cell line was provided by Dr. Daniel Junqueira Dorta (Department of Chemistry, Faculty of Philosophy, Sciences and Letters of Ribeirão Preto-Brazil). Cells were cultured in Minimum Essential Medium (MEM) containing 10% fetal bovine serum, 1% penicillin/streptomycin-stabilized solution, 2 mM L-glutamine, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate. Growing conditions were 5% CO_2 at 37 °C until reaching a confluence suitable to commence testing. Prior the experiments, adequate amounts of cells were plated and incubated for an additional 24 h, in order to ensure adequate adhesion.

2.3. Cell proliferation assay

For cell proliferation studies, HepG2 cells were seeded at a density of 5×10^4 cells/well into 96-well culture plates and the effects of the DZN were assessed using the sulforhodamine B (SRB) colorimetric assay [70]. After 24 or 48 h of treatment with DZN (25–150 μM), the medium was discarded and cells were washed with phosphate-buffered saline (PBS). The range of DZN concentrations was chosen based on responses seen in our previous study using isolated rat liver mitochondria [44]. Next, the cells were fixed with absolute methanol for 2 h. The fixed cells were then washed with PBS, stained with 0.5 % SRB in 1 % acetic acid solution for 1 h and then washed with a 1% acetic acid solution to remove excess probe. The SRB attached to the cell membranes was extracted using 1 mL of a 10 mM Tris solution, pH 10.0. Finally, the absorbance of the dye was measured at 540 nm in a MultiSkan microplate reader (Thermo Fisher Scientific, Vantaa, Finland). Each test was performed in at least three replicates.

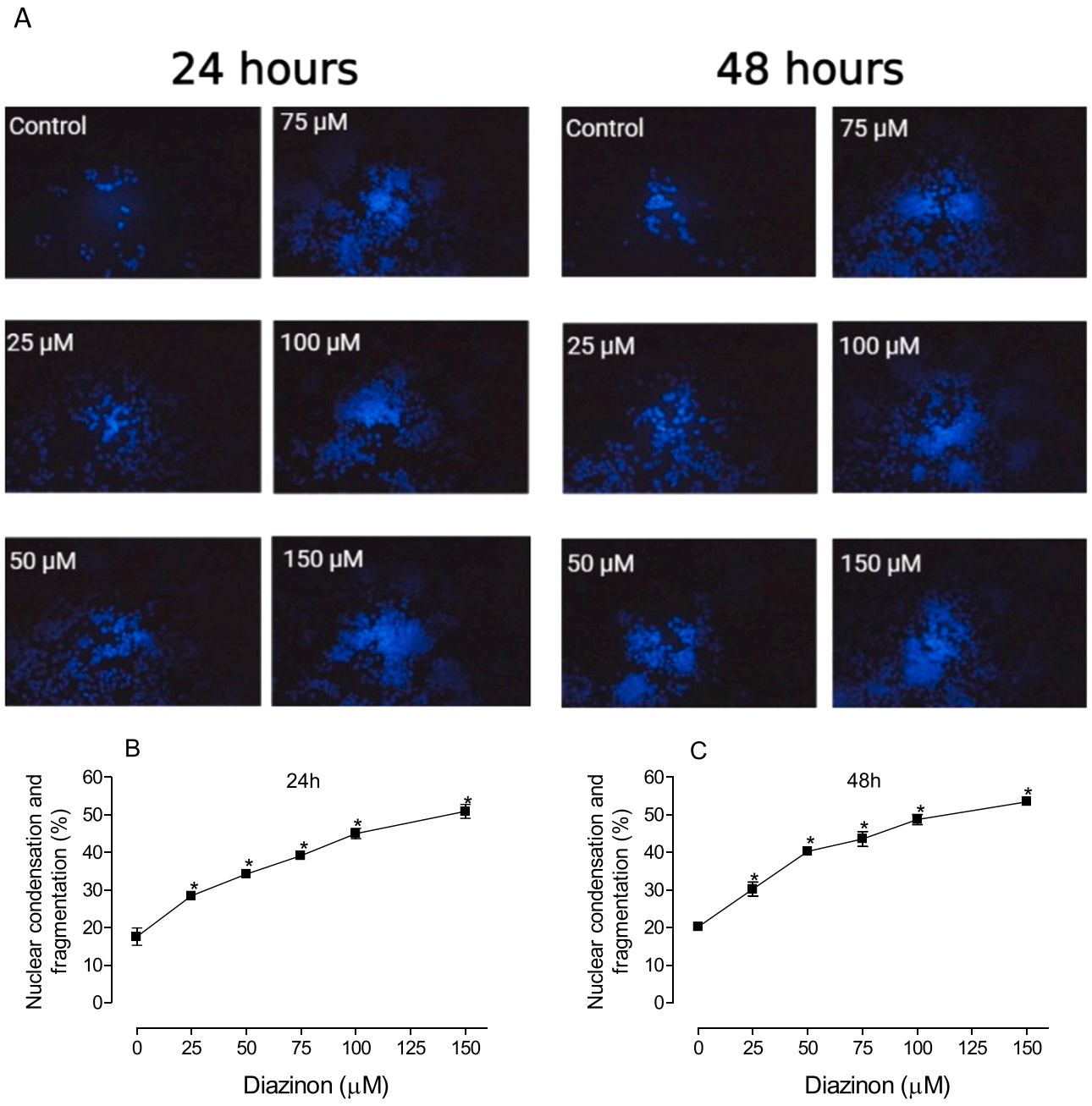

Fig. 2. Effect of diazinon on nuclear condensation and fragmentation of the HepG2 cell line after 24 (A) and 48 (B) h of exposure evaluated by fluorescence microscopy. The graphs represent the mean ± SEM of three independent experiments. *$p < 0.05$ in relation to the control group. C = control, 0.1% DMSO only. (C) Hoechst 33258 fluorescence staining in HepG2 cells (magnification, ×20).

2.4. Cell viability assay

The effect of DZN on HepG2 cell viability was determined using MTT (3-(4,5-dimethylthiazol-2-yl)− 2,5-diphenyl tetrazolium bromide) assay as described by us previously [31]. Briefly, 2×10^3 HepG2 cells/well were seeded in 96-well plates and treated with DZN (25–150 μM). After 24 and 48 h of incubation, MTT solution (20 μL of 5 mg/mL in PBS) was added to each well and incubated at 37 °C in an atmosphere containing 5% CO_2 for 3 h. The formazan crystals formed were dissolved in 200 μL of DMSO solution in 0.2 M glycine buffer, pH 10.2, and the absorbance of the supernatant was measured at 570 nm using a MultiSkan microplate reader (Thermo Fisher Scientific, Vantaa, Finland).

2.5. Condensation and nuclear fragmentation

Apoptotic cells were quantified by nuclear morphological changes (chromatin with visible signs of marginalization and fragmented nuclei), detected by fluorescence microscopy of cells stained with Hoechst 33342, at excitation wavelengths of 330–380 nm and an emission wavelength of 480 nm. HepG2 cells (5×10^4 cells/mL) were cultured and treated on coverslips in 6-well plates. After 24 or 48 h of treatment with DZN (25–150 μM), the cells were washed with culture medium and fixed on coverslips with methanol for 2 h at − 20 °C, stained with Hoechst 33342 (5 μg/mL) for 30 min at 37 °C and mounted on glass slides. Nuclear fragmentation was evaluated and photographed using a Leica model DM 2500 fluorescence microscope. Cell counts were performed using Leica QWin 3.0 software. Cells in several randomly chosen

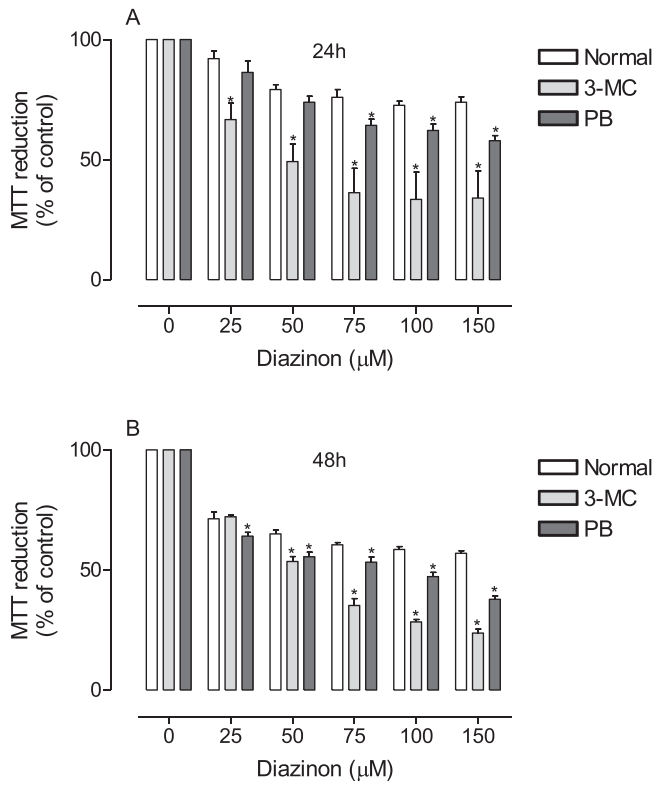

Fig. 3. Effect of pretreatment with 3-methylcholanthrene (3-MC, 2 μM) and phenobarbital (PB, 1 mM) on diazinon-induced cytotoxicity in HepG2 cells after 24 (A) and 48 (B) h of exposure. The graphs represent the mean ± SEM of three independent experiments. *$p < 0.05$ in relation to the effect of diazinon on normal cells (no previous treatment). 0 = control, 0.1 % DMSO only.

fields were evaluated and the number of cells showing nuclear fragmentation was expressed as a percentage of the total number of cells.

2.6. Effect of biotransformation on DZN cytotoxicity

HepG2 cells were exposed to DZN (25–150 μM) after previous treatment with cytochrome P450 monooxygenase inducers: 1 mM phenobarbital (PB) (dissolved in sterile PBS) and 2 μM 3-methylcholanthrene (3-MC) (dissolved in DMSO) for 48 h. These final concentrations of the inducers were chosen based on responses seen in previous experiments performed by [15]. For cytochrome P450 monooxygenase induction, the medium was changed every 24 h. Cell viability was monitored by MTT as described in item 2.4.

2.7. Comparative effect of cytotoxicity of DZN and DZO

HepG2 cells were exposed to DZN and DZO (25–150 μM) for 24 and 48 h. The cytotoxicity of the compounds was evaluated as cell viability monitored by MTT as described in item 2.4.

2.8. Effect of THC on DZN toxicity

THC was incubated with HepG2 cells at concentrations of 25, 50 or 100 μM for 30 min before the cells were exposed to 75 μM DZN. The THC concentrations used was chosen based in the cell viability tests performed in a previous study using HepG2 cells [27]. After 24 or 48 h of exposure to the insecticide, cell viability was evaluated using MTT dye, as described in item 2.4.

2.9. Effect of THC on DZN-induced RONS production

HepG2 cells was incubated with three different concentrations of THC (25, 50 and 100 μM) for 30 min before the addition of 75 μM DZN. After 24 or 48 h of exposure to the insecticide, RONS production was evaluated using CM-H_2DCFDA, a non-fluorescent compound that becomes fluorescent in the presence of intracellular oxidation [14]. Following the treatment, the cells were further incubated with a solution CM-H_2DCFDA (2 mM) at 37 °C for 1 h. The fluorescence intensity was detected at an excitation wavelength of 503 nm and at an emission of 528 nm using an RF-5301PC fluorescence spectrophotometer (Shimadzu, Tokyo, Japan). The results were expressed as the difference in fluorescence intensity in relation to the control group. A tert-butyl hydroperoxide (100 μM) solution was used to induce oxidative stress.

2.10. Statistical analysis

The data were presented as the mean ± SEM. Statistical analysis was performed with GraphPad Prism, v 4.0 for Windows (GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Dunnett's test were used to the multiple comparisons. Individual groups were compared using the unpaired Student's t-test. The values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Effect of DZN on cell proliferation and viability

The results showed that DZN inhibited cell proliferation in a dose-dependent manner. A significant effect was observed at concentrations above 50 μM at both time points (24 and 48 h) (Fig. 1A). DZN induced a decrease in cell viability in a time- and dose-dependent manner. The insecticide affected the ability of HepG2 cells to reduce MTT at the concentrations above 50 μM following 24 h of exposure and above 25 μM following 48 h of exposure (Fig. 1B).

3.2. Effect of DZN on nuclear condensation and fragmentation

The effect of DZN on chromatin condensation was evaluated by staining with Hoechst 33342 and is shown in Fig. 2. DZN induced a dose-dependent effect, with a significant increase in the number of cells with DNA condensation and fragmentation, characteristic of cells undergoing apoptosis, at concentrations above 25 μM at both time points (24 and 48 h).

3.3. Effect of biotransformation on DZN cytotoxicity

To investigate the role of metabolism in the toxicity of DZN, the influence of the inducers of cytochrome P450 monooxygenase (CYP) 3-MC and PB was studied. CYP-induced HepG2 cells were more sensitive with regard to the decrease of cell viability promoted by DZN after 24 or 48 h of exposure (Fig. 3). The effect of 3-MC was more pronounced than that of PB at both time points (24 and 48 h).

3.4. Comparative effects of DZN and DZO on cell proliferation and viability

DZO reduced cell proliferation and viability in a dose and time-dependent manner. A significant effect was observed for DZO concentrations above 25 μM following 24 or 48 h of exposure (Fig. 4).

In comparison with the parent compound, DZO reduced cell proliferation and viability more effectively at both incubation times. Treatment of HepG2 cells with the different concentrations of DZN resulted in a dose- and time-dependent inhibition of cell proliferation as 42 % and 56 % for 24 and 48 h exposure, respectively. Treatment of HepG2 cells with different concentrations of DZO also resulted in a dose- and time-

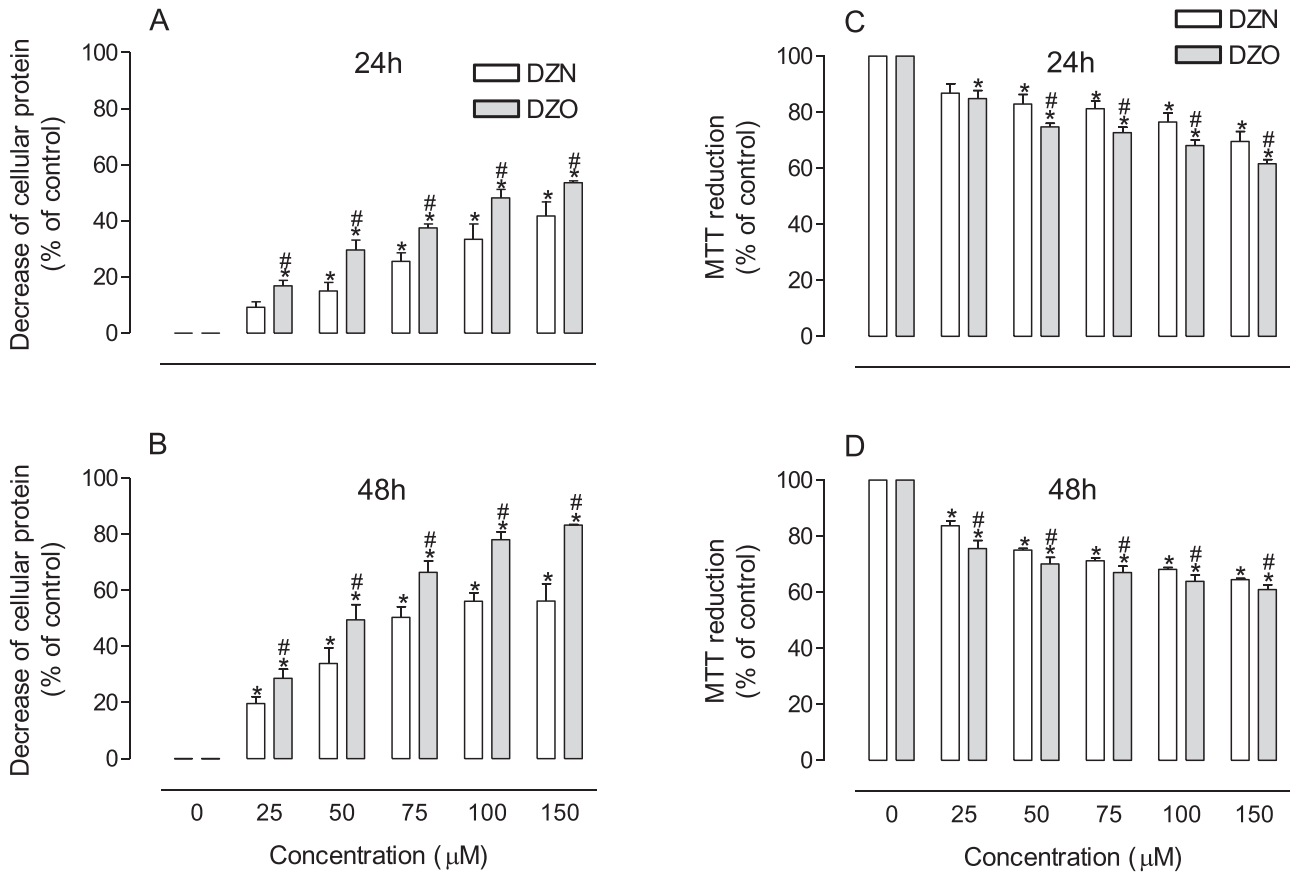

Fig. 4. Comparative effect of diazinon (DZN) and its metabolite diazoxon (DZO) on HepG2 proliferation (amount of cell protein) after 24 (A) and 48 (B) h and viability (MTT reduction) after 24 (C) and 48 (D) h of exposure. The graphs represent the mean ± SEM of three independent experiments. *$p < 0.05$ in relation to the control group. #$p < 0.05$ in relation to DZN at the corresponding concentration. 0 = control, 0.1% DMSO only.

dependent inhibition of cell proliferation amounting to 54 % and 83 % for 24 and 48 h exposure, respectively (Fig. 4A and B). Moreover, DZN inhibited cell viability as 30 % (for 24 h exposure) and 36 % (for 48 h exposure) while DZO inhibited cell viability as 37 % (for 24 h exposure) and 40 % (for 48 h exposure) (Fig. 4C and D).

3.5. Effect of THC on DZN-induced cytotoxicity

The protective effect of THC on the DZN-induced loss of viability of HepG2 cells was evaluated by their ability to reduce MTT (Fig. 5). For this study, a concentration of 50 μM of DZN was selected, as it showed a significant effect at both time points (24 and 48 h) in the MTT assays performed previously, and three concentrations of THC (25, 50 and 100 μM). THC reduced the metabolic changes induced by DZN in a dose-dependent manner, showing significant protection at concentrations of 50 and 100 μM following 24 h and 48 h of incubation.

3.6. Effect of THC on DZN-induced RONS production

The effect of THC on DZN-induced RONS production by HepG2 cells is presented in Fig. 6. For this study, a concentration of 75 μM of DZN was selected, as it showed a significant effect at both time points (24 and 48 h) in the MTT assays performed previously, and the concentration of THC 50 μM was chosen. THC reduced the RONS production induced by DZN following both 24 h and 48 h of incubation.

4. Discussion

Our results demonstrate that DZN was able to cause cytotoxicity in HepG2 cells after 24 and 48 h of exposure. Inhibition of cell proliferation was observed by the decrease in cellular protein that occurred at concentrations above 50 μM for both incubation times, presenting a dose-dependent effect. Another factor that contributed to the DZN toxicity was the reduction of cell viability as observed in the MTT assay, which indicates mitochondrial damage, this effect being dose- and time-dependent.

Apoptosis is a regulated form of cell death that is characterized by morphological changes such as chromatin condensation, nuclear fragmentation and DNA degradation, a reduction in cell volume and the appearance of bubbles in the plasma membrane as well as the formation of apoptotic bodies (vesicles surrounded by fragments of the plasma membrane that store cell content) [47]. In the present study, using fluorescence microscopy with Hoechst 33342, a fluorescent dye permeable to the cell membrane, capable of binding to the nucleic acids that make up the cellular DNA and therefore widely used for the determination of nuclear fragments formed in apoptosis [52], a significant increase in chromosomal condensation and fragmentation was observed in relation to control cells, after either 24 or 48 h of exposure to DZN, indicating that the insecticide induces cell death by apoptosis. This result is in agreement with previous studies that demonstrated DZN-induced apoptosis in primary murine cortical cultures [56], the NTera2-D1 cell line [6] and rat cardiac and liver tissues [41,54].

DZN is rapidly metabolized in vivo by liver CYP enzymes, mainly into its analogue, DZO [25,37], which is known to be a strong inhibitor of AChE, responsible for most of the acute neurotoxicity induced by DZN [17,62]. Thus, since HepG2 cells have a limited P450 profile [59], we used two CYP activators to test the effect of the biotransformation in DZN toxicity. Pre-incubation of HepG2 cells with 3-MC, an inducer of the CYP1A2 isoform, and PB, an inducer of CYP2B6 and CYP3A4 isoforms [15], all involved in the biotransformation of DZN in humans

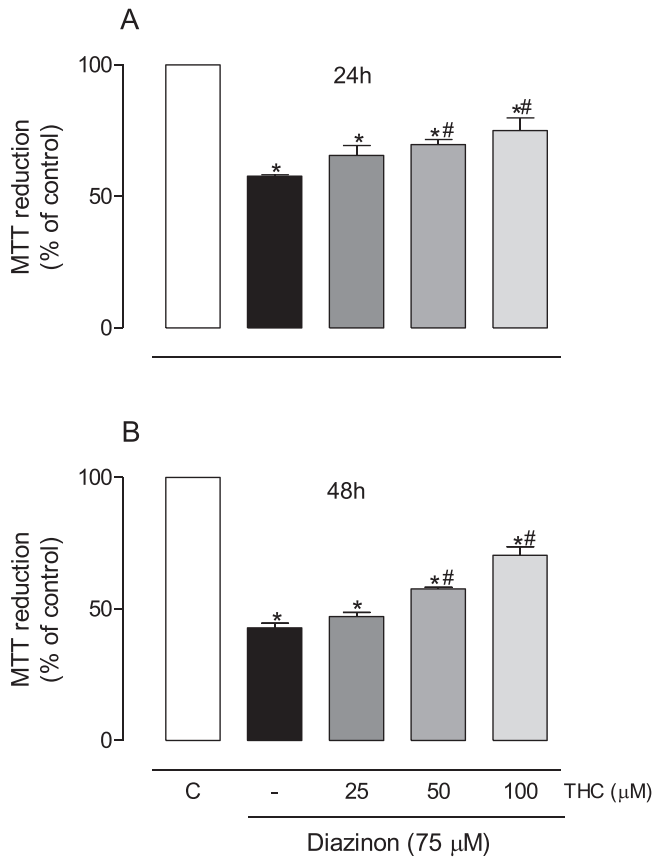

Fig. 5. Effect of tetrahydrocurcumin (THC) on diazinon-induced cytotoxicity in HepG2 cells after 24 (A) and 48 (B) h of exposure. The graphs represent the mean ± SEM of three independent experiments. *p < 0.05 in relation to the control group, without diazinon or THC. #p < 0.05 in relation to the diazinon without THC. C = control, 0.1% DMSO only; - = 75 μM diazinon only, in the absence of THC.

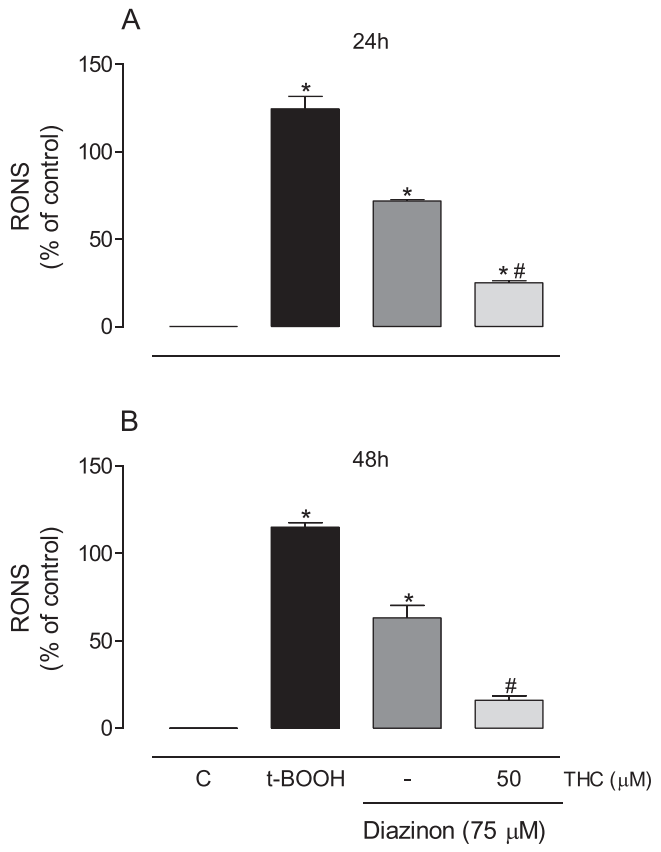

Fig. 6. Effect of tetrahydrocurcumin (THC) on diazinon-induced reactive oxygen and nitrogen species (RONS) production in HepG2 cells after 24 (A) and 48 (B) h of exposure. The graphs represent the mean ± SEM of three independent experiments. *p < 0.05 in relation to the control group, without diazinon or THC. #p < 0.05 in relation to diazinon without THC. C = control, 0.1% DMSO only; t-BOOH = 100 μM tert-butyl hydroperoxide; - = 75 μM diazinon only, in the absence of THC.

[58], potentiated the toxic effect of the insecticide, since greater cytotoxicity was observed after 24 h and 48 h of incubation with DZN, suggesting that the P450-formed DZO metabolite is the cytotoxic component in HepG2 cells.

To test this hypothesis, we compared the toxicity of DZO with the effects presented by DZN on HepG2 cells. The results showed that DZO presented greater toxicity in HepG2 cells after 24 and 48 h of exposure compared to DZN. The inhibition of cell proliferation was observed by the decrease of cellular protein induced by DZO that occurred from a concentration of 25 μM for both incubation times (24 and 48 h), with a dose and time-dependent effect. At all concentrations tested, DZO had a significantly greater effect than that presented by DZN. Another factor evaluated was cell viability, based on the MTT assay, in which both compounds induced cytotoxicity in HepG2 cells in a dose- and time-dependent manner with the greater effects being at 48 h post-incubation. Therefore, our results are in agreement with those presented by Ueyama et al. [68] who, in experiments carried out with diabetic rats, in which the hepatic metabolism mediated by CYP1A2, responsible for the production of DZO, is accelerated, demonstrated that DZN is more toxic when its metabolite is produced.

Studies have revealed that oxidative stress can be an important component of the mechanism of DZN intoxication [1,28,66,69]. Treatment of rats with DZN significantly increases renal lipid peroxidation, which is accompanied by a decrease in the activity of renal antioxidant enzymes (CAT, GSH-Px, glutathione reductase and glutathione S-transferase), in addition to promoting a decrease in the level of GSH, events that may be related to the renal toxicity of DZN, mediated by oxidative stress [60]. Tang et al. [65], in a study carried out with crucian carp, observed that the exposure of animals to DZN was capable of causing oxidative stress in addition to histopathological damage. In this way, additional experiments were carried out to assess the protective role of THC, a potent antioxidant and anti-apoptotic agent, against the toxic effects of DZN.

The antioxidant activity of THC has been attributed to its ability to scavenge free radicals and also to increase levels of non-enzymatic antioxidants (vitamin C, vitamin E and GSH) and enzymatic antioxidants (SOD, CAT and GSH-Px) [49]. Muthumani and Miltonprabu [45] demonstrated that THC reduces oxidative stress, dyslipidemia and mitochondrial damage and improves mitochondrial structure and function in arsenic-exposed rat liver, attributing this effect to the antioxidant activity of THC. In the present study, THC was able to partially inhibit the DZN-induced loss of cell viability and RONS production, reinforcing the important role of RONS production in cell death induced by the insecticide. It is also worth noting that in experiments performed with mitochondria isolated from rat liver, it was observed that DZN is able to oxidize protein thiol groups [44].

Because of the widespread use in agricultural production systems, humans can be exposed to eating food treated with DZN. The insecticide may also be present in surface or well water as a result of run-off and movement through the soil from areas where it is used in farming [8]. In addition, the use of DZN in the surroundings of human dwellings and its inadequate use in farm animals indicate its great availability and potential exposure, which can lead to acute, subacute or chronic and even fatal poisoning. In this way, several cases of human accidental [18,21,55,64,71] or suicidal [12,19,2,51,57,64] poisoning by DZN have been

reported, some of which were fatal. Also, cases of fatal intoxication in animals are reported [10,13,26,36,40,61]. The dose of DZN observed to cause a cytotoxic effect in the present study (25–150 μM) is in line with the human blood concentration of 28 mg/dL (0.91 mM) achieved following acute self-poisoning [51].

As noted throughout this manuscript, oxidative stress has been proposed to play a pathological role in DZN poisoning and the liver is an important target organ of this pesticide. In this way, natural antioxidants compounds from different sources have been studied as protective agents of the DZN-induced liver toxicity [42,46,5,11]. The present study demonstrated that THC prevented HepG2 cell death induced by DZN. Its protective mechanisms occur through inhibition of accumulation of intracellular RONS as an antioxidant property. Therefore, THC may represent a potential therapeutic approach, in particular for the protection of DZN-mediated liver damage in cases of animal and human intoxication.

5. Conclusion

The present study shows that DZO metabolite presented greater toxicity than DZN, which reinforces the hypothesis that biotransformation is an important factor for DZN toxicity in the liver. In addition, THC, an antioxidant and anti-apoptotic agent, protected HepG2 cells against the toxic effects of DZN.

Funding

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP [grant numbers 2018/22002-9, 2019/12497-3].

CRediT authorship contribution statement

D.J. Dorta and F.E. Mingatto conceived and designed of the experiments. C.A. Miranda, E.M. Beretta, L.A. Ferreira, E.S. Silva, B.Z. Coimbra, P.T. Pereira, R.G. Miranda and F.T.V. Rodrigues performed the experiments and analyzed the data. C.A. Miranda and E.M. Beretta wrote the original draft. D.J. Dorta and F.E. Mingatto revised the manuscript. All authors participated in reading and agreed to the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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