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Involvement of Oxidative Stress in Methyl Parathion and Parathion-Induced Toxicity and Genotoxicity to Human Liver Carcinoma ($HepG_2$) Cells

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Abstract

Methyl parathion ($C_8H_{10}NO_5PS$) and parathion ($C_{10}H_{14}NO_5PS$) are both organophosphate insecticides (OPI) widely used for household and agricultural applications. They are known for their ability to irreversibly inhibit acetylcholinesterase which often leads to a profound effect on the nervous system of exposed organisms. Many recently published studies have indicated that human exposure to OPI may be associated with neurologic, hematopoietic, cardiovascular, and reproductive adverse effects. Studies have also linked OPI exposure to a number of degenerative diseases including Parkinson's, Alzheimer's, and amyotrophic lateral sclerosis. Also, oxidative stress (OS) has been reported as a possible mechanism of OPI toxicity in humans. Hence, the aim of the present investigation was to use human liver carcinoma ($HepG_2$) cells as a test model to evaluate the role of OS in methyl parathion- and parathion-induced toxicity. To achieve this goal, we performed the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay for cell viability, lipid peroxidation assay for malondialdehyde (MDA) production, and Comet assay for DNA damage, respectively. Results from MTT assay indicated that methyl parathion and parathion gradually reduce the viability of $HepG_2$ cells in a dose-dependent manner, showing 48 h-LD_{50} values of 26.20 mM and 23.58 mM, respectively. Lipid peroxidation assay resulted in a significant increase ($p<0.05$) of MDA level in methyl parathion- and parathion-treated $HepG_2$ cells compared to controls, suggesting that OS plays a key role in OPI-induced toxicity. Comet assay indicated a significant increase in genotoxicity at higher concentrations of OPI exposure. Overall, we found that methyl-parathion is slightly less toxic than parathion to $HepG_2$ cells. The cytotoxic effect of these OPI was found to be associated, at least in part, with oxidative cell/tissue damage.

Keywords

methyl parathion; parathion; cytotoxicity; oxidative stress; DNA damage; $HepG_2$ cells

INTRODUCTION

Organophosphate compounds such as malathion, parathion, methyl parathion, diazanon, and chlorpyrifos are widely used in agriculture as insecticides and acaricides. Residual amounts of organophosphate insecticides (OPI) have been detected in the soil, water bodies,

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vegetables, grains and other food products (IARC 1993; John et al., 2001; Poet et al., 2004, Choudhary and Sharma, 2008). They are also the most frequently used class of insecticides in household applications (Racke 1992). However, the increased use of these compounds has caused concern for not only the environment but also the health of the public (Bhanti et al., 2007). Acute or chronic exposure to these compounds may result in impaired memory and concentration, disorientation, severe depression, irritability, confusion, headache, speech difficulties, delayed reaction time, nightmare, sleepwalking, and drowsiness (Hayes and Wayland 1982; USEPA 1992, 2000). A recent study from our laboratory has demonstrated that malathion (an OPI) induces toxicity and DNA damage in $HepG_2$ cells through oxidative stress by increasing malondialdehyde (MDA) level, a byproduct of lipid peroxidation (Moore et al., 2009).

Methyl parathion is an OPI that was initially registered in 1954 in the United States (U.S. EPA 2000). Its use was restricted in 1978 as a result of detrimental effects to humans (Garcia et al., 2003). Although methyl parathion was restricted specifically to outdoor use and application by a licensed applicator, there have been several incidences of illegal applications of methyl parathion across the United States. These illegal applications have occurred in the homes of families living in Alabama, Arkansas, Illinois, Louisiana, Michigan, Mississippi, Ohio, Tennessee, and Texas (U.S. EPA, 2000) as well as in collected soil samples from 11 Atlanta homes (Riederer et al., 2010). Parathion is also an OPI with a weak cholinesterase inhibitor activity; it is responsible for tissue injury through an unknown mechanism, although some studies have reported that such pesticides generate reactive oxygen species (Bagchi et al., 1995). Several studies have shown that parathion is one of the most toxic insecticides studied.

Published research has indicated that OPI exert their toxicity through inhibition of acetylcholinesterase (AChE); an enzyme responsible for the degradation of the cholinergic neurotransmitter acetylcholine (Chambers et al., 1994). The involvement of oxidative stress leading to the generation of free radicals or reactive oxygen species (ROS) formation has been implicated in the toxicology of OPI (Bagchi et al., 1992; Yang and Dettbarn 1996). Recent investigations have pointed out that oxidative stress and DNA damage are possibly linked to pesticides-induced adverse health effects in agricultural workers (Muniz, 2008). However, the literature is scarce regarding the involvement of oxidative stress in methyl parathion- and parathion-induced toxicity. Therefore, the aim of the present investigation was to use $HepG_2$ cells as a test model to determine whether their cytotoxicity and genotoxicity are mediated through oxidative stress.

MATERIALS AND METHODS

Chemicals and Media

Test chemicals, methyl parathion (CAS No. 298-00-0, Lot No. 332-52B) and parathion (CAS No. 56-38-2, Lot No. 341-35A) were purchased from ChemService, Inc. (West Chester, Pennsylvania).Growth medium, Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (Lot No. 3000427) and supplements, G418 Sulfate and fetal bovine serum (FBS) were purchased from American Type Culture Collection -ATCC (Manassas, VA)

Cell Culture

Human liver carcinoma ($HepG_2$) cells were obtained from ATCC, and stored in liquid nitrogen until use. They were thawed by gentle agitation of their containers (vials) for 2 min in a water bath at 37°C. After thawing, the content of each vial was transferred to a 75cm^2 tissue culture flask, diluted with Dubelcco's Modified Eagle's Minimum Essential Medium

(DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin and penicillin, and incubated for 24 hr at 37°C in a 5% CO_2 incubator to allow the cells to grow, and form a monolayer in the flask. The growth medium was changed two times per week. Cells grown to 75–85% confluence were washed with phosphate buffer saline (PBS), trypsinized with 3mL of 0.25% (v) trypsin-0.0.3% /v) EDTA, diluted with fresh medium, and counted using a hemocytometer.

Cell Viability/Cytotoxicity Assay

For this experiment, 1×10^4 cells plated in each well of 96-well plates were placed in the humidified 5% CO_2 incubator at 37°C and allowed to attach to the substrate for 24h period. Cells grown to 70–85% confluence were exposed to various concentrations (0 mM, 6 mM, 12 mM, 18 mM, 24 mM, and 30 mM) of parathion and methyl parathion, respectively, and subsequently incubated for 48 hr. The cells incubated in culture medium alone served as a control for cell viability (untreated wells). After incubation the cell viability was determined using the MTT [3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay according to the standard test protocol commonly used in our laboratory (Mosmann 1983; Tchounwou et al. 2003; Brown et al. 2008).

Lipid Peroxidation Assay

Briefly, 2×10^6 $HepG_2$ cells/mL were placed in a total volume of 10ml growth medium and treated with different concentrations (0–30mM) of methyl parathion and parathion, respectively. Cells were incubated for 48 h in the humidified 5% CO_2 incubator at 37°C. After the incubation period, cells were collected in test tubes and centrifuged. The cell pellets were re-suspended in 0.5 ml of Tris-HCl, pH 7.4, and lysed using a sonicator (W-220; Ultrasonic, Farmingdale, NY) under the conditions of duty cycle 25% and output control 40% for 5 sec on ice. All samples were centrifuged at 15,000 rpm and 200 μl aliquot of the sample or 2 mg of cell lysate protein was assayed for malondialdehyde (MDA) according to the lipid peroxidation assay kit protocol (Calbiochem-Novabiochem, San Diego, CA). The absorbance of the sample was monitored at 586 nm, and the concentration of MDA was determined from a standard curve (Halliwell 1985, Moore et al., 2009).

Genotoxicity Assay

The Comet Assay was performed as recently described in our laboratory (Stevens et al., 2010; Trivergen, Inc., 2010). Briefly, 1×10^6 $HepG_2$ cells were cultured in 6-well tissue culture plates and exposed to various concentrations (0 – 30mM) of parathion and methyl parathion for 48 hr and stored in a 37°C, 5% CO_2 incubator. Following chemical treatment, the medium was removed, and the cells were washed three times with cold PBS, trypsinized with 1 mL of 0.25% trypsin-EDTA, harvested, and counted. The cells were spun down at 3000 rpm for 5 min. The pellet was re-suspended in PBS at a cell density of 1×10^5. The cells were combined with molten LMAgarose (at 37°C) at a ratio of 1:10 (v/v), and 75 μL was immediately pipetted onto CometSlide™. The slides were placed flat in a refrigerator at 4 °C for 30 min, and then immersed in prechilled lysis solution on ice for 1 h. Excess buffer from slides was removed, and the slides were immersed in freshly prepared alkaline solution, pH > 13 (0.6 g of NaOH pellets, 250 μL of 200 mM EDTA and 49.75 mL of dH_2O) for 1 h. Slides were washed twice for 5 min with 1X TBE electrophoresis buffer (Tris base, boric acid, and EDTA) and electrophoresed in a horizontal gel apparatus at 1 V/cm (22 V) for 10 min. Slides were placed in 70% ethanol for 5 min, removed, and tapped to remove excess ethanol. Slides were air dried overnight, stained with SYBR Green, and allowed to set for 12 h. Photographs were taken to illustrate the changes in DNA morphology associated with chemical exposure. A total of 150 comets were scored per chemical dose, and 75 comets randomly selected from three replicated slides, examined with an Olympus Epifluorescence Microscope, and analyzed using the LAI's Automated Comet Assay

Analysis System (Loates Associates, Inc. Westminster, MD). The experiment was repeated at least three times.

Statistical Analysis

Experiments were performed in triplicates. Data were presented as means ± SDs. Where appropriate, one-way ANOVA or Student paired t-test was performed using SAS Software available in the Biostatistics Core Laboratory at Jackson State University. *P*-values less than 0.05 were considered statistically significant. Cell viability, DNA damage, and MDA levels were presented graphically in the form of histograms, using Microsoft Excel computer program.

RESULTS

Measurements of Cell Viability

The cytotoxic effects of methyl parathion and parathion to $HepG_2$ cells are represented in Figures 1 and 2, respectively. As shown in these figures, methyl parathion and parathion gradually decrease the viability of $HepG_2$ cells in dose-dependent fashion, showing 48h-LD_{50} values of 26.20 mM and 22.11 mM, respectively. The percentages of cell viability were 100.00 ± 9.00, 91.25 ± 12.00, 75.11 ± 13.00, 71.25 ± 7.00, 61 ± 12.00, and 43 ± 9.00% for 0, 6, 12, 18, 24 and 30 mM methyl parathion (Figure 1), 100 ± 3.00, 74.53 ± 11.00, 68.82 ± 4.00, 51.31 ± 8.00, 53.96 ± 3.00, and 41.75 ± 7.00 % for 0, 6, 12, 18, 24, and 30 mM parathion (Figure 2). Based on the cell viability measurement, methyl-parathion appears to be slightly less toxic to $HepG_2$ cells than parathion. This reduction of toxicity may be associated with the presence of methyl group in methyl parathion.

Measurements of Malondialdehyde (MDA)

Lipid peroxidation assay resulted in a significant increase ($p<0.05$) of MDA level in both methyl parathion and parathion treated-$HepG_2$ cells compared to the controls. The amounts of MDA produced were 0.5 ± 0.05, 2.1 ± 0.5, 4.4 ± 0.01, 5.4 ± 0.01, 7.2 ± 0.3 μM, and 8.7 ± 0 for 0, 6, 12, 18, 24 and 30 mM methyl parathion (Figure 3), and 0.52 ± 0, 2.1 ± 0.1, 4.5 ± 0.1, 5.3 ± 0.1, 7.3 ± 0.2, and 9.0 ± 0.5 μM for 0, 6, 12, 18, 24 and 30 mM parathion (Figure 4). We observed a gradual increase of MDA level (an end product of lipid peroxidation) in $HepG_2$ cells with increasing concentrations of both methyl parathion and parathion.

Measurements of DNA Damage

Selected comet assay images of methyl parathion- and parathion-treated $HepG_2$ cells are presented in Figures 5 and 6, respectively. Our data shows that exposure to both compounds significantly increases the degree of genotoxicity in $HepG_2$ cells, as evidenced by the graduate increase in the percentage of DNA damage and the length of comet tail.

DISCUSSION

Measurement of Cell Viability

Cytotoxicity, the degree to which a chemical can cause cell damage, was assessed in the present study by the means of MTT assay. As shown in Figure 3, the MTT assay results reveal that methyl-parathion and parathion are cytotoxic to human liver carcinoma ($HepG_2$) cells, showing 48 h-LD_{50} values of 26.20 mM and 22.11 mM for methyl parathion and parathion, respectively. Hence, methyl-parathion is slightly less toxic to $HepG_2$ cells than parathion. Overall, both organophosphate insecticides significantly decrease ($p<0.05$) the viability of $HepG_2$ cells in dose-dependent fashion. Consistent with our finding, Budreau and Singh (1973) reported that organophosphate pesticides are embryotoxic and can induce

fetal anomalies in experimental animals. Several studies have indicated that methyl parathion exposure has toxic effects to mammals, fishes, birds, and non-target invertebrates (Solecki et al. 1996; Fanta et al. 2003; Roque et al., 2005).

Published studies have also demonstrated that organophosphate pesticides/insecticides exposure can affect many organ systems including the nervous, hematopoietic, cardiovascular, and reproductive systems (Handy et al., 2002; Neishabouri et al., 2004; Gokalp et al., 2005, Kalender et al., 2005). Both methyl parathion and parathion primarily affect the nervous system through the inhibition of cholinesterase (an enzyme required for proper nerve functioning), and the reproductive system by crossing the placenta to induce toxicity to the fetus (Kearney and Kaufman 1975; Occupational Health Services 1991, Hayes and Laws 1990). Laboratory reports have indicated that organophosphate pesticides exert their toxicity by inhibiting acetylcholinesterase (AChE), enzyme responsible for the degradation of the cholinergic neurotransmitter acetylcholine (Chambers et al. 1994; John et al., 2001; Yavuz et al., 2005). Due to parathion high toxicity and risks of exposure to agricultural workers and to birds, EPA announced the cancellation of all uses on fruits, nuts, and vegetable crops (U.S. EPA 1992). Although the present study reveals that methyl parathion exposure is slightly less toxic than parathion to $HepG_2$ cells, both organophosphate insecticides are highly cytotoxic and may cause severe damage to the liver cells/tissues. A recent study of rats treated with a single dose (6.25, 12.5, or 50 mg/kg) of methyl parathion resulted in severe signs of acute toxicity within 24 hr of dosing and died within 72 hr (Zhu et al., 2001).

Measurement of Malondialdehyde (MDA)

To investigate the hypothesis that methyl parathion and parathion exposure induces oxidative stress in humans, $HepG_2$ cells were treated with different doses of methyl parathion and parathion for 48 hr and assessed for the degree of MDA production. Our results indicate that methyl parathion and parathion exposure caused significant increase ($p < 0.05$) in the concentration of MDA, an end product of lipid peroxidation. The lipid peroxidation is an autocatalytic process which is caused by free radicals. The increase of MDA level in this study is an indicator of oxidative stress or free radical formation caused by methyl parathion and parathion in liver cells. Consistent with our finding, recent studies have indicated that organophosphorus compounds cause an increase of lipid peroxidation level in rats (Kalender et al., 2004; Sulak et al., 2005).

A previously published study has reported that pesticides induce the generation of reactive oxygen species, DNA damage, and lactate dehydrogenase leakage in cell and animal models (Bagchi et al. 1995). Ranjbar and his collaborators have also reported an association between induction of oxidative stress and acetylcholinesterase inhibition in organophosphate pesticide manufacturing workers with a minimal exposure of one year (Ranjbar et al., 2003). Research has also pointed out that the mechanism by which organophosphate insecticides exert their toxic effects is through the accumulation of acetylcholine resulting from the inhibition of acetylcholinesterase (AChE). This accumulation often leads to the onset of side effects depending on the interaction of the substrate with cholinergic receptors. Interaction with these receptors often leads to the onset of lipid peroxidation (Hazarika et al., 2003). The release of phospholipids has therefore been reported to be very important in the action of acetylcholinesterase (AChE) (Datta et al., 1994).

Measurements of DNA Damage

The single cell gel electrophoresis (SCGE) or Comet assay, measures DNA damage from individual cells based on the migration of DNA towards the negative anode. This migration takes place through an electrophoretic field. Damaged DNA containing strand breaks

migrate farther in the gel than intact DNA thus giving the appearance of a comet. DNA is stained with a fluorescent dye and viewed utilizing an epifluorescent microscope. The extent of the damage is determined by the length of the comet tail and percentage of migrated DNA.

A review of the Comet assay outlines a number of factors effecting DNA damage (Møller et. al, 2000). Several studies assessing DNA damage as a consequence of pesticide exposure have been conducted using the Comet assay. These studies have involved exposure to various pesticides. The Comet assay has been used to assess DNA damage in blood leukocytes of twenty nine Pakistani manufacturing workers exposed to various mixtures of OPIs, carbamates, and pyrethroids. The mean comet tail lengths and DNA damage were found to be significantly higher in exposed workers (Bhalli, et. al 2006). In a more recent study of immigrant farm-workers in Oregon exposed to various pesticides, significantly higher percentages of damaged DNA and comet tail moment were recorded in oral leukocytes of agricultural workers compared to nonagricultural workers (McCauley et. al., 2008). Urine analysis showed the presence of tetrahydrophthalimide, a Captan metabolite, dialhylphosphate metabolites, and Ops. Additionally, *in vitro* studies with lymphocyte cultures treated with OPs also resulted in a significant induction of DNA damage; validating and supporting the *in vivo* studies. Also, a recent study conducted in our laboratory has shown that malathion exposure induces DNA damage in $HepG_2$ cells (Moore et al., 2009).

CONCLUSIONS

The present *in vitro* study demonstrated that both methyl parathion and parathion exposure decreases cell viability, increases MDA levels resulting from reactive oxygen species formation, and induces DNA damage in human liver carcinoma ($HepG_2$) cells. These findings suggest that oxidative stress plays an important role in organophosphate insecticides (OPI)-induced toxicity and genotoxicity in $HepG_2$ cells. Overall, the present research demonstrated that OPI exposure is associated with cytotoxic and genotoxic effects on human liver carcinoma ($HepG_2$) cells. Further investigations are needed to identify the intracellular processes and mechanisms of oxidative stress resulting from methyl parathion and/or parathion exposure.

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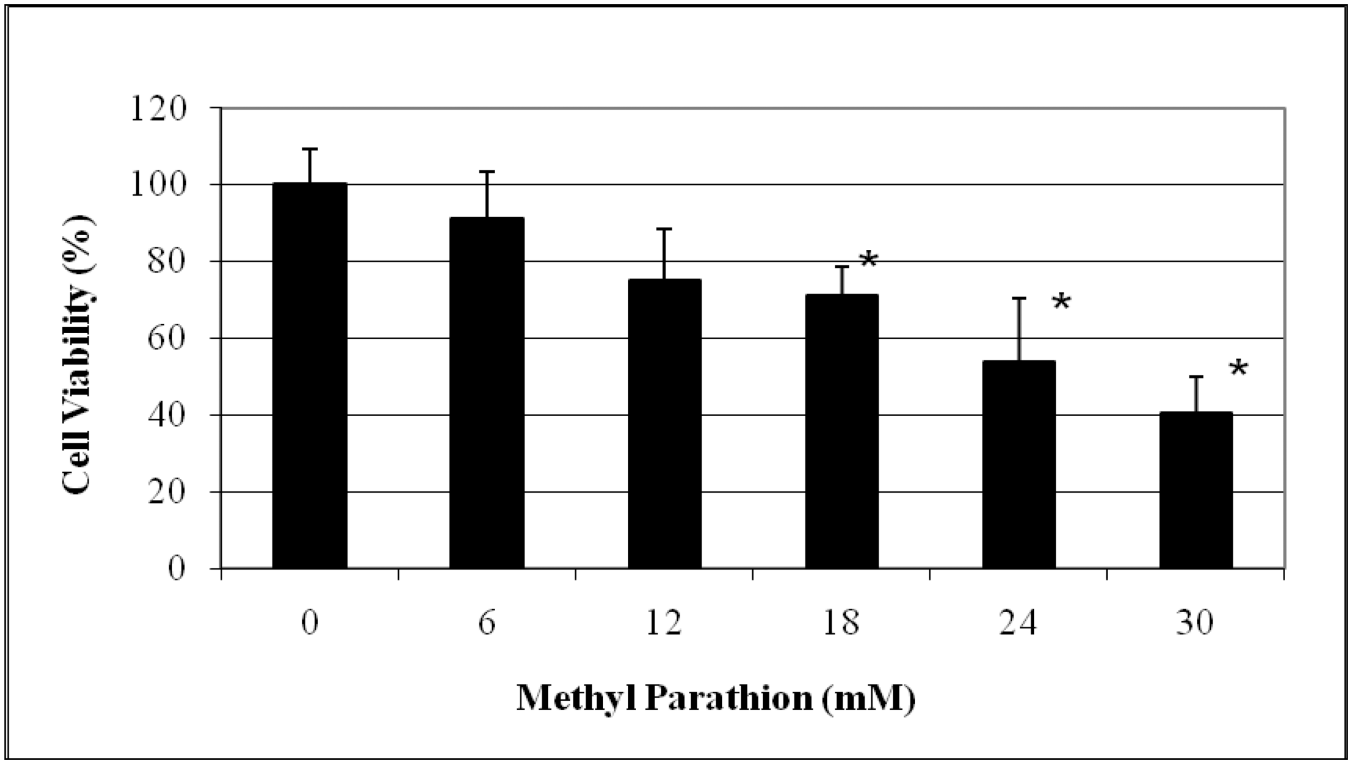

Fig. 1.

Cytotoxic effect of methyl parathion to human liver carcinoma ($HepG_2$) cells. $HepG_2$ cells were exposed to different doses (0–30 mM) of methyl parathion as indicated in Materials and Methods. Cell viability was determined based on the MTT assay. Each point represents a mean value and standard deviation of 3 experiments. *Significantly different from the control by ANOVA Dunnett's test; $p < 0.05$.

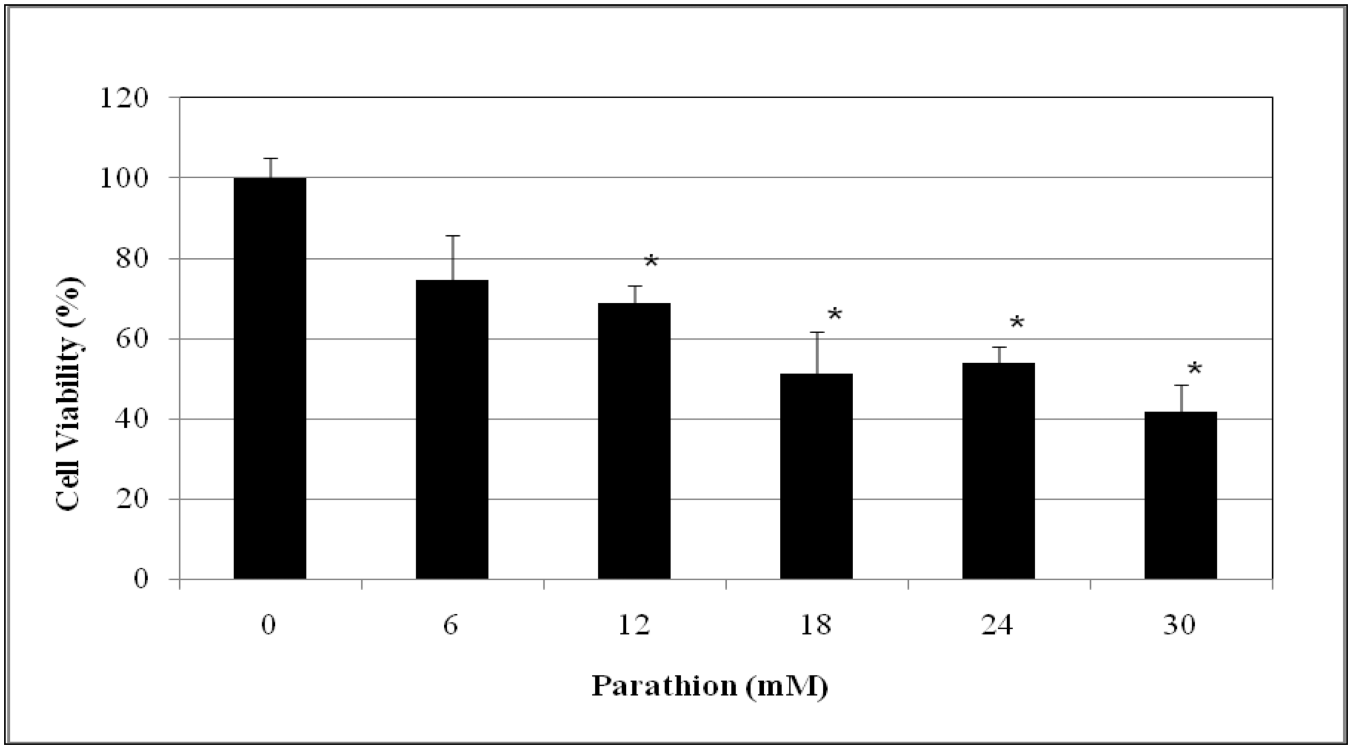

Fig. 2.

Cytotoxic effect of parathion to human liver carcinoma ($HepG_2$) cells. $HepG_2$ cells were exposed to different doses (0–30 mM) of parathion as indicated in Materials and Methods. Cell viability was determined based on the MTT assay. Each point represents a mean value and standard deviation of 3 experiments. *Significantly different from the control by ANOVA Dunnett's test; $p < 0.05$.

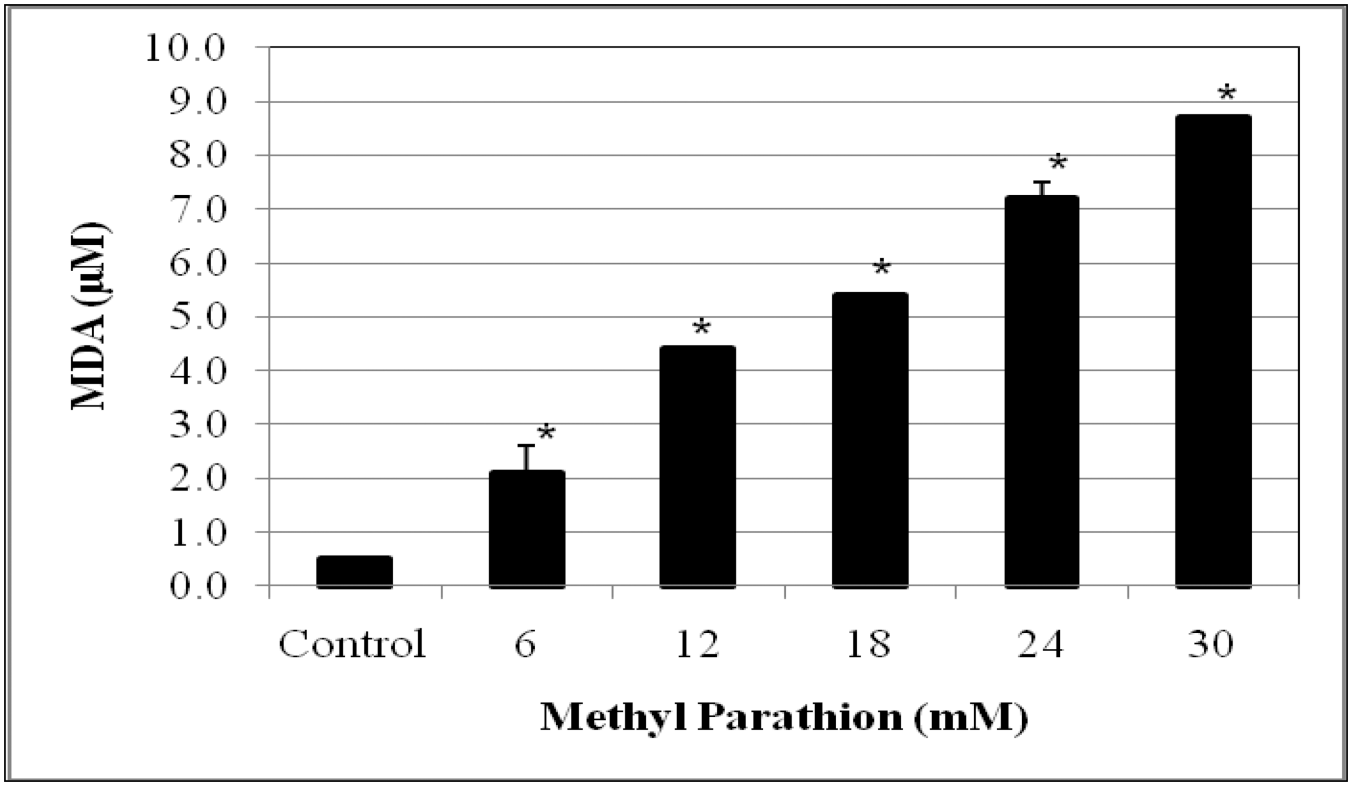

Fig. 3.

Effects of methyl parathion on MDA generation in $HepG_2$ cells. Cells were exposed to different doses (0–30 mM) of methyl parathion, and malondialdehyde formation was determined as described in Materials and Methods. *Significantly different from the control by ANOVA Dunnett's test; $p < 0.05$.

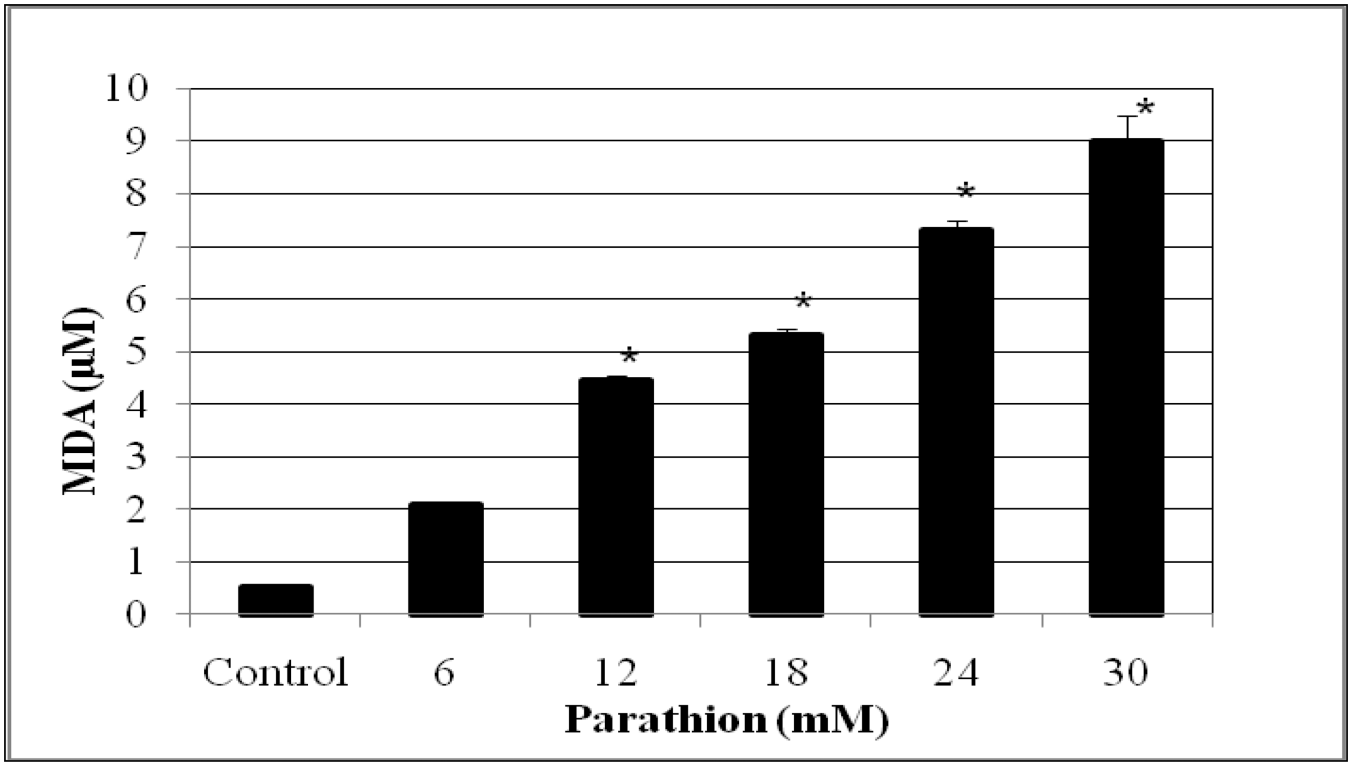

Fig. 4.

Effect of parathion on MDA generation in $HepG_2$ cells. Cells were exposed to different doses (0–30 mM) of parathion, and malondialdehyde formation was determined as described in Materials and Methods. *Significantly different from the control by ANOVA Dunnett's test; $p < 0.05$.

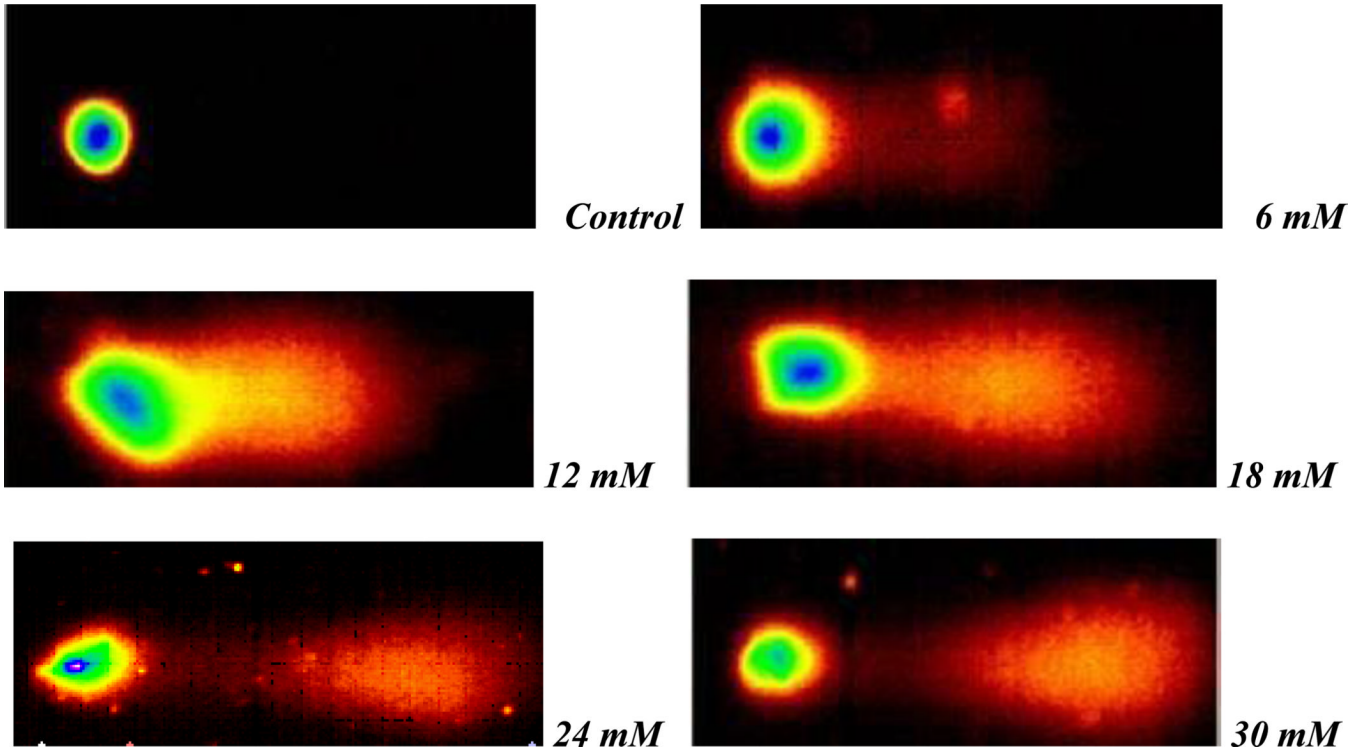

Fig. 5.

Representative Comet assay SYBR Green images of $HepG_2$ cells exposed to different doses (0–30 mM) of methyl parathion. Cells were exposed to different doses of methyl parathion, and single cell gel electrophoresis (Comet) assay was performed as described in Materials and Methods.

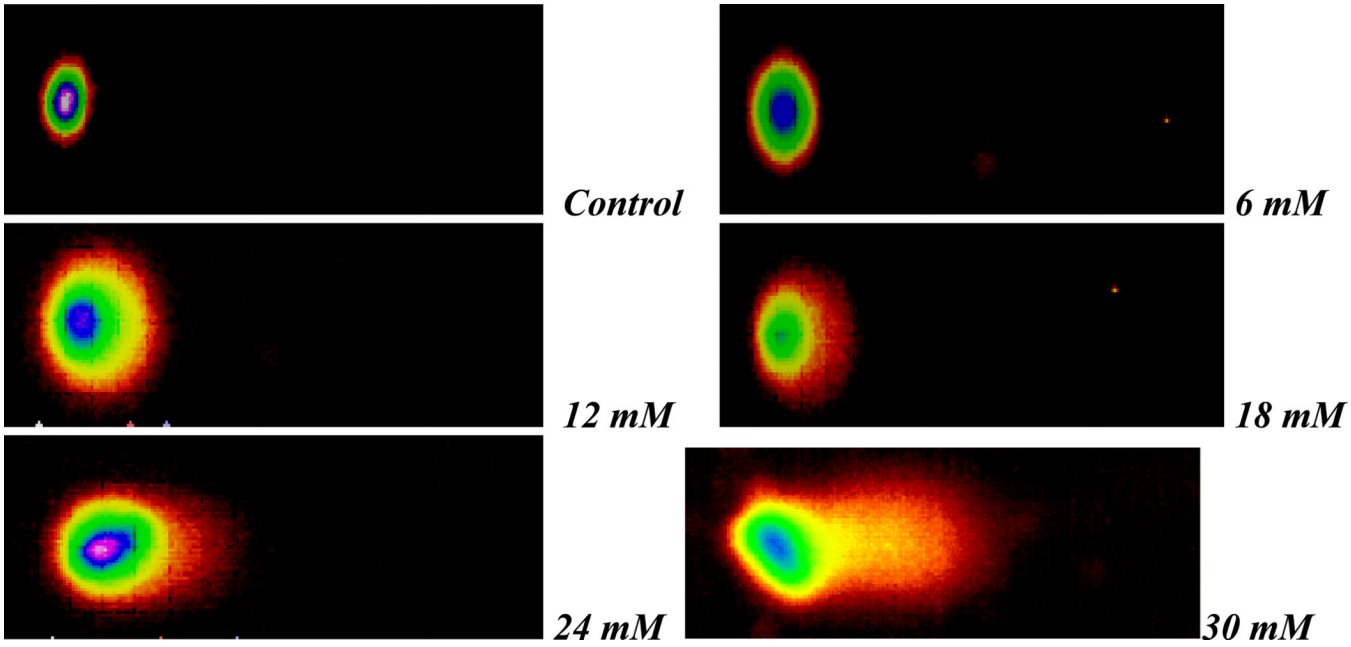

Fig. 6.

Representative Comet assay SYBR Green images of $HepG_2$ cells exposed to different doses (0–30 mM) of parathion. Cells were exposed to various doses of parathion, and single cell gel electrophoresis (Comet) assay was performed as described in Materials and Methods.