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Research Article

Fenthion and Terbufos Induce DNA Damage, the Expression of Tumor-Related Genes, and Apoptosis in HepG2 Cells

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This study investigates the effects of fenthion and terbufos, two organophosphorous pesticides, on DNA damage, tumor-related gene expression, and apoptosis in HepG2 cells. We found that exposure to concentrations ranging from 50 to 200 μM of fenthion and terbufos for 2 hr caused significant death in HepG2 cells. Both compounds induced DNA damage in a concentration-dependent manner as measured using the alkaline comet assay. Tumor-related genes (*jun*, *myc*, and *fos*) and apoptosis-related genes (*socs3*, *tnfaip3*, *ppp1r15a*, and *nr4a1*) were up-regulated by both compounds. Finally, both compounds induced apoptosis. The results demonstrate that both terbufos and fenthion induce DNA damage and should be considered potentially hazardous to humans. Environ. Mol. Mutagen. 52:529–537, 2011.

Key words: fenthion; terbufos; comet assay; apoptosis; tumor-related genes

INTRODUCTION

Fenthion and terbufos are organophosphate pesticides (OPs). OPs are the most widely used insecticides worldwide [Hreljac et al., 2008]. The toxicity of OPs is reported to be related to the inhibition of acetylcholinesterase (AChE) in the central and peripheral nervous system, causing a variety of short-term and chronic effects, such as nausea, headache, confusion, depression, memory loss, and chronic fatigue syndrome [Gupta, 2006]. In addition to occupational poisoning in industrial production and agricultural application, acute OP poisonings resulting from suicide, homicide and accidental overdose have occurred [Kamiya et al., 2001; Zhou et al., 2002].

Previous studies have revealed that fenthion may be carcinogenic in male mice [Shepard, 1986]. However, no carcinogenic effects were observed in a 2-year feeding study in rats and mice [ACGIH, 1986]. Available data are insufficient to draw conclusions regarding the carcinoge-

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nicity of fenthion [EXTOXNET, 1996]. Terbufos has been classified by the United States Environmental Protection Agency (USEPA) as a noncarcinogen due to a lack of observed carcinogenic effects in two animal studies, and a lack of mutagenicity in several short-term genotoxicity assays [McCall, 1995]. However, Bonner et al. [2010] suggested that cancers of the prostate and lung as well as leukemia and non-Hodgkin's lymphoma may be associated with terbufos use.

Tests for the mutagenicity and genotoxicicity of fenthion have produced inconsistent results. Negative results were observed in vitro for bacterial mutagenicity, chromosome aberrations in Chinese hamster ovary and lung cells, and sister chromatid exchanges in human lymphoid cells [Ma, 1995]. However, other studies have shown that fenthion induces genotoxicity in vitro including chromosomal aberrations and sister chromatid exchanges in human lymphocytes, and unscheduled DNA synthesis in rat primary hepatocytes, and in vivo micronucleus formation in mouse bone marrow cells [Ma, 1995]. Terbufos has been reported to be nonmutagenic using several tests including: A dominant lethal study in rats, a bacterial reversion mutation test, a chromosomal aberration test, and a test for DNA repair in rat liver [Moretto, 1990].

In this study, we investigate the genotoxicity of terbufos and fenthion in HepG2 cells using the comet assay. The comet assay has been validated in HepG2 cells as a reliable method for predicting genotoxic agents [Uhl et al., 2000]. In addition, the effects of fenthion and terbufos on the expression of various tumor-related and apoptosis-related genes in HepG2 cells were examined to provide insight into the genotoxic mechanisms involved in response.

MATERIALS AND METHODS

Materials

L-Glutamine, phosphate buffered saline (PBS; Ca^{2+}/Mg^{2+}-free), RPMI 1640 medium and fetal bovine serum were purchased from Gibco-Invitrogen (Carlsbad, CA). Low melting-point agarose (LMPA), normal melting-point agarose (NMPA), Tris buffer, ethidium bromide, trypsin, antibiotic solution, trypan blue, terbufos ($C_9H_{21}O_2PS_3$, Cas No. 13071-79-9), and fenthion ($C_{10}H_{15}O_3PS_2$, Cas No. 55-38-9) were obtained from Sigma (St. Louis, MO). High-purity water was prepared from a Milli-Q gradient water purification system (Millipore). All of these chemicals were of analytical reagent grade. Tested chemicals were freshly prepared by dissolving in DMSO and were kept in the dark. The final concentration of DMSO was less than 0.5% of the reaction mixtures.

Cell Culture and Treatment

HepG2 cells were grown in DMEM medium including 20% heat-inactivated fetal bovine serum, 2% phytohemagglutinin, 50 U/ml penicillin, 100 μg streptomycin, and 2 mM L-glutamine at 37°C under 5% CO_2 atmosphere (HyClone, USA). The cells were diluted down to a concentration of 2.5×10^5 cells/ml prior to use. At doses over 200 μM, the solubility of the tested chemicals dissolved in 0.5% DMSO was limited. Thus, fenthion and terbufos at doses below 200 μM were used separately for the determination of their genotoxicity with the comet assay. The cells were incubated with different concentrations of fenthion and terbufos (50, 100, and 200 μM) dissolved in DMSO (0.5% as a final concentration) at 37°C for 2 hr in a dark incubator. Subsequently, the cells were centrifuged at 200*g* for 3 min at 4°C and mixed with low melting-point agar for the comet assay as described below.

Cell Viability Analysis

The assays were conducted following the procedures in Chen et al. [2003]. After the cells' incubation with 50 μM, 100 μM, and 200 μM terbufos and fenthion, respectively, for 2 hr, the cells were mixed with 10 μl 0.4% trypan blue solution for 5 min. Cells were analyzed through microscopic observation to determine the percentage of viable cells.

Comet Assay

The comet assay was performed under alkaline conditions following previously published methods [Chen et al., 2008]. Cells treated with 100 μM H_2O_2 were used as the positive control, and the solvent, 0.5% DMSO, was used as the negative control. Conventional microscope slides were dipped into a solution of 85 μl 0.5% NMPA and 0.5% LMPA in PBS (pH 7.4), and allowed to dry on a flat surface at room temperature. Ten microliters of cell suspension (2.5×10^5 cells/ml) was mixed with 75 μl 0.5% (w/v) LMPA in PBS (pH 7.4). Seventy-five microliters of this suspension was rapidly layered onto the slides precoated with the mixtures of 0.5% NMPA and 0.5% LMPA and covered with a cover slip. The slides were maintained at 4°C for 5 min, the cover slip was removed, and cells were immersed in a freshly made lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, and 1% (v/v) Triton X-100 at pH 10) at 4°C for 1 hr. The slides were then placed in a double row in a 260-mm wide horizontal electrophoresis tank containing 0.3 M NaOH and 1 mM Na_2EDTA for 10 min. Electrophoresis (1 V/cm, 300 mA) was conducted for 15 min at 4°C. After the electrophoresis, the slides were soaked in a cold neutralizing buffer (400 mM Tris buffer, pH 7.5) at 4°C for 10 min. Slides were dried in 100% methanol for 5 min and stored in a low humidity environment before staining with 40 μl propidium iodide (PI; 2.5 μg/ml).

Quantification of the Comet Assay

Images from 300 random cells (3 slides/each experiment, 100 random cells/ slide) per sample were analyzed and divided by three based on 100 random cells as the scoring unit. This experiment was independently repeated three times. One hundred comets on each slide were scored visually according to the relative intensity of the tail [Chen et al., 2008]. An intensity score from class 0 (undamaged) to class 4 (severely damaged) was assigned to each cell. Thus, the total score for the 100 comets could range from 0 to 400 because 100 cells were observed individually in each comet assay. The extent of DNA damage was analyzed and then scored by the same experienced person, using a specific pattern when moving along the slide. The observer had no knowledge of the identity of the slide when scoring the comets.

Total RNA Isolation

Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's protocol. Briefly, after fenthion or terbufos exposure, the cells were washed with cold 1 × PBS, and 1 ml Trizol reagent was added to the cells, followed by the addition of chloroform. After mixing, the solution was centrifuged at 13,000*g* for 20 min. The supernatant was isolated, an equal volume of isopropanol was added, and the mixture was incubated for 10 min after gently mixing. This mixture was centrifuged at 13,000*g* for 10 min. The supernatant was discarded, and the pellet was treated with 70% alcohol with the addition of 1 ml diethylpyrocarbonate (DEPC). After drying, RNA was dissolved in DEPC. RNA

quantity and purity was measured by spectrophotometry (BioPhotometer, Eppendrof), and the RNA was considered suitable for further processing if A_{260}/A_{280} ratios were between 1.8 and 2.0. RNA integrity was determined by 1.8% agarose gel electrophoresis.

Real-Time Reverse Transcription PCR

Total RNA (5 μg) was reverse transcribed using MMLV reverse transcriptase (Promega), and the resulting samples were diluted with DNase-free water. Each cDNA solution was stored at −20°C until further real-time PCR analysis. In each real-time PCR run, two-independent samples were analyzed, and each experiment was performed twice independently. The specificity of each primer pair was validated by performing an RT-PCR using common reference RNA (Stratagene, USA) as a DNA template, and the size of the PCR product was assessed using a DNA 1000 chip (Agilent Technologies, USA) run on a Bioanalyzer 2100 (Agilent Technologies, USA). Primer pairs that generated the predicted product size and no other side product were chosen to conduct real-time RT-PCR. RT-PCR was performed on a Roche LightCycler Instrument 1.5 using a LightCycler® FastStart DNA Master^{PLUS} SYBR Green I kit (Roche, Castle Hill, Australia). Briefly, 10-μl reactions contained 2 μl Master Mix, 2 μl 0.75 μM forward primer and reverse primer (primer pairs for each target gene are shown in Table I), and 6-μl cDNA sample. The RT-PCR program consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles of amplification and quantification at 95°C for 10 sec, 60°C for 15 sec, and 72°C for 10 sec. At the end of the program, a melt curve analysis was done. At the end of each RT-PCR run, the data were automatically analyzed by the system and an amplification plot was generated for each cDNA sample. From each of these plots, the LightCycler3 Data Analysis software automatically calculated the CP value (crossing point, which corresponds to the first maximum of the second derivative curve), which indicates the beginning of exponential amplification. The fold expression or repression of the target gene relative to the internal control gene in each sample was calculated by the following the ΔΔCt model.

As described by Yuan et al. [2006], the ratio of target gene expression in treatment vs. control was derived from the ratio between target gene efficiency (E_{target}) to the power of target ΔCt (ΔCt_{target}) and reference gene efficiency ($E_{reference}$) to the power of reference ΔCt ($\Delta Ct_{reference}$). The ΔΔCt model can be derived from the efficiency-calibrated model, if both target and reference genes reach their highest PCR amplification efficiency. In this circumstance, both target efficiency (E_{target}) and control efficiency ($E_{control}$) equals 2, indicating amplicon doubling during each cycle, then there would be the same expression ratio derived from $2^{-\Delta\Delta Ct}$.

TABLE I. Sequences of Primers

Gene	Primer sequences
Fos	Forward 5′-CTCGTCTCCTCTGTG-3′
	Reverse 5′-TTCGGATTC-3′
Jun	Forward 5′-CAAGTGGCAGAGT-3′
	Reverse 5′-CGCTCGCCCAAGTTCAA-3′
Myc	Forward 5′-AGCAAACCTCCTCAC-3′
	Reverse 5′- TGTTCGCCTCTTGACATTC -3′
Gadd45a	Forward 5′-TCGGCTGGAGAGCAGAA-3′
	Reverse 5′- GGCACAACACCACGTTA -3′
Ppp1r15a	Forward 5′-GAGGAAGAGGGAGTTGCT-3′
	Reverse 5′- TGGAGACAAGGCAGAAGTAGA -3′
Socs3	Forward 5′-GACGGGACATCTTTCACC-3′
	Reverse 5′- CCCATCCAGGCTGAGTAT -3′
Tnfaip3	Forward 5′-GTTCAGGACACAGACTTGGT-3′
	Reverse 5′- TTCATCATTCCAGTTCCGAGTAT -3′
Tbp	Forward 5′-TGTTGTGAGAAGATGGATGTTGA -3′
	Reverse 5′-CCAGATAGCAGCACGGTA-3′

Detection of Apoptotic Cells

Annexin V/PI staining was carried out according to the manufacturer's protocol (BD Bioscience). After treatment with different concentrations

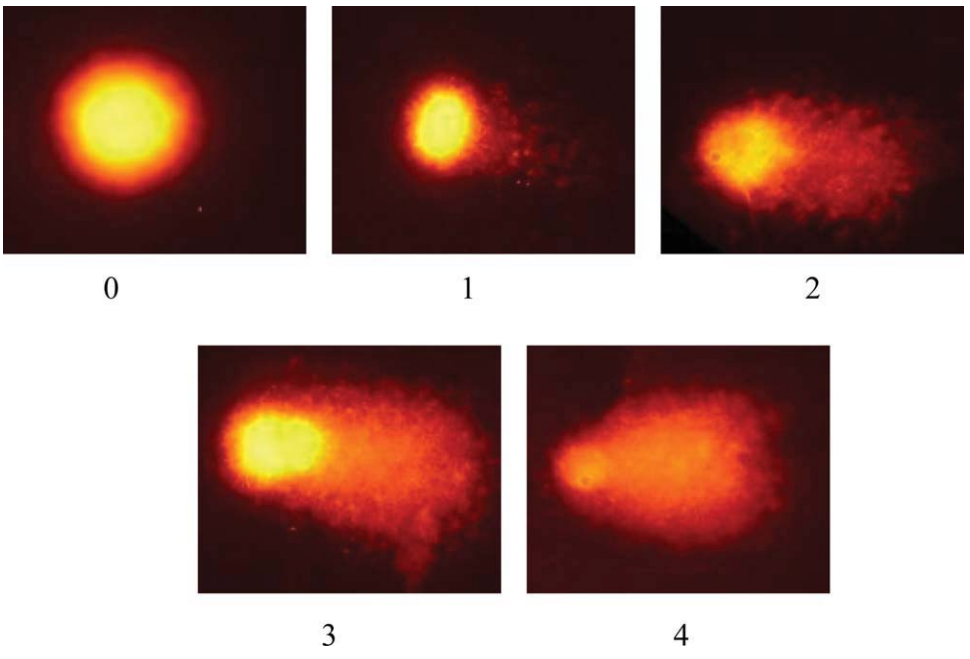

Fig. 1. Comet images of HepG2 cells, illustrating the visual scoring classification. (**A**) Class 0. (**B**) Class 1. (**C**) Class 2. (**D**) Class 3. (**E**) Class 4. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

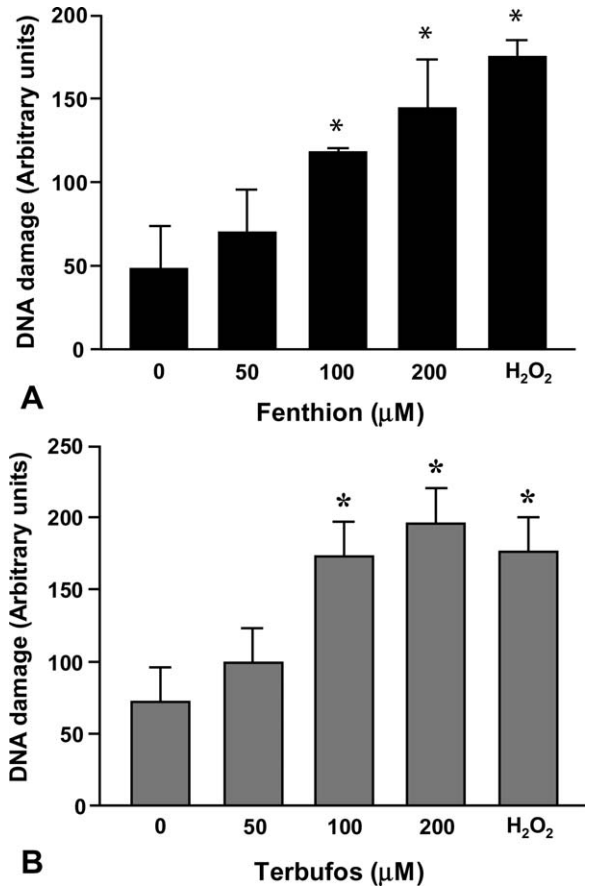

Fig. 2. DNA damage in HepG2 cells exposed to different concentrations of (**A**) fenthion and (**B**) terbufos for 2 hr using the alkaline comet assay. Asterisk (*) indicates $P < 0.01$. Cells treated with 100 μM H_2O_2 were used as the positive control, and 0.5% DMSO was used as the negative control.

of fenthion and terbufos (0, 100, and 200 μM) for 2 hr, the cells were washed with cold PBS and resuspended with a binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Next, 1×10^6/ml cell suspension was transferred to the mixture of 5 μl annex-V-FITC and 10 μl PI (50 μg/ml) and incubated at room temperature in the dark for 15 min. Binding buffer was added, and the cells were analyzed by flow cytometry (Cell Lab Quanta™ SC, Beckman). This assay was repeated twice for each experiment, and each experiment was performed twice independently.

Statistical Analysis

For the comet assay, we followed the statistical method of Baipayee et al. [2006]. The mean comet data (DNA damage scores) from three slides in each experiment was obtained, and the differences in the mean comet scores for the control and test groups from the three independent experiments were analyzed using a one-way analysis of variance (ANOVA), with DNA damage as the dependant variable and concentrations of the tested compounds as the independent variable. If a significant F-value was obtained, Dunnett's multiple comparison tests were conducted. $P < 0.05$ was considered to be significant. The homogeneity of variance between treatment groups was ascertained prior to the statistical analysis of the comet assay data.

TABLE II. Effects of Different Concentrations of Fenthion on Gene Expression in HepG2 Cells

	Fenthion (μM)		
Gene	50	100	200
Fos	2.27 ± 0.25[a]	26.17 ± 7.31[a]	72.50 ± 23.7[a]
Jun	1.20 ± 0.07	1.87 ± 0.13	2.43 ± 0.43[a]
Myc	1.63 ± 0.16	3.93 ± 0.91[a]	14.73 ± 3.56[a]
Gadd45a	1.78 ± 0.21	1.84 ± 0.18	2.73 ± 0.32[a]
Ppp1r15a	3.39 ± 0.78[a]	4.35 ± 0.76[a]	6.48 ± 1.54[a]
Socs3	1.04 ± 0.08	1.31 ± 0.06	6.19 ± 1.49[a]
Tnfaip3	1.43 ± 0.14	2.00 ± 0.23[a]	9.26 ± 1.70[a]

Each data point ($n = 4$) represents the mean ± SD from two-independent experiments, and was normalized against TATA box binding protein as a housekeeping gene.
[a]The values of mRNA in fenhion-treated cells relative to those of the controls (without fenthion treatment).

TABLE III. Effects of Different Concentrations of Terbufos on Gene Expression in HepG2 Cells

	Terbufos (μM)		
Gene	50	100	200
Fos	15.70 ± 2.74[a]	18.42 ± 1.81[a]	69.79 ± 8.19[a]
Jun	1.53 ± 0.30	2.22 ± 0.37[a]	4.79 ± 0.33[a]
Myc	2.29 ± 0.13[a]	2.05 ± 0.12[a]	4.73 ± 0.09[a]
Gadd45a	11.99 ± 3.30[a]	10.37 ± 2.36[a]	15.90 ± 1.94[a]
Ppp1r15a	3.16 ± 0.62[a]	5.68 ± 1.22[a]	8.51 ± 1.33[a]
Socs3	8.02 ± 2.02[a]	4.05 ± 0.26[a]	5.41 ± 0.69[a]
Tnfaip3	4.66 ± 0.88[a]	6.64 ± 1.05[a]	26.32 ± 3.86[a]

Each data point ($n = 4$) represents the mean ± SD from two-independent experiments, and was normalized against TATA box binding protein as a housekeeping gene.
[a]The values of mRNA in terbufos-treated cells relative to those of the controls (without terbufos treatment).

RESULTS

DNA Damage

The viability of HepG2 cells treated with fenthion and terbufos (50–200 μM) for 2 hr was evaluated. The cell viability after the treatment was >90% using trypan blue dye assays (data not shown). Genotoxicity measured by the alkaline comet assay was determined using the visual scoring method [Chen et al., 2008] as shown in Figure 1. Figures 2A and 2B summarizes the DNA damage measured in HepG2 cells treated with varying concentrations of fenthion and terbufos at 37°C for 2 hr. The positive control group (cells pretreated with 100 μM H_2O_2) showed a significant DNA damage score, while the negative control (0.5% DMSO) displayed a very low DNA damage score. At concentrations of 100 and 200 μM, fenthion and terbufos exhibited significant DNA damage compared to the negative control group ($P < 0.05$). This result indicates that these chemicals can induce DNA damage in HepG2 cells.

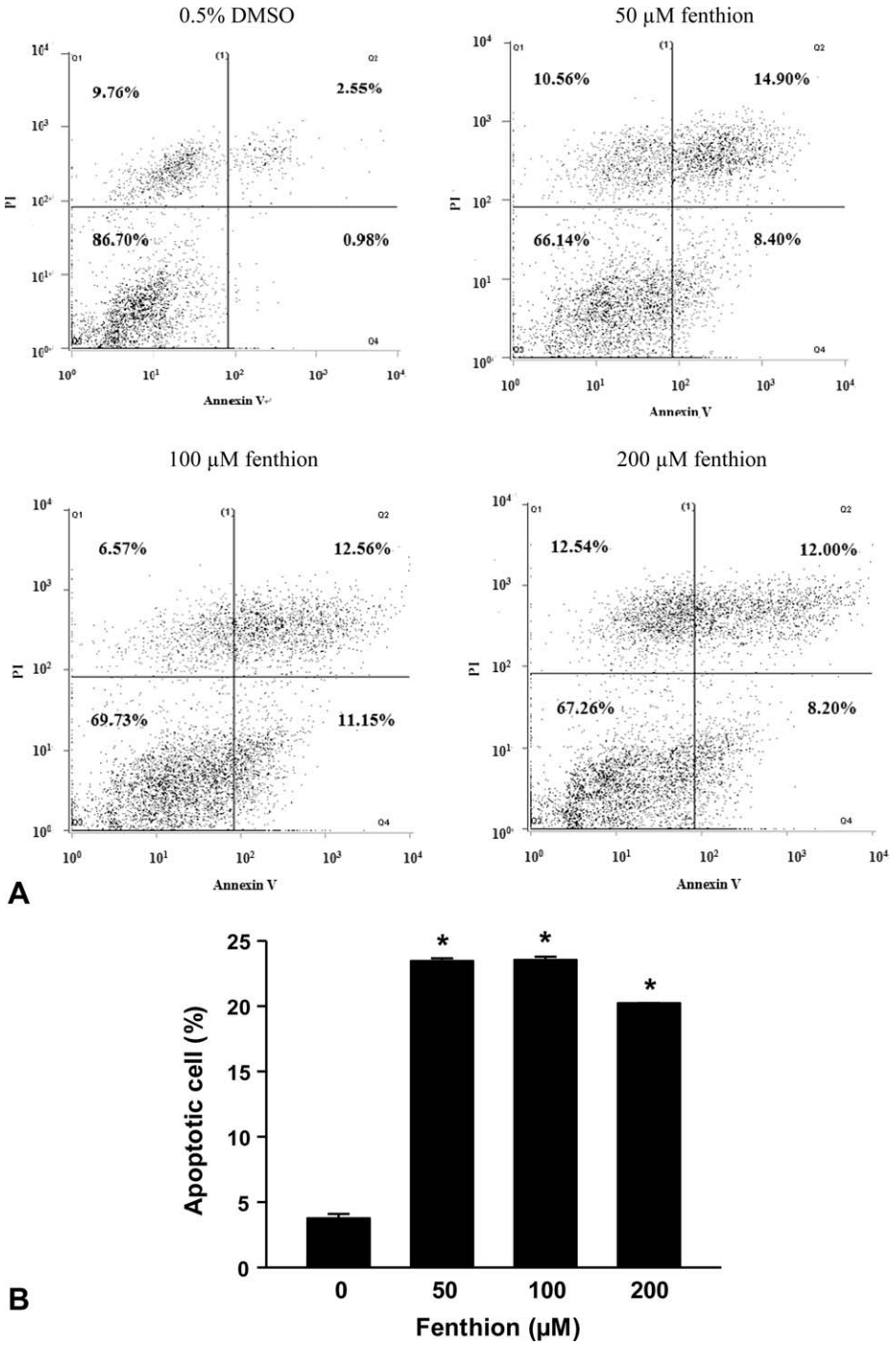

Fig. 3. Fenthion-induced apoptosis in HepG2 cells. **A**: Dot plot of FITC-Annexin-V/PI in control cells vs. fenthion-treated cells. **B**: The percentages of apoptotic cells were derived from the sum of the percentages of quadrant 2 and 3, which show FITC-Annexin-V^+/PI^+ (late apoptosis) and FITC-Annexin-V^+/PI^- (early apoptosis), respectively. Data are presented as the mean ± SD ($n = 4$). *Significantly different from the control cells at $P < 0.05$.

Expression of Tumor-Related and Apoptosis-Related Genes

As shown in Tables II and III, exposure to 200 μM fenthion or terbufos caused a statistically significant increase in the expression of the tumor-related genes *fos, jun*, and *myc* and the apoptosis-related genes *gadd45a*, *ppp1r15a*, *soc3*, and *tnfaip3*. At concentrations of 100 μM and below, terbufos resulted in the up-regulation of a greater

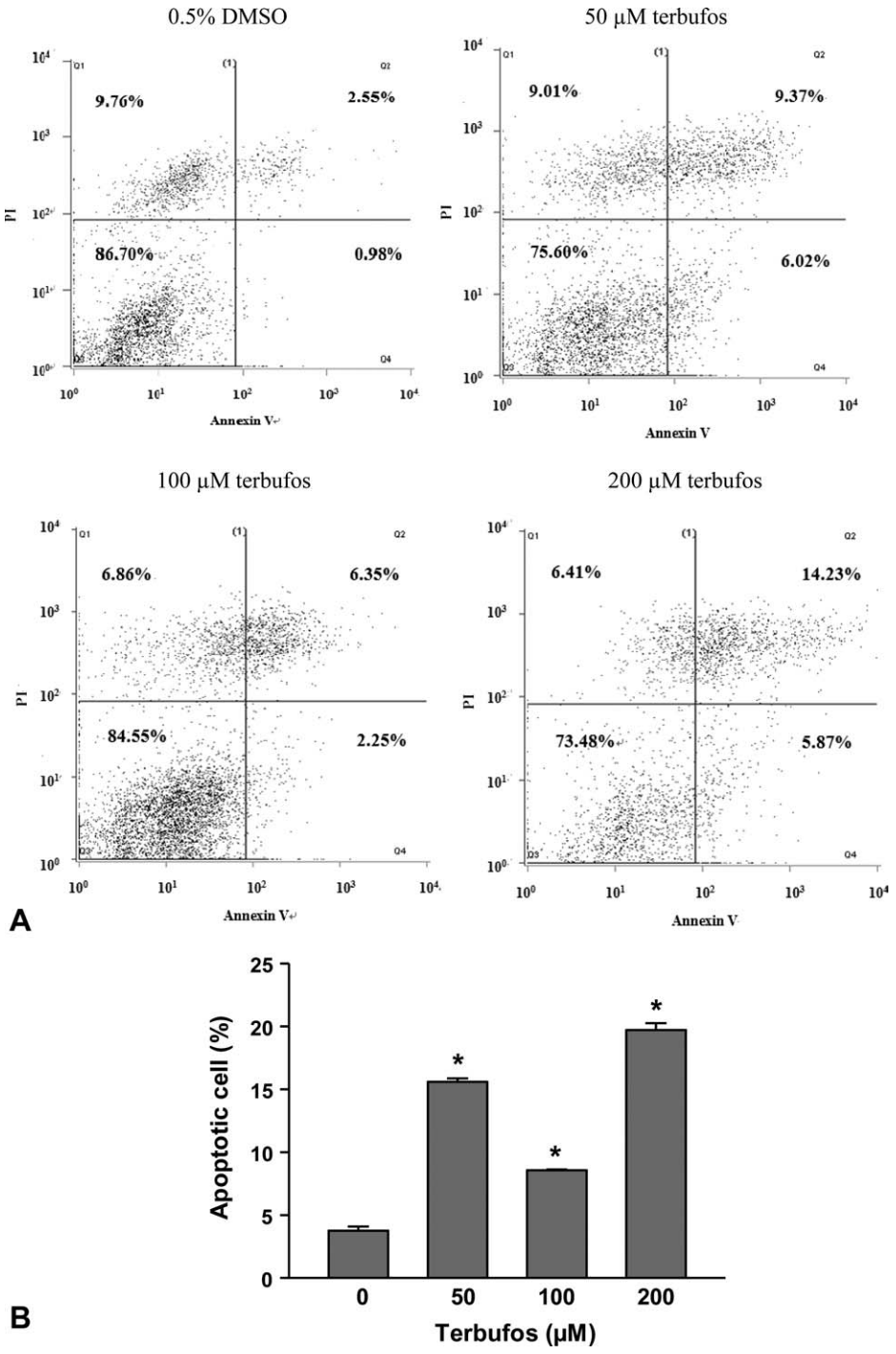

Fig. 4. Terbufos-induced apoptosis in HepG2 cells. **A**: Dot plot of FITC-Annexin-V/PI in control cells vs. terbufos-treated cells. **B**: The percentages of apoptotic cells were derived from the sum of the percentages of quadrant 2 and 3, which show FITC-Annexin-V^+/PI^+ (late apoptosis) and FITC-Annexin-V^+/PI^- (early apoptosis), respectively. Data are presented as the mean ± SD ($n = 4$). *Significantly different from the control cells at $P < 0.05$.

numbers of genes than fenthion. The expression of some genes was modulated more than fivefold, irrespective of the concentration of terbufos tested. These genes included: *myc, ppp1r15*a, *sosc3*, and *tnfaip3* by fenthion and *fos*, *gadd45a*, *ppp1r15a*, *sosc3*, and *tnfaip3*s.

Apoptosis Assay

To evaluate the ability of fenthion and terbufos to induce apoptosis, we stained the cells with PI and annexin V. In the absence of test compound, few cells exhibiting

PI or annexin V staining were observed with no PI or annexin V staining (lower right quadrants of the dot plots) (Figs. 3A and 4A) and low PI staining and annexin V staining (upper right quadrants of the dot plots). PI was used to stain for early apoptotic cells, and annexin V was used to stain for late apoptotic cells. The ratio of apoptotic cells (the sum ratio of early and late apoptotic cells) in fenthion- and terbufos-treated cells was increased relative to control cells (Figs. 3B and 4B) ($P < 0.05$).

DISCUSSION

Genotoxins can elicit a variety of types of DNA damage, including base modification, DNA adduction, single-strand breaks, double-strand breaks, and intra or interstrand crosslinks, among which double-strand breaks are regarded as the most severe type of damage [Yu et al., 2006]. The comet assay is a sensitive, reliable, and rapid method for the detection of DNA double- and single-strand breaks, alkaline-labile sites and delayed repair site detection in eukaryotic individual cells [Rojas et al., 1999]. The comet assay has been used to detect the genotoxicity of various OPs in different tissues of fish [Ali et al., 2008], in rats [Mehta et al., 2008], and in mouse retina [Yu et al., 2008], as well as in human peripheral blood lymphocytes [Prabhavathy Das et al., 2006]. In this study, we have applied the comet assay to demonstrate the genotoxicity of fenthion and terbufos in HepG2 cells, a cell line of human origin that expresses xenobiotic-metabolizing enzymes [Knasmuller et al., 1998].

A group of immediate-early genes that encode the transcriptional activator proteins c-fos, c-jun, and c-myc are associated with cell growth, differentiation, and malignancy [Schutte et al., 1989; Vogt and Bos, 1989]. Interaction of c-fos with any one of the various members of the Jun family forms the activating protein-1 (AP-1) transcription factor required for cell proliferation [Abate et al., 1990]. Blazka et al. [1996] suggested that enhanced expression of AP-1 and related genes may be a molecular biomarker of chemically induced hepatotoxicity. For example, benzo[*a*]pyrene [B(a)P], a mutagen and suspected human carcinogen, induced the expression of c-fos in rat liver [Parrish et al., 1998], and the combination of B(a)P with SO_2 caused an increase of *c-fos* and *c-jun* gene expression in rat liver [Qin and Meng, 2009]. Cadmium and selenium induced the over-expression of c-fos, c-jun, and c-myc genes in rat hepatocytes [Yu et al., 2006a,b, 2007], and 2,4-dichlorophenoxyacetic acid (2,4-D) elicited c-myc expression in Syrian hamster embryo (SHE) cells [Maire et al., 2007]. The deregulation of c-myc has been reported to be associated with increased cell proliferation and has been demonstrated to contribute to cell transformation [Nilsson and Cleveland, 2003] and hepato-carcinogenesis in rodents treated with peroxisome proliferators [Dzhekova-Stojkova et al., 2001]. In contrast, over-expression of c-myc did not result in an increased number of HepG2 cells caused by the treatment of SHE cells with 2,4-D [Maire et al., 2007]. However, c-myc is known to play an important role in tumorigenesis [Nesbit et al, 1999; Nilsson and Cleveland, 2003].

Up-regulation of four genes that respond to DNA damage (*gadd45a, ppp1r15a, socs3*, and *tnfaip3*) was observed in HepG2 cells after fenthion and terbufos treatment (Tables II and III). The induction of *gadd45a* was also reported in HepG2 cells exposed to 1, 10, and 100 μg/ml of the OPs methyl parathion and methyl paraoxon for 24 hr [Hreljac et al., 2008]. GADD45A, a growth arrest and DNA damage-inducing protein, plays an important role in inducing G2/M arrest following genotoxic stress [Hollander et al., 1993; Wang et al., 1999]. *Ppp1r15a* (gadd34) also belongs to a member of the growth arrest and DNA damage-inducible gene family and is related to the induction of apoptosis in hepatocellular carcinoma with gold-1a treatment [Lum et al., 2006]. SOCS3 regulates p21 expression and cell cycle arrest in response to DNA damage elicited by radiation in MEF cell lines [Sitko et al., 2008]. TRIFAIP3 (A20) has crucial functions as a dual inhibitor of nuclear factor-κB (NF-κB) and apoptosis in the tumor necrosis factor receptor 1 signaling pathway [Won et al., 2010]. Increased expression of these genes correlates with the DNA damaging potential of fenthion and terbufos observed with the comet assay. Analysis of gene expression is useful to distinguish genotoxic compounds from nongenotoxic ones [Hreljac et al., 2008]; as such, the induced expression of these genes further supports that fenthion and terbufos are genotoxic compounds.

It has been shown that environmental stressors (metals, particulate matter, and pesticides) can induce apoptotic cell death [Ayed-Boussema et al., 2008; Gong et al., 2009; Hanzel and Verstraeten, 2009; Kobayashi et al., 2009]. Thus, fenthion- and terbufos-induced apoptosis was investigated. Both compounds caused increased ratios of apoptotic cells. However, cells with DNA damage that escape apoptosis may eventually undergo malignant transformations [Kumari et al., 2009]. Thus, the results of our study lead us to hypothesize that cells with significant DNA damage that escape apoptosis, and exhibit increased expression of tumor-related genes, may be predisposed to malignant transformation following exposure to terbufos and fenthion.

In summary, the results suggest that fenthion and terbufos should be considered potentially hazardous to humans based on their ability to induce genotoxicity, apoptosis, and the expression of genes in cancer-related pathways in HepG2 cells.

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