Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(5):203-214

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

The protective effect of creatine supplements against malathion-induced neuro and liver toxicity

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ABSTRACT

The aim of this study was to investigate the effect of treatment with creatine on the neuro and hepatotoxic effects of acute malathion exposure. Rats received malathion (150 mg/kg, i.p. injection) for two successive days either alone or combined with creatine at doses of 160, 360 or 720 mg/kg, orally. Serum acetylcholine esterase (AChE), butyryl cholinesterase (BuChE) and paraoxonase-1 (PON1) activities were determined in addition to comet assay. Malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD) activity and nitric oxide (NO) were determined in brain and liver tissues. Serum BuChE, AChE and PON1 activities were inhibited after the administration of malathion. Malathion resulted in an increase in MDA, NO; a decrease in GSH level and SOD activity in both brain and liver tissues. Malathion also caused marked increase in DNA damage of peripheral blood lymphocytes. These effects of malathion were ameliortaed with the administration of creatine. Our data indicate that creatine protects against malathion neuro and hepatic adverse effects, most likely through direct antioxidant mechanism and up regulation of antioxidant defense systems.

Key words: Malathion; creatine; cholinesterase; oxidative stress; comet assay

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INTRODUCTION

Organophosphate (OPs) pesticides poisoning continues to be a major health problem. Pesticide poisoning can result from occupational, accidental or intentional exposure. The uncontrolled use results from ease of access in addition to low level of regulations governing their use especially in developing countries [1]. Malathion (O, O-dimethyl-S-1, 2-bis ethoxy carbonyl ethyl phosphorodithionate) is the most commonly used OP pesticide throughout the world because of its low persistence in the environment compared to other OPs [2]. Exposure to malathion is linked to a variety of chronic diseases including respiratory (e.g., chronic obstructive respiratory disease), metabolic (e.g., obesity, diabetes) and neurologic (e.g., Alzheimer, Parkinsonism) [3]. OPs, including malathion, manifest their effect through inhibition of carboxylic ester hydrolase such as acetylcholinesterase (AChE), butyryl cholinesterase (BuChE), paroxonase-1 (PON1) and carboxyl esterase [4]. However, the most prominent clinical effect results from inhibition of cholinesterases at synapses and neuromuscular junctions. Cholinesterases hydrolyse the neurotransmitter acetylcholine (ACh) in synapses of the autonomic nervous system and at neuromuscular junctions of the voluntary nervous system. AChE is essential for ACh hydrolysis and transmission of nerve impulses and its activity. Upon inhibition of AChE, acetylcholine accumulates at cholinergic sites, causing overstimulation and alteration of the normal nervous system function [5, 6]. Acute excessive stimulation of cholinergic receptors causes cholinergic neuronal excitotoxicity and dysfunction, which are largely responsible for the cholinergic crisis in the

acute phase of the OP exposure (within minutes) and could subsequently cause secondary neuronal damage and chronic neuropsychiatric disorders [7, 8]. BuChE, also known as pseudo cholinesterase, rapidly hydrolyses the naturally occurring choline esters, ACh, propionylcholine and butyryl choline. Animal studies suggest that BuChE serves as a backup for ACh in nerve impulse transmission [9].

PON1 is the main means of protecting the nervous system against the neurotoxicity of OPs [10]. Upon exposure to OPs, PON1 protects AChE by enzymatically degrading the pesticide and thus preventing inhibition of AChE [11]. PON has obtained its name by the ability of the enzyme to hydrolyze paraoxon (the oxidized in vivo derivative of parathion) to the metabolite p-nitro phenol [12]. Studies in knockout mice have demonstrated that PON1 is a key determinant in the detoxification of OP pesticides, such as chloripyrifos. Rats that were injected with PON1 showed increased resistance to chloripyrifos-oxon acute cholinergic toxicity than controls [13].

Creatine (Cr) is an endogenous molecule found in all cells in the body and is synthesized in the kidney, liver, and pancreas using the amino acids arginine, glycine and methionine before entering the bloodstream [14, 15]. This reaction is catalyzed by the rate-limiting enzyme, guanidinoacetate-methyltransferase (GAMT). Patients with GAMT deficiency exhibit clear development delays, extrapyramidal movement disorders and seizures [16]. Administration of creatine supplements to GAMT-deficient patients improved the neurological impairments [17].

Creatine exists in the cell in both free Cr and phosphocreatine (PCr) which together comprise the total Cr pool. The Cr/ PCr system is assumed to play a critical function in neuronal ATP metabolism. Several studies reported that this circuit plays a key role in the energy metabolism of the brain and spinal cord [17, 18, 19]. Consequently, Cr depletion in brain is associated with disruption of neuronal functions, e.g., loss of hippocampal mossy fiber connection and changes in mitochondrial structure [20].

Creatine has important implications in antioxidant mechanisms, controlling intracellular calcium concentrations, regulating extracellular glutamate concentrations, and preventing the opening of the mitochondrial permeability transition pore [20]. The combination of these benefits has made creatine a leading candidate in the fight against neurological disorders that have marked impairment in energy metabolism, such as Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, long-term memory impairments associated with the progression of Alzheimer's disease, and stroke [15, 17, 19, 20, 22]. Therefore, it was tempting to study whether creatine will combat possible adverse effect of malathion on brain and liver tissues.

EXPERIMENTAL SECTION

Animals

Male albino rats weighing 120–140 g of body weight were used. Standard laboratory food and water were provided *ad libitum*. Animal procedures were performed in accordance with the Ethics Committee of the National Research Centre and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

Drugs and chemicals

Malathion (Commercial grade, Nasr Chemical Co., A.R.E), and creatine (Biopharma, A.R.E) were used. Other chemicals and reagents were obtained from Sigma, USA.

Study design

Rats were randomly allocated into five groups, six rats each. Group 1 (normal control) received saline (0.2 ml/rat, i.p.). Group 2-5 received malathion (150 mg/kg, i.p.). Following malathion injection, groups 3-5 received creatine at doses of 160, 360 or 720 mg/kg, orally. On the second day, rats received another dose of the above treatments and were euthanized by decapitation 2h after drug administration. Brain and liver tissues were then removed, washed with ice-cold saline solution (0.9 % NaCl), weighed and stored at-80ºC for the biochemical analyses. The brain and liver tissues was homogenized with 0.1M phosphate buffer saline at pH 7.4 to give final concentration of 20% w/v for the biochemical assays.

Biochemical analyses

Serum acetylcholinesterase activity:

The procedure used for the determination of cholinesterase activity in the serum was a modification of the method of Ellman et al. [23] as described by Gorun et al. [24]. The principle of the method is the measurement of thiocholine produced when acetylcholine is hydrolyzed, the colour was read immediately at 412 nm.

Serum butyrylcholinesterase activity

Butyrylcholinesterase activity was measured kinetically in serum using commercially available kit (Ben Biochemical Enterprise, Milan, Italy). In this assay, cholinesterase catalyzes the hydrolysis of butyrylthiocholine, forming butyrate and thiocholine. The thiocholine reacts with dithiobis-nitrobenzoic acid (DTNB) forming a colored compound. The increase in absorbance in the unit time at 405 nm is proportional at the activity of the cholinesterase in the sample.

Serum paraoxonase activity

Arylesterase activity of paraoxonase was measured kinetically in serum using phenylacetate as a substrate [25].

Lipid peroxidation

Lipid peroxidation in the brain and liver homogenates was assayed by measuring the level of malondialdehyde (MDA) using the method of Uchiyama and Mihara [26] where the thiobarbituric acid reactive substances react with thiobarbituric acid to produce a pink colored complex having a peak absorbance at 532 nm.

Reduced glutathione

Reduced glutathione (GSH) was determined in brain and liver homogenates using the method of Ellman et al [27]. The procedure is based on the reduction of Ellman's reagent by –SH groups of GSH to form 2-nitro-s-mercaptobenzoic acid, to produce intense yellow colour and measured at 412 nm.

SOD activity

The ability of SOD to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye, measured at 560 nm [28]. SOD activity was expressed as % inhibition from control samples.

Nitric oxide

Nitric oxide was determined using Griess reagent, according to the method of Moshage et al. [29], where nitrite, stable end product of nitric oxide radical.

Liver function tests

Serum ALT, AST activities were determined by colorimetric methods according to provided procedures.

Comet assay

Peripheral blood lymphocytes were isolated by centrifugation (15min, 280 g) in a density gradient of Gradisol L (Aqua Medica, Lodz, Poland). The concentration of the cells was adjusted to (1-3) x 10^5 cells/ ml by adding RPMI 1640 without glutamine to the single cell suspension. A freshly prepared suspension of cells in 0.75% low melting point agarose dissolved in phosphate buffer saline (PBS; sigma chemicals) was cast onto microscope slides precoated with 0.5% normal melting agarose. The cells were then lysed for 1h at 4°C in a buffer consisting of 2.5M NaCl, 100 mM EDTA, 1% Triton X-100, 10mM Tris, pH 10. After the lysis, DNA was allowed to unwind for 40 min in electrophoretic solution consisting of 300mM NaOH, 1mM EDTA, pH>13. Electrophoresis was conducted at 4°C for 30 min at electric field strength 0.73 V/cm (30mA). The slides were then neutralized with 0.4M Tris, pH 7.5, stained with 2ug/ml ethidium bromide and covered with cover slips. The slides were examined at 200 x magnification fluorescence microscope (Nikon Tokyo, Japan) to a COHU 4910 video camera (Cohu, Inc., San Diego, CA, USA) equipped with a UV filter block consist an excitation filter (359nm) and barrier filter (461nm) and connected to a personal computer –based image analysis system, Lucia-Comet v.4.51. Fifty images were randomly selected from each sample and the comet tail DNA was measured [30]. Endogenous DNA damage measured as the mean comet tail DNA of peripheral blood lymphocytes of five mice groups (10 mice each). The number of cells scored for each animal was 100.

Statistical analysis

Data are expressed as mean ± SEM. Statistical significance was determined using an ANOVA, followed by Tukey-

HSD test using SPSS software (SAS Institute Inc., Cary, NC). A probability value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

In the present study, administration of malathion inhibited serum AChE and BuChE activities by 42% and 34.6 %, respectively. Serum AChE and BuChE activities were restored after treatment with creatine (720 mg/Kg), (Figure 1-a, b).

Malathion and other OPs are known to manifest their acute biological actions primarily through inhibiting the various forms of cholinesterases [31]. Due to the highly reactive nature of OPs, it is not surprising that they might alter the function of important enzymes and proteins. Toxicity to target organs is then mediated through elevation of synaptic levels of ACh in tissues innervated by cholinergic neurons and subsequent overstimulation of postsynaptic cells [32]. Since activity of both enzymes is usually inhibited upon their measurement is widely used as an indication of exposure and/or biological effect of OPs [33]. The protective role of creatine can be attributed to possible antioxidant mechanism of creatine that helped to replenish active enzymes.

Injection of malathion resulted in significant decrease in serum PON1 activity by 39%. Co-treatment with creatine resulted in marked increase in PON1 activity up to control values (Figure 1-c).

The decrease in serum PON-1 activity in the current study can be explained by inactivation of the enzyme by the pesticide or possible over consumption of the enzyme in conjugation reactions. PON1 is involved in the hydrolysis of OPs and many xenobiotics [10, 33]. Also, PON1 is known to protect cells from a buildup of ACh by its peroxidase activity [25, 34].

The PON-1 detoxification capacity is considered by several studies as an important link between environmental exposure to pesticides and diseases [10]. Recent study demonstrated significant associations between paroxonase genotype status and pathology in populations exposed to pesticides [35]. The protective role of PON-1 against pesticide mediated toxicity is mainly dependent on the presence of Q and R isoforms in the population. In vivo studies have shown that high levels of PON-1 activity in serum, due to the presence of the R isoform, are consistent with resistance to toxic pesticide metabolites, such as chlorpyrifos oxon [36].

PON-1 is known to play a pivotal role in many neurological disorders. For example, PON-1 activity was found to be lower in autistic patients [37]. PON-1 polymorphism was considered as a reliable marker to distinguish patients with Alzheimer's from patients with vascular dementia [38].

In the present study, malathion produced a relatively large increase in brain MDA content. MDA had increased by 224.5 % in brain, and by 81% in liver. A decrease in the GSH concentration by 50.6% and 14.4% was found in brain and liver after injection of malathion, whereas marked increase in NO levels by 310%, and 62% was observed in brain and liver tissues. Meanwhile, malathion injection produced a decrease in SOD activity by 47% and 60% in both brain and liver, respectively. Creatine co-administered with malathion reduced the brain concentration of MDA, and markedly increased GSH levels and SOD activity up to control values. NO levels showed significant decrease after creatine treatment (Figure 2 a-d, Figure 3 a-d).

Inhibition of AChE is known to result in excessive stimulation of nervous tissue and muscle, which in turn causes depletion of high energy phosphates, ATP and PCr [39]. This results in reducing energy, activation of phospholipases and preoteases / caspases that increase generation of reactive oxygen species (ROS). Moreover, nitric oxide synthase is activated with the subsequent generation of nitric oxide (NO). NO can cause neurotoxicty by reacting with the superoxide O^{2-}leading to the formation of $OONO^{-}$. Also, NO impairs mitochondrial/cellular respiration causing ATP depletion [31].

Oxidative stress and the subsequent damage to lipids, proteins and nucleic acids in acute response to malathion is well established in literature [40-47]. This can be manifested by changes in the activity of antioxidant enzymes and/or altered levels of non enzymatic antioxidants, such as GSH and SOD. Thereby, this decrease would render the brain cells more vulnerable to oxidative and excitotoxic insults, and initiating neuronal cell death. Due to high concentration of substrate polyunsaturated fatty acids in cells, lipid peroxidation is a major event of free radical-

mediated injury. Two major outcomes of lipid peroxidation are structural damage to cellular membranes and generation of oxidized products, some of which are chemically reactive and may covalently modify cellular macromolecules [48].

During the biotransformation reactions of malathion into malaoxon high concentrations of ROS are generated. Furthermore, malathion is detoxified via conjugation reactions with glutathione. It was reported that malathion exposure decreased the levels of GSH, increased the levels of GSSG and finally decreased the ratio of GSH/GSSG [49]. The lack of GSH availability could result from GSH-dependent metabolism and malathion detoxification rate. GSH is an essential antioxidant, which acts as a direct scavenger of oxy radicals as well as antioxidant enzyme substrate. The consequence of the oxidative stress in cells may be the decrease of GSH and increase of its oxidized form (GSSG) [50].

Brain tissue is particularly susceptible to oxidative damage, as it is rich in polyunsaturated fatty acids which can easily undergo peroxidation. In addition, the brain utilizes a relatively large amount of oxygen at rather low activities of antioxidant enzymes [51].

The degree of oxidative damage after malathion exposure is much higher in brain rather than in liver. This can be attributed to the fact that liver is endowed with high antioxidant capacity.

Since the creatine-treated groups presented a reduction in lipoperoxidation and higher GSH and SOD activity, it is possible that creatine may up regulate the antioxidant defense systems.

The antioxidant effects of creatine may derive from different mechanisms of actions such as the indirect mechanism involved in cell membrane stabilization and improved cellular energy capacity [52] and from its direct antioxidant properties [53]. The direct antioxidant mechanism of creatine was proved using in vitro techniques, where Lawler and colleagues [54] found a dose-response relationship between creatine concentration and the ability to remove superoxide anions (O^{2-}) and peroxynitrite (OONO).

The PCr and CK energy pathway represents an extremely efficient energy buffering system for two reasons. First PCr has a slightly higher diffusion capacity than ATP making PCr transport a more efficient energy delivery system to different cellular locations. Second, the subcellular localizations of cytosolic and mitochondrial CK couples areas of energy generation with energy production. Thus, the CK/PCr essentially serves as a vital energy circuit within the cell [53].

The importance of the PCr system for brain function has been shown using genetically altered mice that lack the brain isoform of cytosolic CK which show that deficits in open field behavior, slower learning, and a loss of hippocampal mossy fiber connections [21].

The protective role of creatine in neurological disorders was demonstrated in several studies. Oral supplementation with Cr resulted in significant protection against MPTP-induced dopamine depletion in mice [55]. Cr supplements are also proved to be protective against glutamine and β-amyloid toxicity in rat hippocampal neurons [56].

In the present study, acute administration of malathion resulted in marked increase in serum liver function enzymes. Co treatment with creatine significantly lowered serum levels of ALT, AST and ALP (Figure 4 a-c). The increase in liver oxidative status was accompanied by increase in serum enzymes related to liver function. The profound increase in transaminases activities resulted from the impairment of cell permeability and necrosis of hepatocytes with the subsequent liberation of enzymes into the circulation from damaged tissues.

Malathion exposure has been associated with hepatotoxicity [57]. Moreover, recent investigations have shown that malathion is a hepatotoxin [58-66]. It has been shown that OP insecticides can elevate the enzymatic activities of ALP, ALT, and AST, and LDH. The observed increase in transaminases resulted from the impairment and necrosis of the function of tissues with the subsequent liberation of enzyme into the circulation from damaged tissues. When the liver cell membrane is damaged, several enzymes located in the hepatocyte cytosol, including ALP, ALT, AST, and LDH, are secreted into the blood. Consequently, these serum enzymes are markers of liver damage. In the present study, creatine ameliorated the rise in ALT, AST and ALP. This is in line with recent report by Bassit et al. [67] who demonstrated that creatine in a dose of 5 g/day/5 days prevented the rise in ALT and AST.

The level of DNA damage of peripheral blood lymphocytes was assayed by alkaline single cell gel electrophoresis (Comet assay), a sensitive technique for detecting DNA stand breaks in single mammalian cells. The technique is widely used for monitoring of genotoxicty testing and to evaluate DNA damage / repair.

In the current study, malathion injection induced DNA fragmentation in a large number of peripheral blood lymphocytes as indicated by the many comets that were produced. The comet percentage of peripheral blood lymphocytes was increased after malathion injection compared with the saline control group. Creatine administered at 160, 360 or 720 mg/kg resulted in a dose-dependent decrease in the % of damaged cells by 39%, 51 and 76%. (Figures 5, 6).

Moore and his collaborators [68] showed that malathion injected for 5 days at different doses increased mean percentages of DNA damage. Also, Muniz et al. [69] reported an elevated level of oxidative stress and DNA damage in human lymphocytes of agricultural workers exposed to OPs pesticides. Reus el al. [70] demonstrated that malathion induced oxidative stress that led to alterations in DNA molecules of malathion-treated rats. The authors concluded that malathion can be classified as a potential mutagen/carcinogenic compound.

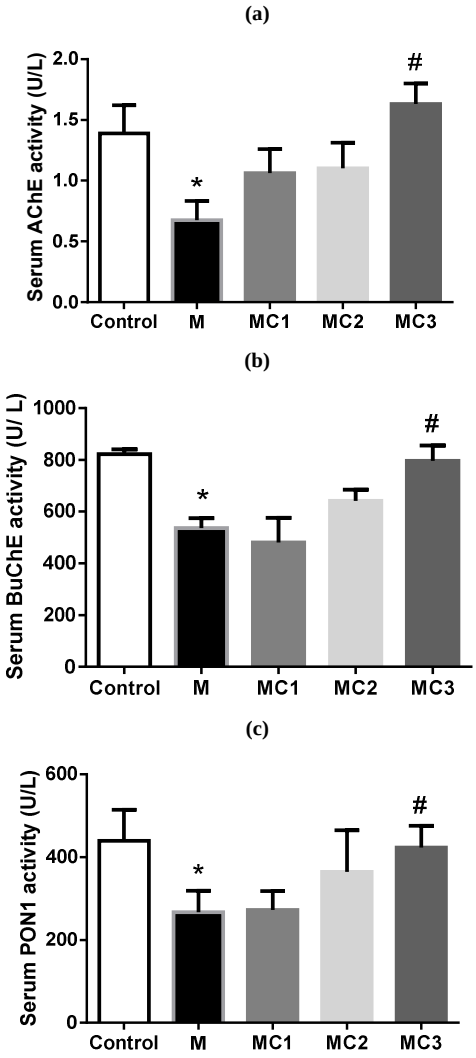


Figure 1. Effect of creatine on serum BChE, AChE and PON1 in rats exposed to malathion

*The data are presented as means ±SE. Statistical analysis was done using one way ANOVA followed Tukey-HSD test. *p<0.05 vs. saline group. # p<0.05 vs. malathion only group*

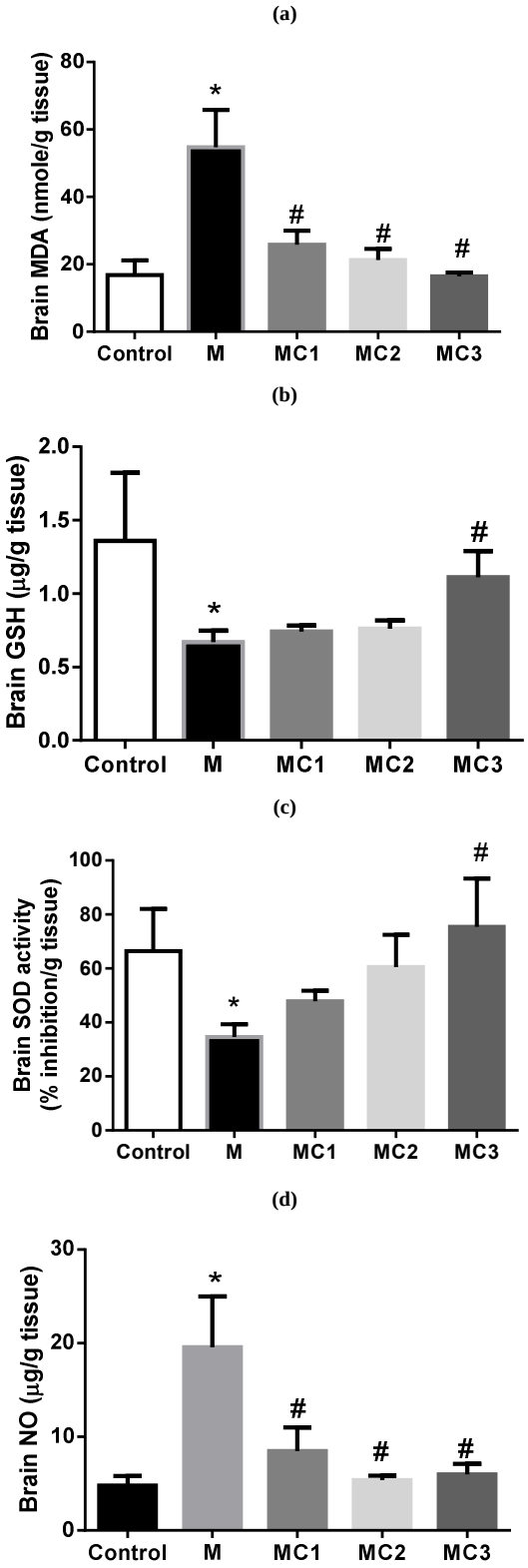


Figure 2. Effect of creatine on brain oxidative stress in rats exposed to malathion (a) MDA, (b) GSH, (c) SOD and (d) NO

*The data are presented as means ± SE. Statistical analysis was done using one way ANOVA followed Tukey-HSD test. *p<0.05 vs.saline group. # p<0.05 vs. malathion only group*

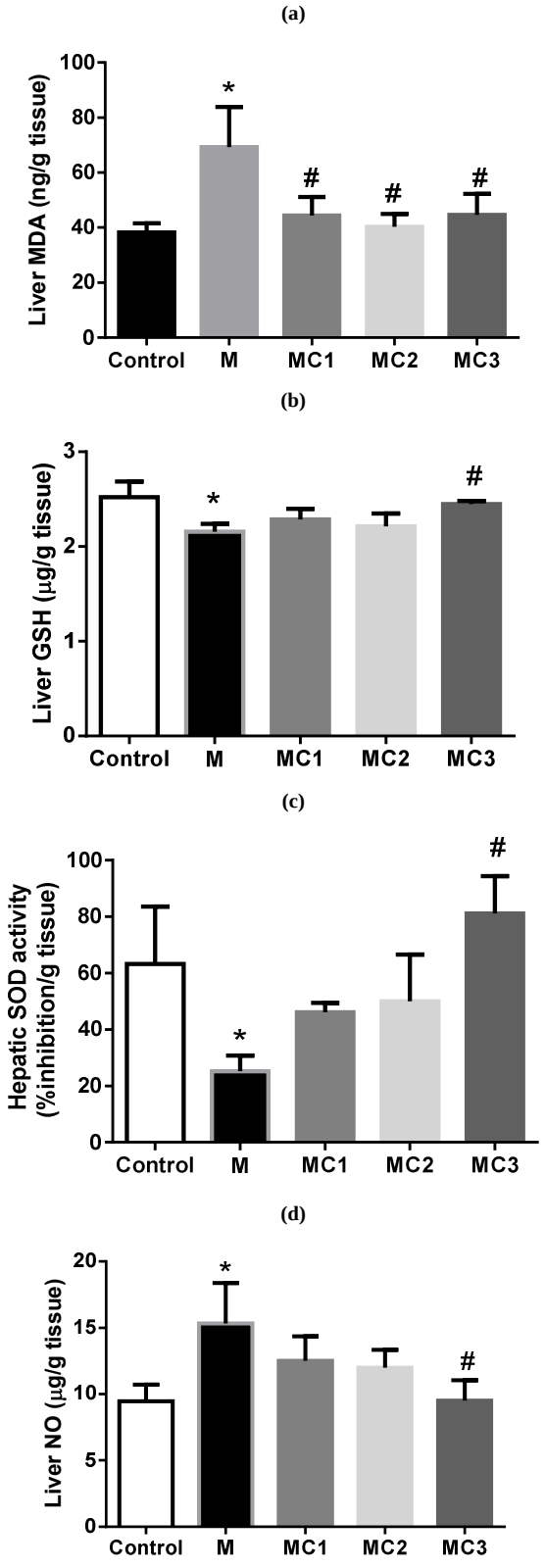


Figure 3. Effect of creatine on liver oxidative stress in rats exposed to malathion (a) MDA, (b) GSH, (c) SOD and (d) NO

*The data are presented as means ± SE. Statistical analysis was done using one way ANOVA followed Tukey-HSD test. *p<0.05 vs.saline group. # p<0.05 vs. malathion only group*

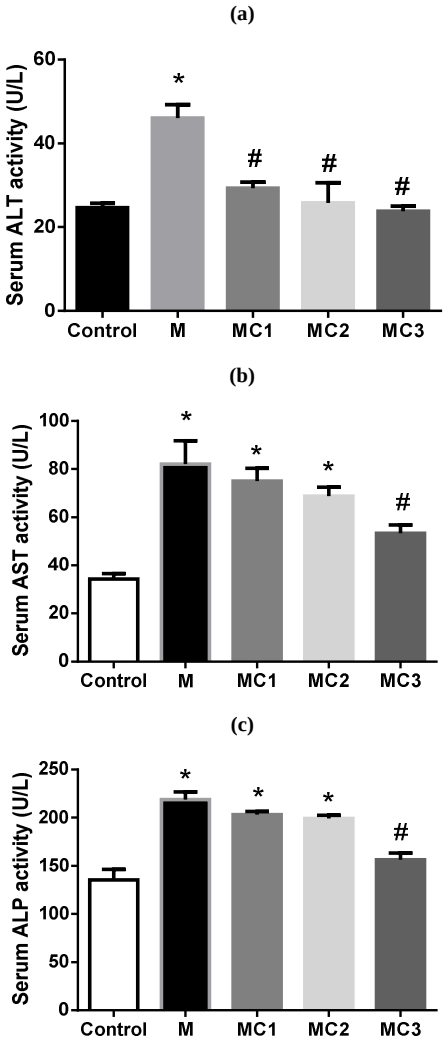


Figure 4. Effect of creatine on serum GOT, GPT and ALK in rats exposed to malathion

*The data are presented as means ± SE. Statistical analysis was done using one way ANOVA followed Tukey-HSD test. *p<0.05 vs. saline group. # p<0.05 vs. malathion only group*

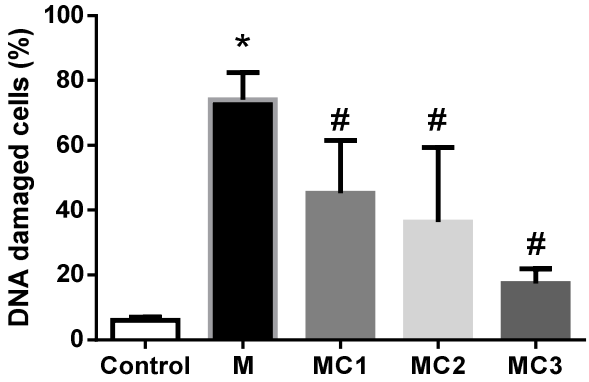
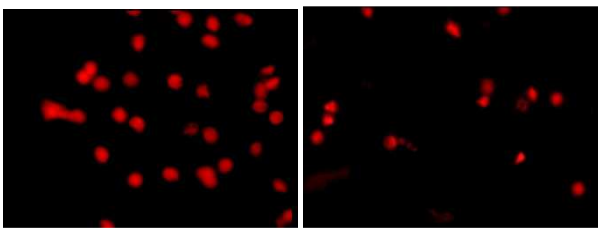


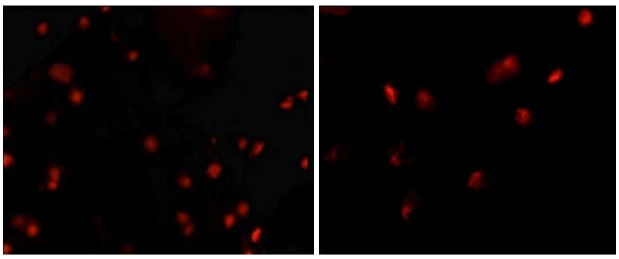
Figure 5. The comet percentage of peripheral lymphocytes in malathion-treated rats and the effect of creatine

*The data are presented as means ± SE. Statistical analysis was done using one way ANOVA followed Tukey-HSD test. *p<0.05 vs.saline group. # p<0.05 vs. malathion only group*

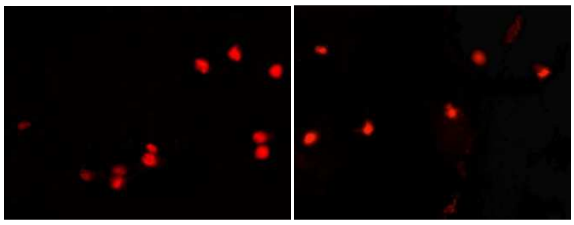
(a) Control group



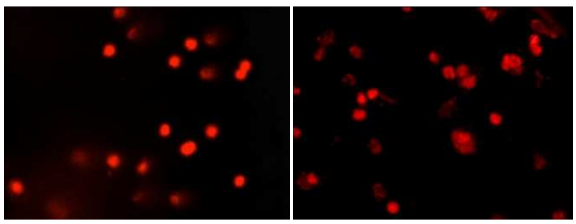
(b) Malathion-treated group



(c)Malathion+ creatine (160mg/kg)



(d) Malathion+ creatine (320mg/kg)



(e) Malathion+ creatine (720 mg/kg)

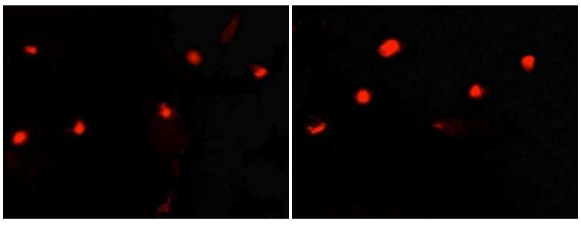


Figure 6. The comet percentage of peripheral lymphocytes in malathion-treated rats and the effect of creatine.
*The data are presented as means ±SE. Statistical analysis was done using one way ANOVA followed Tukey-HSD test. *p<0.05 vs. saline group. # p<0.05 vs. malathion only group*

CONCLUSION

In conclusion, these results indicate that acute exposure to malathion leads to inhibitory effect on major enzymes as AChE, BuChE and PON-1. These manifestations were accompanied with marked increase in brain and liver oxidative status, along with increase in release of liver enzymes. In addition, malathion increased DNA

fragmentation in peripheral lymphocytes. This study provides evidence for the first time that creatine supplements do protect against malathion –induced brain and liver damage.

Acknowledgements
The authors thank Prof. Dr. Omar M. Abdel-Salam for providing technical advice and support throughout this work.

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