

Protective effect of amlodipine on diazinon-induced changes on oxidative/antioxidant balance in rat hippocampus

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Abstract

Oxidative stress (OS) is a main mechanism in organophosphorus poisoning. The effects of calcium channel blockers have been confirmed in decreasing of oxidative stress. In the current study, the effects of amlodipine (AM), as a calcium channel blocker, were evaluated on oxidative damages induced by diazinon (DZN) in hippocampus tissue of Wistar rats. Forty-two rats were divided into six groups and treated intraperitoneally for two weeks. Group 1 served as control received vehicle, group 2 was treated with 9 mg/kg of AM, group 3 (positive control) received DZN (32 mg/kg), Groups 4, 5, and 6 were treated with 3, 6, and 9 mg/kg of AM adjunct with DZN (32 mg/kg), respectively. After 14 days, all the animals were sacrificed under anesthesia and hippocampus tissue and blood samples were collected for biochemical analysis and histopathology experiments. The results showed that DZN caused significant increase in lipid peroxidation ($P < 0.001$), nitric oxide ($P < 0.001$) and lactate dehydrogenase ($P < 0.001$) levels, depletion of total antioxidant capacity ($P < 0.01$), and structural changes in hippocampus tissues. Following AM administration, a significant improvement was observed in oxidative biomarkers in hippocampus tissues. Additionally, our biochemical findings were related well with histopathological examinations. In conclusion, the data of this study indicated that AM administration may prevent oxidative damages via improving of energy production and preventing of free radical formation in DZN-exposed animals.

Keywords: Amlodipine; Calcium channel blocker; Diazinon; Hippocampus.

INTRODUCTION

Organophosphorus compounds (OPCs) as effective insecticides are commonly used in agriculture. It is predicted that using of pesticides such as OPCs has been increased at least two to three times from 1995 to 2020 years (1). This will increase the incidence of OPCs poisoning and their consequent toxicities in humans. For instance, the epidemiological studies have shown that different neurobehavioral deficits in children, such as impaired intelligence quotient (IQ) and increased incidence of attention deficit hyperactivity disorder can be related to the toxic effects of OPCs (2,3).

Diazinon (DZN), O,O-diethyl O-[4-methyl-6-(propan-2-yl) pyrimidin-2-yl] phosphorothioate, is one of the most widely used OPCs in agricultural, veterinary, medical and public health practices (4). The main mechanism of acute poisoning with DZN is irreversible inhibition of the acetylcholinesterase (AChE) activity in blood and synaptic terminals of nervous system. It results in the accumulation of acetylcholine and extra stimulation of nicotinic and muscarinic receptors, which may lead to life-threatening bronchospasm and respiratory failure (5).

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In OPCs exposure, it has been reported that the oxidative stress and mitochondrial dysfunction are the chief mechanism of their toxicity (6). Oxidative stress is directly involved in the incidence of neurodegenerative disorders such as Parkinson's, Alzheimer's diseases, and atherosclerosis (7). The extent of the damages induced by oxidative stress depends on the structure and antioxidant defense capacity of the cells. For instance, brain tissue is very susceptible to oxidative stress, because it is a rich source of polyunsaturated fatty acids which easily undergo peroxidation processes (8). It has found that the oxidative stress is increased in the hippocampus and cerebral cortex following the acute or chronic exposure to OPCs (9).

Overwhelming evidences indicate that calcium ions (Ca^{2+}) play a key role in neural degeneration. Intracellular Ca^{2+} concentration is regulated through link among channels located on the plasma membrane such as L-type calcium channels, endoplasmic reticulum (ryanodine receptor and sarcoplasmic reticulum calcium ATPase), and mitochondria (10).

Oxidative stress leads the Ca^{2+} influx into the cytoplasm from the endoplasmic reticulum or sarcoplasmic reticulum (ER/SR) and from extracellular space through the ER/SR channels and the cell membrane, respectively. Increasing Ca^{2+} level in the cytoplasm causes Ca^{2+} influx into nuclei and mitochondria. In nuclei Ca^{2+} regulates gene transcription that control cell apoptosis. In mitochondria Ca^{2+} disrupts normal metabolism that it can lead to cell death (11).

Several reports have demonstrated the antioxidant properties of dihydropyridine calcium channel blockers. It is related to their direct scavenging effect or the protection of the mitochondria in pathologic conditions such as ischemia and/or hypobaric hypoxia (12, 13). Among these drugs, amlodipine (AM) is a lipophilic dihydropyridine that inhibits L-type calcium channels in the different organs such as brain (14).

Based on existing evidences, the present study was designed to evaluate the effects of AM on oxidative damages induced by DZN in hippocampus tissue of Wistar rats.

MATERIALS AND METHODS

All chemicals were obtained from Merck, Darmstate, Germany unless otherwise stated. 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB), N-(1-naphthyl) ethylenediamine dihydrochloride (NED), 2-thiobarbituric acid (TBA), and 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) were purchased from Sigma-Aldrich chemical company (St. Louis, MO, USA).

Animals

Wistar male rats (200-250 g) were provided by animal house of Hamadan University of Medical Sciences, Hamadan, I.R. Iran. They housed in polypropylene cages and fed a standard laboratory diet and water *ad libitum*. Animals were kept in 12-h light/dark cycles and at a room temperature of 22-25 °C. The study protocol was approved by Ethics Committee of Hamadan University of Medical Sciences (HUMS) under ethical ID of 940215632.

Determination of optimum dose

Optimum dose of DZN was determined as 32 mg/kg from commercial grade on the basis of pilot studies (data are not shown). Indeed, at this dose, DZN showed explanatory changes in biochemical biomarkers such as AChE inhibition, increased lipid peroxidation (LPO) and nitric oxide (NO), and decreased total antioxidant capacity (TAC) in hippocampus tissues. In addition, the histopathological studies were confirmed our biochemical findings.

Experimental design

Forty-two rats were randomly divided into six groups of seven animals each. The animals were treated for 14 consecutive days with intraperitoneal (i.p) injection as follows: Group 1 (control group) received vehicle, Group 2 received 9 mg/kg of AM, Group 3 received 32 mg/kg of DZN as positive control (dose was determined by a pilot study), and groups 4-6 were treated with 3, 6, and 9 mg/kg of AM 1 h before DZN (32 mg/kg) administration.

Twenty four h after the completion of treatment, animals were anesthetized by diethyl ether and scarified immediately. Blood

samples were collected by cardiac puncture, and animals' brains were removed for isolation of hippocampus tissue as described previously (15). Briefly, animals' brains were carefully removed by sterile scissors from the skull and washed in ice-cold sterile saline solution to remove any surface blood. Immediately, it was cut along the longitudinal fissure to divide both hemispheres on an ice-chilled plate. The exposed hippocampus tissue was resected by using scissors and microsurgical forceps (No. 5) from the neocortex. A part of hippocampus tissue was placed quickly in liquid nitrogen for biochemical analysis. Another portion of hippocampus was fixed in 10% of buffered formalin and subsequently used for staining by hematoxylin and eosin (H&E).

After thawing the hippocampus, a piece of this tissue was homogenized in tris buffer (pH 7.4). The tissue homogenate (10%) was made (w/v) and centrifuged at 3000 g for 10 min at 4 °C. Then, the supernatants were separated and kept at -80 °C until the biochemical analysis.

Acetylcholinesterase and butyrylcholinesterase assay

The AChE activity in supernatant of tissue homogenate and serum butyrylcholinesterase (BChE) activity were determined by modified Ellman's spectrophotometric method according to Ozarowski *et al.* (16). Briefly, the activities of AChE and BChE were determined by measuring the formation of the yellow anions obtained from the reaction between Ellman's reagent and the thiocholine generated by the enzymatic hydrolysis of acetylthiocholine iodide (ATCh) and butyrylthiocholine (BTCh), respectively (sample 0.01 mL, phosphate-buffered saline (PBS) 0.08 mL, DTNB 0.01 mL, ATCh 0.02 mL or BTCh 0.02 mL). The change of absorbance was detected at 412 nm using a microplate reader (Synergy HTX, Biotek, USA). The results were expressed as the percent of control.

Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) activity in the hippocampus homogenate sample was

determined by measuring the rate of oxidation of NADH using an enzymatic colorimetric kit (Pars Azmoon Co., Tehran, Iran). The change of absorbance was detected at 340 nm and results were expressed as unit/mg protein.

Lipid peroxidation assay

Hippocampus homogenate samples were mixed with TBA (0.2%) in H₂SO₄ (0.05 M) and heated for 30 min in boiling water bath. Thiobarbituric acid-reactive substances were extracted by n-butanol and absorbance was determined at 532 nm. Malonaldehyde (MDA) was used as the standard and the results were expressed as nmol/mg protein (17).

Total antioxidant capacity assay

Total antioxidant capacity level was determined in hippocampus homogenate sample by measuring their ability to reduce Fe³⁺ to Fe²⁺ as described previously (18). The complex between Fe²⁺ and TPTZ gives a blue color with a peak absorbance at 593 nm. Results were expressed as nmol/mg protein.

Nitric oxide assay

The tissue level of nitrite, an indicator of the production of NO, was measured using Griess reagent (1% sulfanilamide, 0.1% NED, and 2.5% phosphoric acid). Equal volumes of Griess reagent and hippocampus homogenate sample were mixed and incubated for 10 min at room temperature in the dark place. The absorbance was assayed at 520 nm using a microplate reader. Finally, the level of nitrite was determined from sodium nitrite calibration curve and expressed as nmol/mg protein.

Protein assay

Protein concentrations were determined with Bradford method and the absorbance were evaluated at 595 nm using a microplate reader. Bovine serum albumin (BSA) was used as the standard and results were expressed as mg protein/mL (19).

Data analysis

The data are expressed as the mean ± standard deviation (SD) and analyzed by Graph Pad Prism software, version 6.0. The statistical difference of the values was

determined by one-way analysis of variance (ANOVA) with Tukey's test for multiple comparisons. A P -value of less than 0.05 was considered statistically significant.

RESULTS

Effect of amlodipine on hippocampus acetylcholinesterase and serum butyrylcholinesterase activities

Hippocampus AChE (Fig. 1A) and serum BChE (Fig. 1B) activities are shown as percent of the control. In the serum and hippocampus tissues, a significant decrease in the activity of these enzymes was observed in DZN-treated group and those groups treated with DZN and AM. However, no significant changes were observed when DZN group was compared with AM-treated groups.

Effect of amlodipine on lactate dehydrogenase activity in hippocampus tissue

As shown in Fig. 2, administration of DZN caused a remarkable increase in LDH activity when compared to the control rats ($P < 0.001$). Following administration of AM at doses 3, 6, and 9 mg/kg showed significant decrease in LDH activity compared to DZN ($P < 0.001$, $P < 0.05$, and $P < 0.01$ respectively).

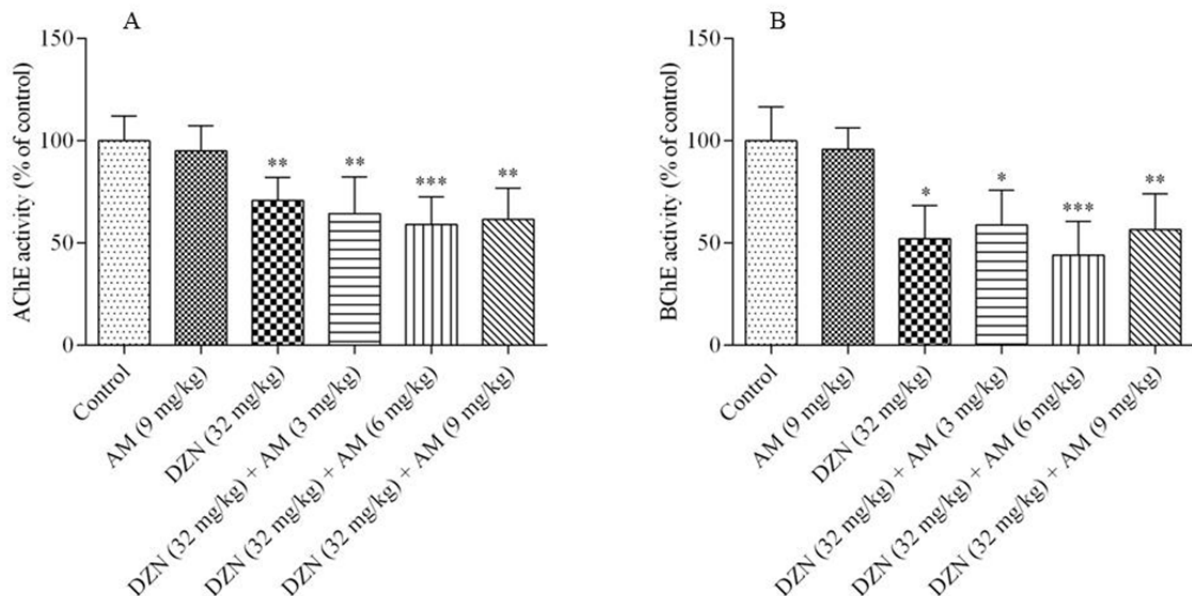


Fig. 1. Effects of amlodipine (AM) on (A) acetylcholinesterase (AChE) activity in hippocampus tissue and (B) serum butyrylcholinesterase (BChE) activity in male rats. One-way ANOVA followed with Tukey's test was used for statistical comparisons. Results are expressed as means \pm SD, $n = 7$ for each group. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs. control group.

Effect of amlodipine on lipid peroxidation level in hippocampus tissue

LPO level in hippocampus was significantly increased by administration of DZN in comparison to the control ($P < 0.001$). However, administration of AM at doses of 3 mg/kg significantly prevented lipid peroxidation in comparison to DZN control ($P < 0.01$). In the other doses of AM, the changes were not statistically significant (Fig. 3).

Effect of amlodipine on nitric oxide level in hippocampus tissue

As shown in Fig. 4, administration of DZN caused a significant increase in tissue level of NO when compared to the control rats ($P < 0.001$). Following AM administration, a remarkable decrease was observed in NO level in treatment groups when compared to DZN group.

Effect of amlodipine on total antioxidant capacity level in hippocampus tissue

DZN significantly reduced TAC content of hippocampus ($P < 0.01$). Administration of AM (3, 9, and 18 mg/kg) restored TAC hippocampus supply when compared to the DZN group but these changes were not significant (Fig. 5).

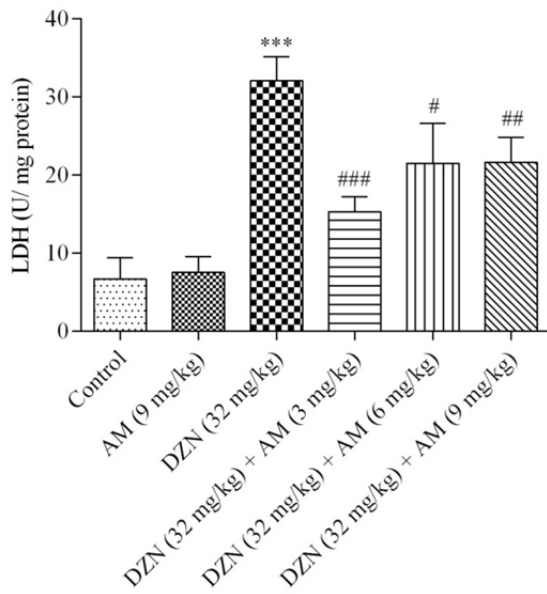


Fig. 2. Effects of amlodipine (AM) on lactate dehydrogenase (LDH) activity in hippocampus of male rats. One-way ANOVA followed with Tukey's test was used for statistical comparisons. Results are expressed as means \pm SD, $n = 7$ for each group. $***P < 0.001$ vs. control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs. diazinon (DZN) group.

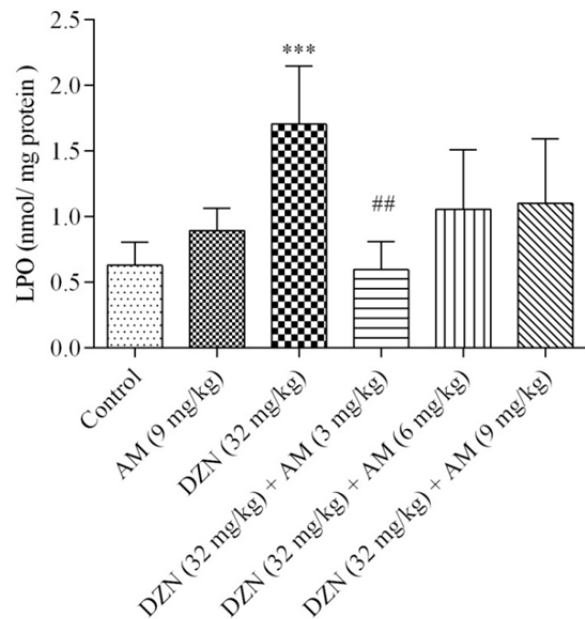


Fig. 3. Effects of amlodipine (AM) on lipid peroxidation (LPO) level in hippocampus of male rats. One-way ANOVA followed with Tukey's test was used for statistical comparisons. Results are expressed as means \pm SD, $n = 7$ for each group. $***P < 0.001$ vs. control group, $^{\#\#}P < 0.01$ vs. diazinon (DZN) group.

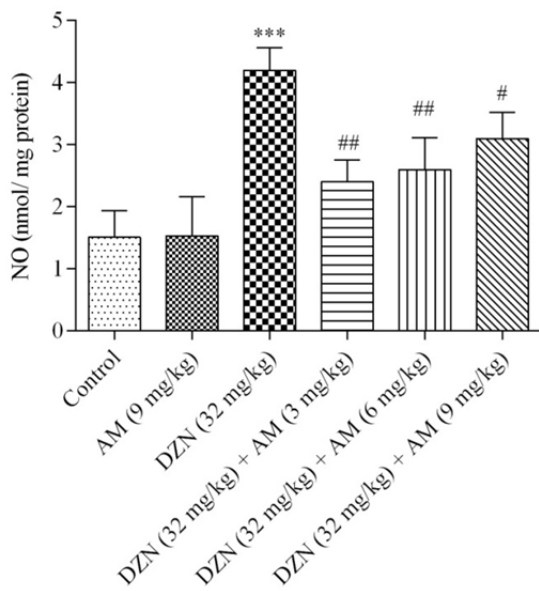


Fig. 4. Effects of amlodipine (AM) on nitric oxide (NO) level in hippocampus of male rats. One-way ANOVA followed with Tukey's test was used for statistical comparisons. Results are expressed as means \pm SD, $n = 7$ for each group. $***P < 0.001$ vs. control group, $^{\#\#}P < 0.01$, and $^{\#}P < 0.05$ vs. diazinon (DZN) group.

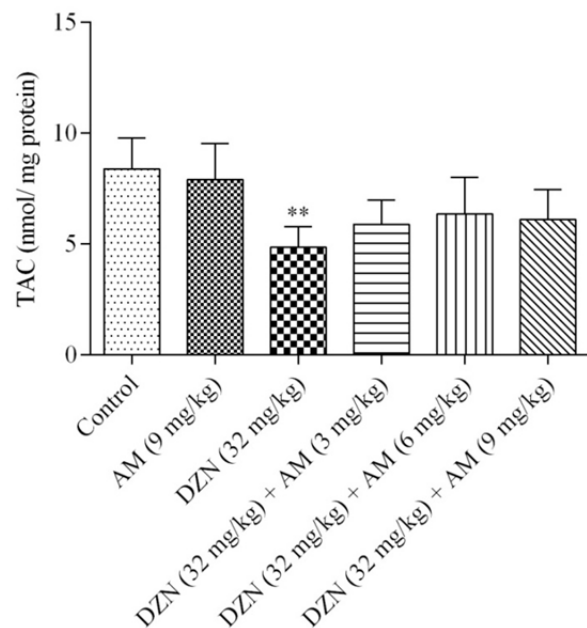


Fig. 5. Effects of amlodipine (AM) on total antioxidant capacity (TAC) level in hippocampus of male rats. One-way ANOVA followed with Tukey's test was used for statistical comparisons. Results are expressed as means \pm SD, $n = 7$ for each group. $**P < 0.01$ vs. control group. DZN, diazinon.

Histopathological assessment

Figure 6 shows histological changes in rat hippocampus following DZN administration and/or AM. By administration of only DZN, some histological changes in hippocampus

such as alteration of vascular and glial cells, increasing of lymphocytes were observed. AM prevented the histological changes of DZN and reduced the number of inflammatory cells in hippocampus tissue (Table 1).

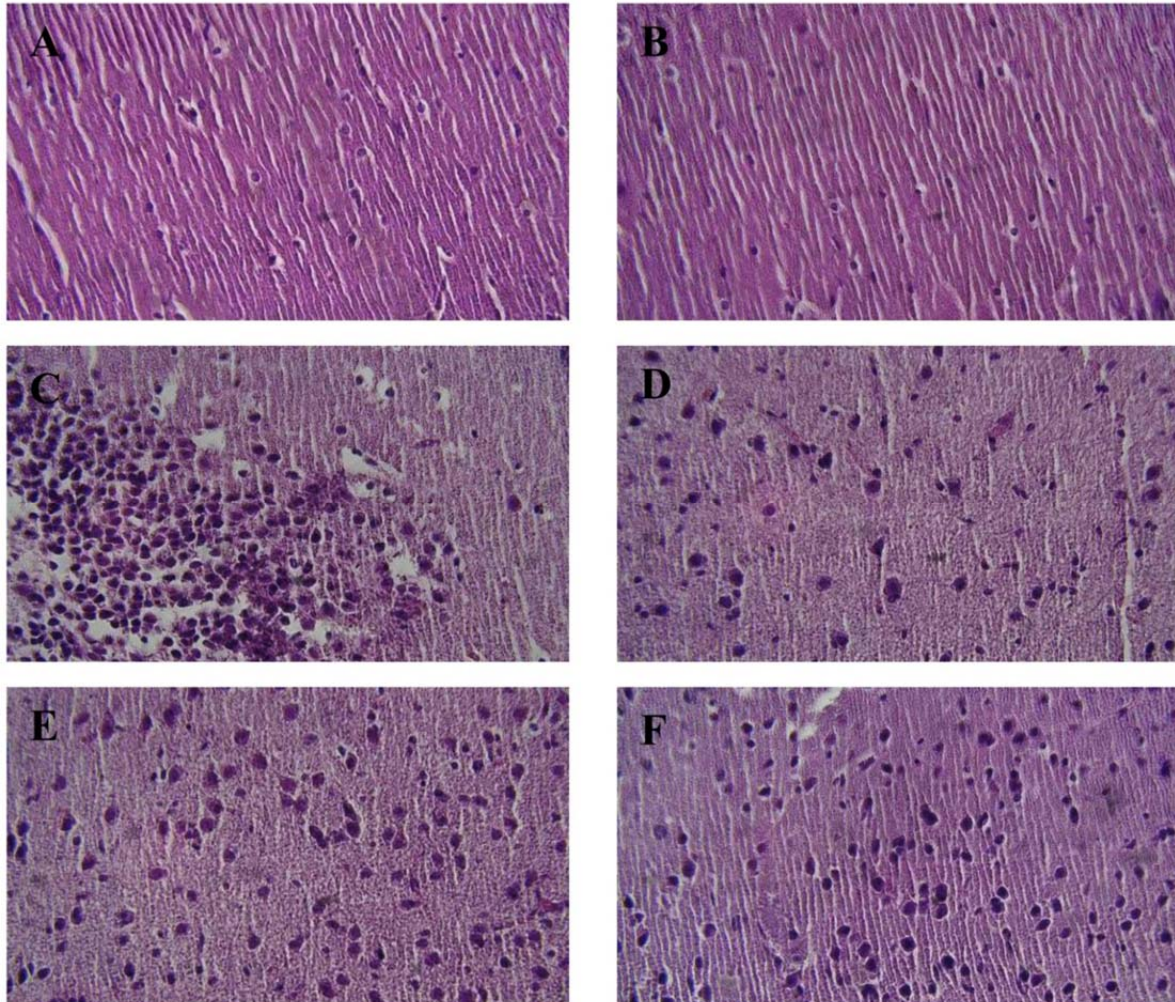


Fig. 6. Photomicrographs of rat hippocampus tissue in different groups. Original magnification of all images is $\times 40$. (A) negative control group, (B) amlodipine (9 mg/kg) group, (C) diazinon (32 mg/kg) group, (D) diazinon (32 mg/kg) + amlodipine (3 mg/kg) group, (E) diazinon (32 mg/kg) + amlodipine (6 mg/kg) group, and (F) diazinon (32 mg/kg) + amlodipine (9 mg/kg) group.

Table 1. Effects of amlodipine (AM) on histopathological changes of hippocampus in male rats.

Groups	Vascular congestion	Number of lymphocytes	Changes in glial cells	Vascular changes
Control	-	-	-	-
AM (9 mg/kg)	-	-	-	-
DZN (32 mg/kg)	+++	+++	+++	+++
AM (3 mg/kg) + DZN (32 mg/kg)	+	+	+	++
AM (6 mg/kg) + DZN (32 mg/kg)	+	++	++	++
AM (9 mg/kg) + DZN (32 mg/kg)	++	+++	+++	+++

DZN, diazinon.

DISCUSSION

Cholinesterase enzymes, especially AChE, are specific biomarkers for confirming toxicity with OPCs. In this study, decreasing of the hippocampus AChE enzyme and serum BChE activities were confirmed as the toxicity induced by DZN. Following administration of AM, a significant change in the activities of these enzymes was not observed in poisoned animals with DZN. According to these findings, it seems that AM doesn't compete with DZN for binding to cholinesterase enzymes.

The present results indicate that AM prevented oxidative damages in hippocampus tissue following subacute exposure with DZN. In previous studies, the neurotoxic effects of OPCs are well documented (20,21). Overall, brain has a high level of oxidative metabolism, and consumes approximately 20% of the cardiac output (22). Since it is rich in polyunsaturated fatty acids and other peroxidisable substrates, the brain is especially susceptible to oxidative damages induced by toxic agents (23,24). Some regions of brain are highly enriched in non-heme iron, which is catalytically involved in the production of free radicals thus increasing the risk of neurodegenerative diseases (23,25). According to previous studies, hippocampus and cortex regions are more vulnerable to oxidative damage when compared to cerebellum (26).

In this work, we showed that DZN (32 mg/kg) was able to induce significant changes in LPO and NO as indices of oxidative degradation of intracellular macromolecules that indicate increasing of free radicals in hippocampus tissue. It is known that NO reacts with high affinity with superoxide anion, thereby producing the highly toxic and pro-apoptotic peroxynitrite (ONOO) that triggers inflammatory processes and contributes directly to neurodegenerative events (27). DZN is detoxified via conjugation reactions with glutathione (GSH). GSH depletion is related to oxidative damages and cytotoxicity (28). In the present study, results show that DZN exposure caused a reduction of the TAC levels in hippocampus. Our findings are in consistence with other studies which have shown that OPCs administration to rats

alter the brain levels of GSH/GSSG and antioxidative enzymes such as glutathione peroxidase (GPx), catalase (CAT), and copper-zinc superoxide dismutase (Cu/ZnSOD) (29). Thus, we propose that reducing the TAC may be associated with decreased levels of antioxidant agents in hippocampus tissue.

Following the administration of AM, the lipid peroxidation level was significantly decreased in DZN-treated animals. The part of AM antioxidant properties could be related to both of its high lipophilicity and a chemical structure that facilitates proton-donating and resonance-stabilization mechanisms that inhibit the oxidative stress process (30). On the other hand, excessive influx of calcium due to overstimulation of N-methyl-D-aspartate (NMDA) and muscarinic acetylcholine receptors (mAChRs) is considered as an essential cause of neuronal cell death following OPCs toxicity (31). The altered calcium influx activates proteases, phosphatases, kinases, lipases, and endonucleases in potentially harmful metabolic cascades, thus arresting protein synthesis and depriving cells of enzymes or trophic factors essential to their survival (11,31). In addition, intracellular calcium overload also results in free radical-related injury and apoptosis by inducing the oxidative stresses and inflammatory responses to damages (6,11,31). Sendrowski *et al.* reported neuroprotective potential of both L-type and T-type calcium channel blockers against glutamate-induced death of cultured hippocampal neurons (32). Based on the available evidences, it seems AM can modify excessive influx of calcium into cells, thereby blocking the oxidative damages in hippocampus tissue.

Increasing of LDH activity, as an index of anaerobic metabolism (33), may be the other reason for occurrence of oxidative damages in hippocampus after DZN poisoning. In the previous studies, metabolic modification have been reported to occur following exposure of different tissues to OPCs such as interference with the tricarboxylic acid cycle (TCA), change of the glycolytic pathway and alterations in mitochondrial respiration (20,34). Therefore, mitochondrial dysfunction can cause aerobic metabolism disruption and remarkable increase in the oxidative stress (6,20).

Calcium regulation into cells has a critical role in mitochondrial function and energy production by aerobic pathway (35). Pronobesh *et al.* showed that pretreatment with AM reduced the extent of mitochondrial damage and reversed the activities of TCA cycle and respiratory marker enzymes towards normalcy (12). These evidences support our idea that AM is able to prevent increase LDH activity in hippocampus following DZN toxicity which further indicate the relation between the role of the AM in intracellular calcium regulation with improving of mitochondria function.

Histopathological changes in different groups also provided an important evidence for confirming of biochemical findings. Under microscopic examination significant alterations in cellular architecture of hippocampus such as alteration of vascular and glial cells, increasing of lymphocytes were observed in DZN-treated rats. AM prevented the histological alterations of DZN and reduced the number of inflammatory cells in hippocampus tissue.

CONCLUSION

Taken collectively, AM exerts its neuroprotective effects in DZN poisoning through improvement of energy production by aerobic pathways and reduction of oxidative stress. These effects can be related to the role of calcium channel blocker in intracellular calcium regulation and AM antioxidative properties. The results of our study are interesting and give a hope to manage neurotoxic adverse effects of OPCs in intoxicated patients.

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