ENZYME INDUCTION AND CYTOTOXICITY IN HUMAN HEPATOCYTES BY CHLORPYRIFOS AND *N,N*-DIETHYL-*m*-TOLUAMIDE (DEET)

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SUMMARY

Xenobiotics, including drugs and environmental chemicals, can influence cytochrome P450 (CYP) levels by altering the transcription of CYP genes. To minimize potential drug-pesticide and pesticide-pesticide interactions it is important to evaluate the potential of pesticides to induce CYP isoforms and to cause cytotoxicity in humans. The present study was designed to examine chlorpyrifos and DEET mediated induction of CYP isoforms and also to characterize their potential cytotoxic effects on primary human hepatocytes. DEET significantly induced CYP3A4, CYP2B6, CYP2A6 and CYP1A2 mRNA expression while chlorpyrifos induced CYP1A1, CYP1A2 and CYP3A4 mRNA, and to a lesser extent, CYP1B1 and CYP2B6 mRNA in primary human hepatocytes. Chlorpyrifos and DEET also mediated the expression of CYP isoforms, particularly CYP3A4, CYP2B6 and CYP1A1, as shown by CYP3A4-specific protein expression, testosterone metabolism and CYP1A1-specific activity assays. DEET is a mild, while chlorpyrifos is a relatively potent,

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inducer of adenylate kinase and caspase-3/7, an indicator of apoptosis, while inducing 15-20% and 25-30% cell death, respectively. Therefore, DEET and chlorpyrifos mediated induction of CYP mRNA and functional CYP isoforms together with their cytotoxic potential in human hepatocytes suggests that exposure to chlorpyrifos and/or DEET should be considered in human health impact analysis.

KEY WORDS

chlorpyrifos, DEET, human hepatocytes, CYP isoforms, cytotoxicity

INTRODUCTION

DEET (*N,N*-diethyl-*m*-toluamide) was developed in 1946 and registered as a repellant for insects and other arthropods in 1957 /1-4/. Many products containing 10-100% DEET are sold annually, and ~75 million people in the US use DEET-containing products on an annual basis /3,5/. There is anecdotal evidence that DEET can cause a number of toxic symptoms /1,5-8/, but these effects appear to be rare. DEET metabolism has been studied *in vivo* in a variety of animals /9-11/. *N*-Dealkylation, ring hydroxylation and ring dealkylation of DEET occur in rat liver microsomes /12,13/, while limited *in vivo* studies have been performed in human volunteers /14/. *In vitro* human studies indicated the involvement of CYP2B6 and CYP1A2 in DEET metabolism /15/.

Chlorpyrifos [*O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridinyl)-phosphorothioate] is a widely used organophosphorus pesticide in agricultural, military and other applications. After metabolic activation to its oxon, it is a potent acetylcholinesterase inhibitor and manifests a variety of pathological symptoms /16/. During development the chlorpyrifos metabolite is concentrated in peripheral tissues, especially in the liver /17/. It affects hepatic adenylyl cyclase signal transduction at a subthreshold level for systemic toxicity /18,19/, and can be hepatotoxic at higher doses /20/. *In vivo*, the toxicity of chlorpyrifos can be further impacted by the status of the human polymorphic enzyme PON1, and

Abbreviations: Apaf-1 = apoptotic protease activating factor-1; DEET = *N,N*-diethyl-*m*-toluamide; E_2 = 17β-estradiol; PXR = pregnane X receptor; RXR = retinoid X receptor; XRE = xenobiotic responsive element.

also from the release of reactive sulfur during oxon formation /21,22/. However, toxicity is primarily determined by CYP-mediated chlorpyrifos-oxon formation /23/. Chlorpyrifos metabolism has been studied in a variety of animals /24/ and is metabolized *in vitro* by human liver microsomes and CYP isoforms, particularly CYP2B6 /25/.

Interaction of Gulf War related chemicals produced greater than additive toxicity in rats and mice /26/ and a correlation has been suggested between Gulf War illnesses and the use of pesticides and repellants, including DEET and chlorpyrifos /27,28/. Recent *in vitro* studies have demonstrated human metabolism of DEET and chlorpyrifos and their interactions with each other, with other chemicals and with endogenous neurochemicals and hormones /15,29-32/. DEET increased the biotransformation of chlorpyrifos to chlorpyrifos-oxon, while on the other hand, chlorpyrifos completely inhibits CYP-mediated metabolism of DEET. However, no studies have been performed to delineate their capability for CYP isoform induction or cytotoxicity in human hepatocytes. Recent studies raise the possibility that pesticide mediated inhibition of drug and hormone metabolism poses a health risk to humans /30-37/. However, there have been only limited studies of pesticide mediated CYP induction in humans /38, 39/. Thus, the present study was designed to examine chlorpyrifos and DEET mediated induction of metabolic CYP enzymes and cytotoxicity in primary human hepatocytes.

MATERIALS AND METHODS

Chemicals and reagents

DEET (*N,N*-diethyl-*m*-toluamide) and chlorpyrifos [*O,O*-diethyl-*O*-(3,5,6-trichloro-2-pyridinyl)-phosphorothioate] were purchased from Chem Service (West Chester, PA). Williams E culture medium and the medium supplements dexamethasone and insulin were obtained from Bio-Whittaker (Walkersville, MD). EME medium without L-glutamine and phenol red, non-essential amino acid solution, L-glutamine solution, and other cell culture related products were purchased from Media-tech, Inc. (Herndon, VA). Certified fetal bovine serum, trypsin-EDTA solution and HBSS buffers were obtained from GIBCO InVitrogen Corporation (Carlsbad, CA). Tissue

culture flasks and culture plates along with other tissue culture related products were purchased from Fisher Scientific, Inc. (Pittsburgh, PA). The ToxiLight™ BioAssay Kit was purchased from Lonza, (Rockland, ME). Caspase-Glo™-3/7 Assay Kit was purchased from Promega Corporation (Madison, WI). Actinomycin D and Z-DEVD-FMK were products of Alexis Biochemicals and supplied by AXXORA, LLC (San Diego, CA). Rifampicin, phenobarbital and all other chemicals, unless specified otherwise, were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Rabbit polyclonal anti-CYP2B6 antibodies and monoclonal anti-human CYP3A4 from mice were purchased from BD Biosciences (Bedford, MA) and R & D Systems (Minneapolis, MN), respectively. All chemical, reagent and biohazard wastes were disposed of according to the safety protocols of NCSU.

Human hepatocyte culture

The details of maintaining human hepatoma HepG2 culture in our laboratory, their viability and experimental use were previously described /37,38/. Primary cultures of human hepatocytes were purchased from ADMET Technologies (Durham, NC). Verification of viability by the trypan blue exclusion assay was also described previously /38,39/.

Hepatocyte and HepG2 cell treatment

For measuring adenylate kinase release and performing the trypan blue exclusion assays human hepatoma HepG2 cells were treated with 0-250 μM DEET or chlorpyrifos for 24, 48 or 72 h. Similarly, HepG2 cells were exposed to 0-250 μM DEET or chlorpyrifos for 24, 48 or 72 h for assaying caspase-3/7 activity. Primary cultures of human hepatocytes, approximately 1.5 x 10^6 cells per well in 6-well plates, were treated in culture medium for 72 h with 100 μM DEET or chlorpyrifos for measuring quantitative mRNA expression of CYP isoforms. Primary hepatocytes in 6-well plates were treated for 72 h with 0-100 μM DEET or chlorpyrifos for semiquantitave measurement of immunoreactive CYP2B6 and CYP3A4 protein expression. Human hepatocytes, 3 x 10^5, in 24-well tissue culture plates were exposed to 50 or 100 μM DEET or chlorpyrifos for 72 h before the DEET- or chlorpyrifos-containing medium was replaced with fresh medium.

Medium containing testosterone was then added into the wells and incubated for 30 min. before measuring the formation of 6β-OH-testosterone. Human hepatocytes in 48-well tissue culture plates, 1.5 x 10^5 cells per well in 1 ml medium, were exposed for 72 h to inducing agents, including 3-methyl cholanthrene (3-MC) (10 μM), rifampicin (Rif) (10 μM) or phenobarbital (PB) (100 μM), and increasing concentrations (0-100 μM) of chlorpyrifos along with a solvent control for CYP1A1 activity assay. Similarly, hepatocytes were treated with increasing concentrations (0-200 μM) of chlorpyrifos for 24, 48 or 72 h for quantitative determination of adenylate kinase and caspase-3/7 activity. In all experiments inducers such as 3-MC, Rif or β-naphthoflavone were used as positive controls.

Hepatocyte sample preparation

Appropriate samples were prepared for the following assays which have been previously described in detail /38,39/.

Branched DNA (bDNA) assay

Changes in the expression of CYP isoform mRNA in human hepatocytes following treatment with DEET or chlorpyrifos were measured quantitatively using the branched DNA (bDNA) assay as described previously /39,40/.

Gel electrophoresis, immunodetection and quantification

7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to resolve microsomal proteins as described by Laemmli /41/. The details of the subsequent steps have also been described previously /39/.

CYP1A1 and CYP3A4 metabolic activity assays

Primary cultures of human hepatocytes in 24-well culture plates were treated for 72 h with increasing concentrations of chlorpyrifos or DEET at 37°C, with the chlorpyrifos- or DEET-containing media changed every 24 h. Chlorpyrifos-treated hepatocytes were used for the determination of CYP1A1 and CYP3A4 activity while DEET-treated hepatocytes were used for CYP3A4 activity assay. The determination of CYP1A1 and CYP3A4 activity was performed according

to the manufacturer's protocol (Promega Corporation, Madison, WI). Other details have already been described /39/. The details of the measurement of testosterone metabolism have also been described /30,39/.

Cell viability and cytotoxicity assay

Following completion of treatment, cells were harvested as a cell suspension in isotonic culture medium and viability was assessed by the trypan blue exclusion method using a hemocytometer (Hausser Scientific, Horsham, PA) as described previously /39/.

ToxiLight BioAssay

The toxic effects of chlorpyrifos and DEET were measured using the ToxiLight™ BioAssay kit, a non-destructive luciferase-based bioluminescence cytotoxicity assay. This quantitatively measures the release of adenylate kinase, a marker of toxicity, into the culture medium. The emitted light intensity, expressed as relative luminescence units (RLU), is linearly related to the adenylate kinase activity. The assay was performed according to the manufacturer's protocol (Lonza. Rockland, ME) and as described previously /39/.

Caspase-3/7 assay

Caspase-3/7 is a well known marker for apoptotic cell death. The activity of caspase-3/7 was measured using a luminometer and a Caspase-Glo™-3/7 Assay kit. Luminescence produced by luciferase as RLU value is proportional to the amount of caspase-3/7 activity present in the sample. The assay was performed according to the manufacturer's protocol (Tech. Bull. No 323, Promega Corporation, Madison, WI) and the details were described previously /39/.

Statistical analysis

The data were summarized and expressed as mean ± SE using Microsoft Excel spreadsheet and Sigma Plot graphics programs (Chicago, IL, USA). The significant differences between control and treated data sets were determined by analysis of variance and Student's t-test.

RESULTS

Effect of DEET and chlorpyrifos on CYP isoform mRNA transcription in human hepatocytes

The data show DEET substantially induced CYP mRNAs, approximately 8-fold for CYP1A1 and CYP2B6, while 4-, 10- and 6-fold induction relative to control was noted for CYP1A2, CYP3A4, and CYP2A6, respectively. However, CYP1A1 induction was variable and very little induction of the CYP3A5 isoform was noted (Fig. 1A). Chlorpyrifos showed ~25-fold induction of CYP1A1, and 8-, 6-, and 4-fold induction of CYP1A2, CYP3A4, and CYP1B1, respectively, relative to control. A low level of induction was noted for CYP2B6 while no induction of CYP2A6 was apparent (Fig. 1B).

Effect of DEET and chlorpyrifos on CYP3A4 and CYP1A1 functional protein expression in human hepatocytes

To confirm DEET and chlorpyrifos mediated translation of CYP3A4 mRNA transcripts into protein expression, treated human hepatocytes were examined by Western blotting. Both DEET and chlorpyrifos dose-dependently increased CYP3A4 protein expression, reaching a maximum of 4-5 fold relative to control at higher doses with some variability (Fig. 2). Verification of functional CYP3A4 protein induced in primary hepatocytes by DEET and chlorpyrifos was probed using testosterone as a substrate. CYP3A4 activity was normalized to total cell protein, and 6β-hydroxytestosterone production essentially mirrored expression levels of immunoreactive proteins. However, the degree of testosterone metabolism was 4.5 to 6-fold at 50-100 μM chlorpyrifos while 2.5 to 3-fold at 50-100 μM DEET relative to controls (Fig. 3).

Induction of CYP1A1 mRNA expression was ~25-fold with 100 μM chlorpyrifos in contrast to that of DEET where it was ~7-fold with some degree of variability relative to their respective controls (Fig. 1). In order to verify the functional activity of induced CYP mRNA, CYP1A1 activity was measured in treated human hepatocytes and the data normalized to total cell protein. CYP1A1 activity exhibited greater than 2-fold induction but at much lower dose (1 μM) of chlorpyrifos which tapered off at higher concentrations (Fig. 4), while CYP1A1 showed very little activity with DEET (data not shown).

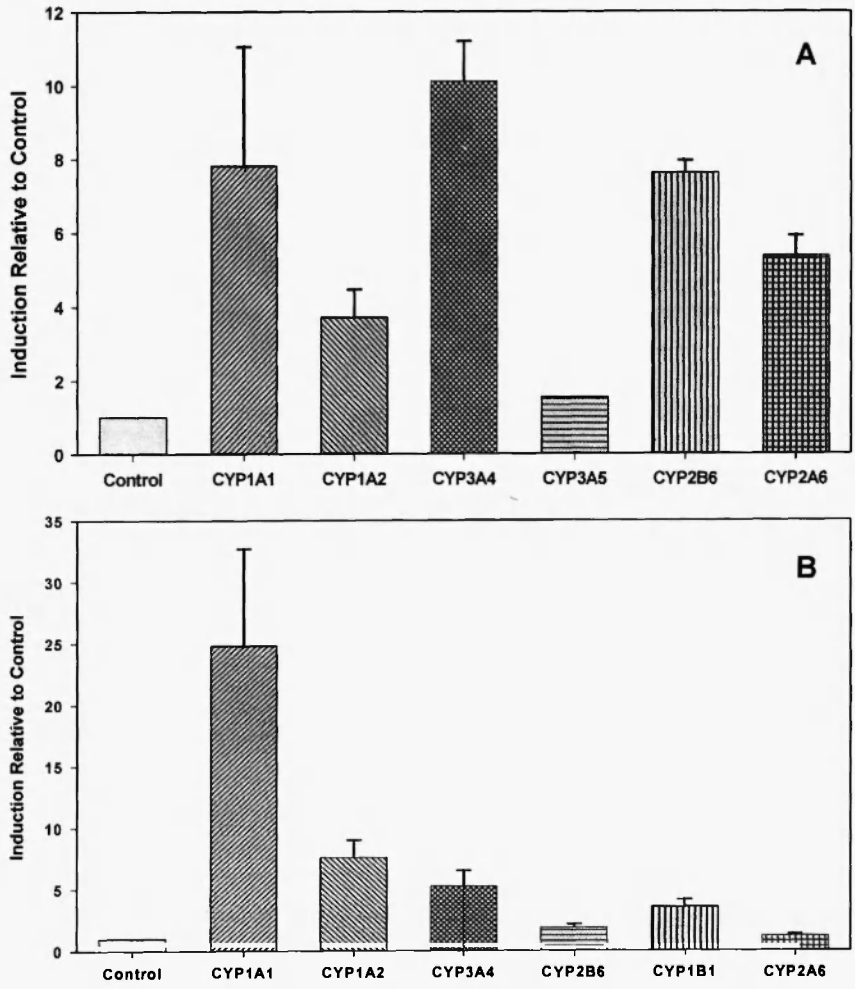


Fig. 1: Effect of DEET (**A**) and chlorpyrifos (**B**) on the expression of mRNA transcripts of different CYP isoforms in human hepatocytes. Freshly prepared human hepatocytes from four individuals were exposed to 100 μM of DEET and chlorpyrifos, respectively, for 72 h and mRNA transcripts were quantitatively measured by the bDNA assay. Each bar represents the mean mRNA value of each isoform from 1 to 4 individuals per group and error bars are the standard error mean in human hepatocytes. Appropriate positive controls were included in the experiment and absence of an error bar indicates a single determination.

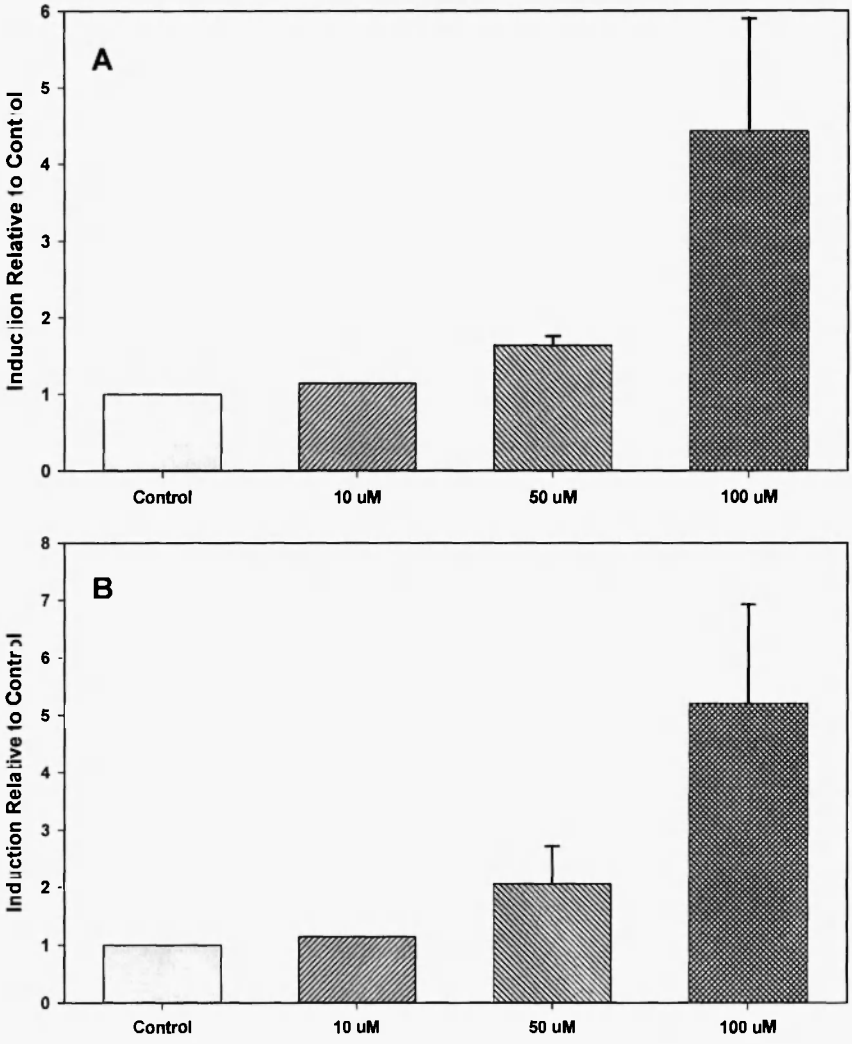


Fig. 2: Dose-response effect of DEET (**A**) and chlorpyrifos (**B**) on the CYP3A4 protein in fresh human hepatocytes. Freshly prepared human hepatocytes from five individuals were exposed to increasing concentrations of (0, 10, 50 and 100 μM) DEET and (0, 10, 50 and 100 μM) chlorpyrifos for 72 h and CYP3A4 protein was determined by Western blot analysis. The graph represents the mean of semi-quantitative densitometric data and error bars are the standard error of the mean at different doses.

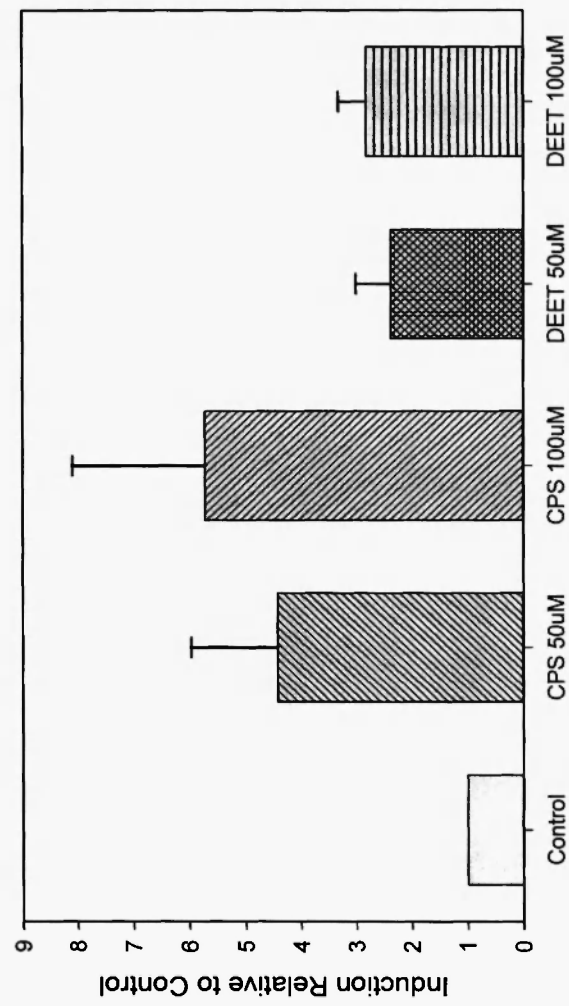


Fig. 3: Dose-response effect of DEET and chlorpyrifos on CYP3A4 activity/function in primary culture of human hepatocytes. Freshly prepared human hepatocytes were exposed to two doses each (50 and 100 μM) of DEET or chlorpyrifos (CPS) for 72 h. CYP3A4 mediated metabolism of testosterone was assayed in these treated human hepatocytes by measuring 6β-(OH)-testosterone production, where the mean control value was 470 ± 251 pmol/mg protein/min. The bar at each dose represents the mean of induction of 6β-(OH)-testosterone production relative to control and the error bar is the standard error of the mean; n = 3.

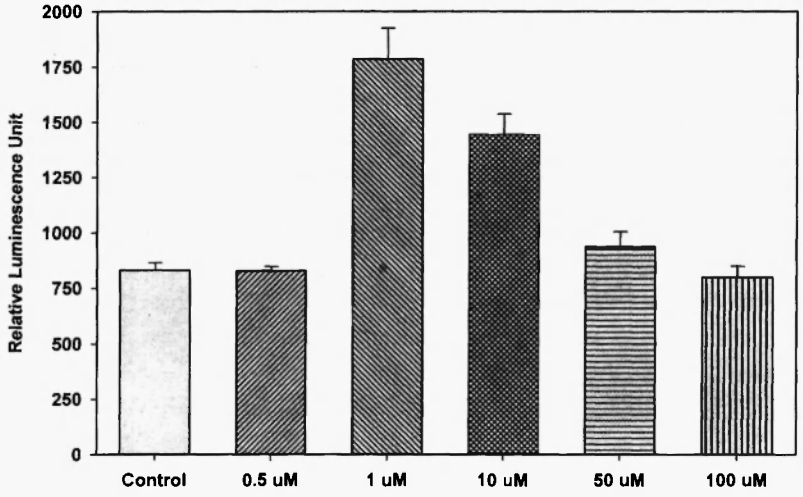


Fig. 4: Dose-response effect of chlorpyrifos on CYP1A1 activity in primary human hepatocytes. Primary cultures of human hepatocytes were exposed to doses of chlorpyrifos for 72 h and CYP1A1 activity was measured according to the manufacturer's protocol. Activity was expressed as relative luminescence units. Each bar represents the mean; n = 2; triplicate determinations were performed for each individual.

Effect of DEET and chlorpyrifos on adenylate kinase activity in human hepatocytes

Adenylate kinase, a determinant of cellular toxicity, was determined. DEET and chlorpyrifos both showed a dose-dependent effect on the release of adenylate kinase activity in HepG2 cells, where 50, 100 and 250 μM DEET induced increased release of 2.5 to 3.5-fold relative to control (Fig. 5A). Similarly, chlorpyrifos at 25 and 50 μM induced a 2-3 fold increase, however, the release of adenylate kinase plateaued at 100 and 200 μM chlorpyrifos (Fig. 5B). Likewise, in primary human hepatocytes both DEET and chlorpyrifos dose-dependently induced adenylate kinase, which peaked at 100 μM DEET and 12.5-25 μM chlorpyrifos, respectively, with plateaus at higher doses at 24 h. A pattern similar to that of 24 h but with a higher response was noted at longer time points, 48 and 72 h (Fig. 6).

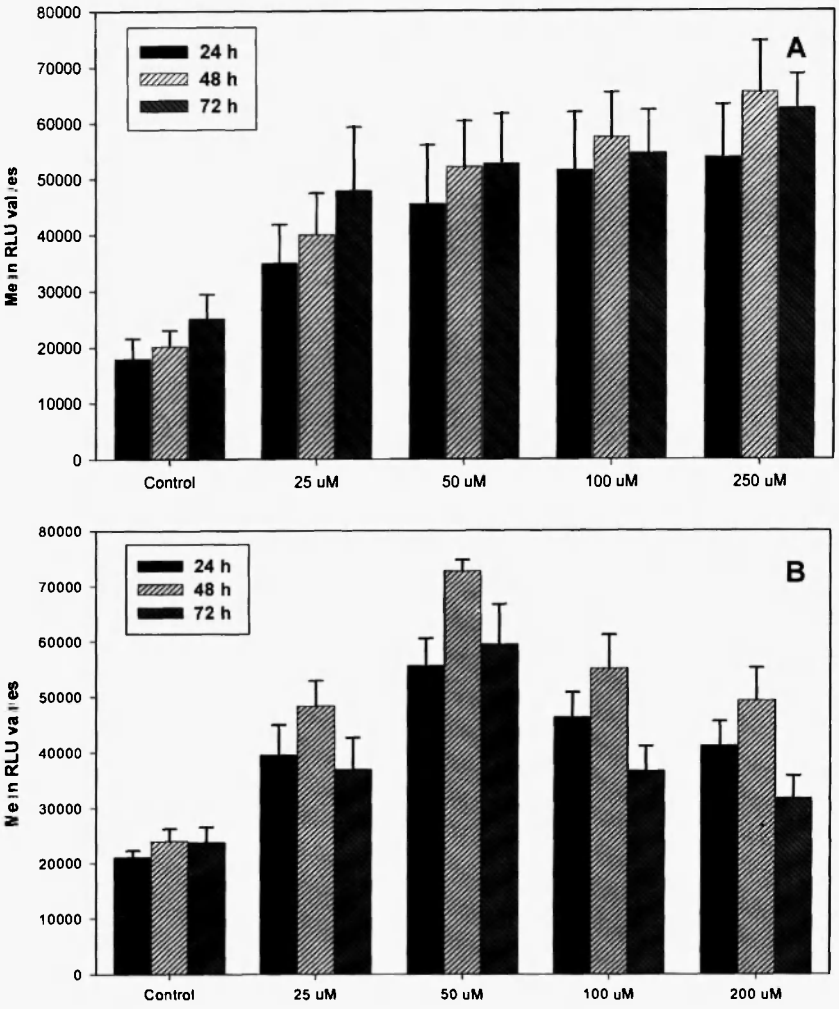


Fig. 5: Dose- and time-dependent effect of DEET (**A**) and chlorpyrifos (**B**) on adenylate kinase activity in HepG2 cells. HepG2 cells were exposed to increasing concentrations of DEET (0, 25, 50, 100 and 250 μM) or chlorpyrifos (0, 25, 50, 100 and 200 μM) for 24, 48 and 72 h and adenylate kinase activity was measured using the ToxiLight Bioassay kit. Each bar represents the mean relative luminescence unit (RLU) value of nine determinations from three independent experiments using HepG2 cells. The error bar represents the standard error of the mean. The data are based on triplicate experiments.

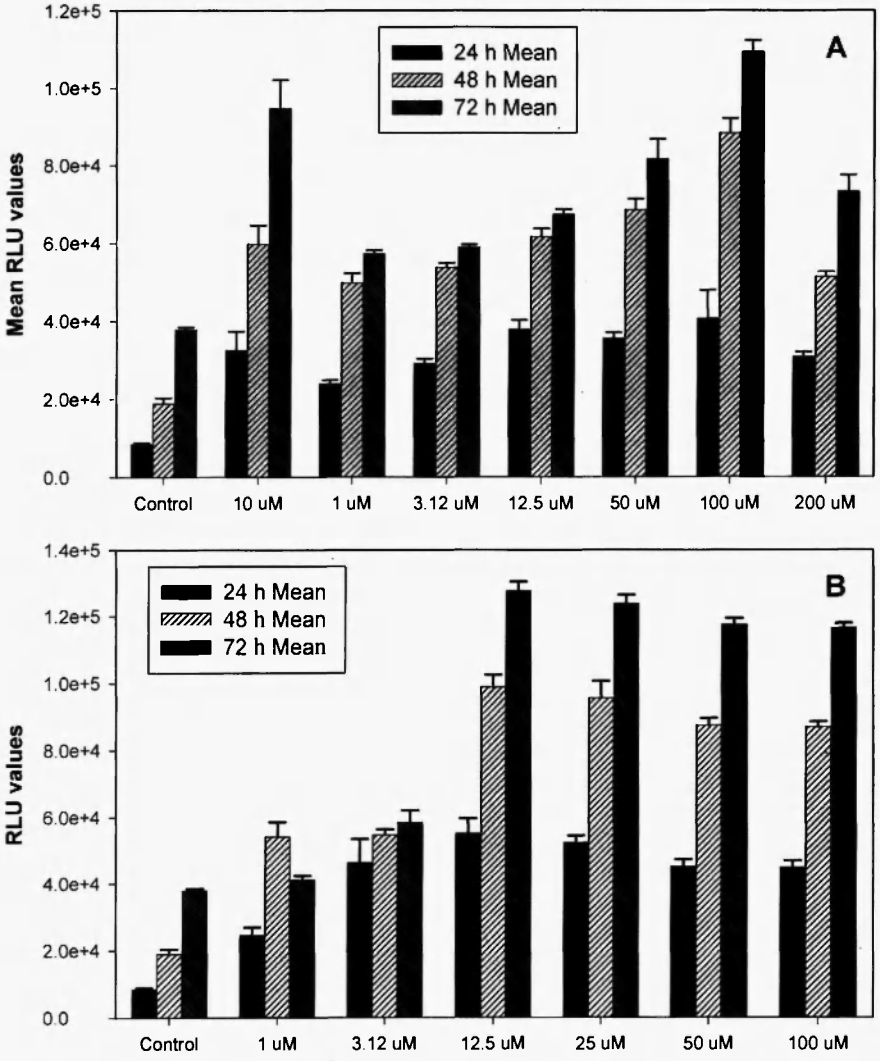


Fig. 6: Dose- and time-dependent effect of DEET (**A**) or chlorpyrifos (**B**) on adenylate kinase activity of primary cultures of human hepatocytes. Cultures of primary human hepatocytes were exposed to increasing concentrations of DEET (0, 1, 3.12, 12.5, 50, 100 and 200 μM) or chlorpyrifos (0, 1, 3.12, 12.5, 25, 50 and 100 μM) for 24, 48 and 72 h and then adenylate kinase activity was measured using the ToxiLight Bioassay kit. Each bar represents the relative luminescence unit (RLU) value mean of six determinations from two individuals. The error bar represents the standard error of the mean and the data are based on triplicate experiments.

Toxicity of DEET and chlorpyrifos in HepG2 cells

In order to ascertain the degree of cell cytotoxicity relative to the release of adenylate kinase the trypan blue exclusion assay was performed on HepG2 cells. Cell death of 15-20% was noted following 48-72 h exposure at 100 and 250 μM DEET (Fig. 7A) while dose- and time-dependent cytotoxic effects of chlorpyrifos were also noted. Initial signs of cytotoxicity were noted at 25 μM reaching approximately 35% at 200 μM chlorpyrifos (Fig. 7B).

Effect of DEET and chlorpyrifos on caspase-3/7 activity inHepG2 cells and human hepatocytes

For initial characterization of cell death the activity of caspase-3/7 was determined. Induction of activated caspase-3/7 is one of the last cascade steps and an identifiable landmark of the cellular apoptotic process. Apparently, DEET and chlorpyrifos have only a minor effect in inducing caspase-3/7 activity in both HepG2 cells and primary human hepatocytes. Induction of 2 to 2.5-fold was noted at 50 and 100 μM DEET at 72 h (Fig. 8A,B), while a dose-dependent increase in induction of activated caspase-3/7 was noted at 3.12 μM which peaked at 12.5-25 μM chlorpyrifos in both cell types at 72 h (Fig. 8C,D).

DISCUSSION

DEET and chlorpyrifos are interacting chemicals used, among other pesticides, in the first Gulf War, and recently studied in *in vitro* human hepatic systems in our laboratory. Chlorpyrifos, which completely inhibits DEET metabolism, also inhibits not only the metabolism of a variety of other xenobiotics but also the metabolism of steroid hormones /42,43/. Studies using human liver microsomes revealed that DEET and chlorpyrifos-oxon stimulate the production of testosterone metabolites /30/. In the rat DEET also increased the metabolism of testosterone /44/. *In vitro* human studies have indicated the involvement of several CYPs in DEET and chlorpyrifos metabolism /30,42/. Studies with rat and human hepatocytes indicated that various pesticides are capable of inducing many metabolic enzymes /45,46/. However, induction of CYPs and cytotoxicity of chlorpyrifos and DEET have yet to be characterized in human hepatocytes. Our

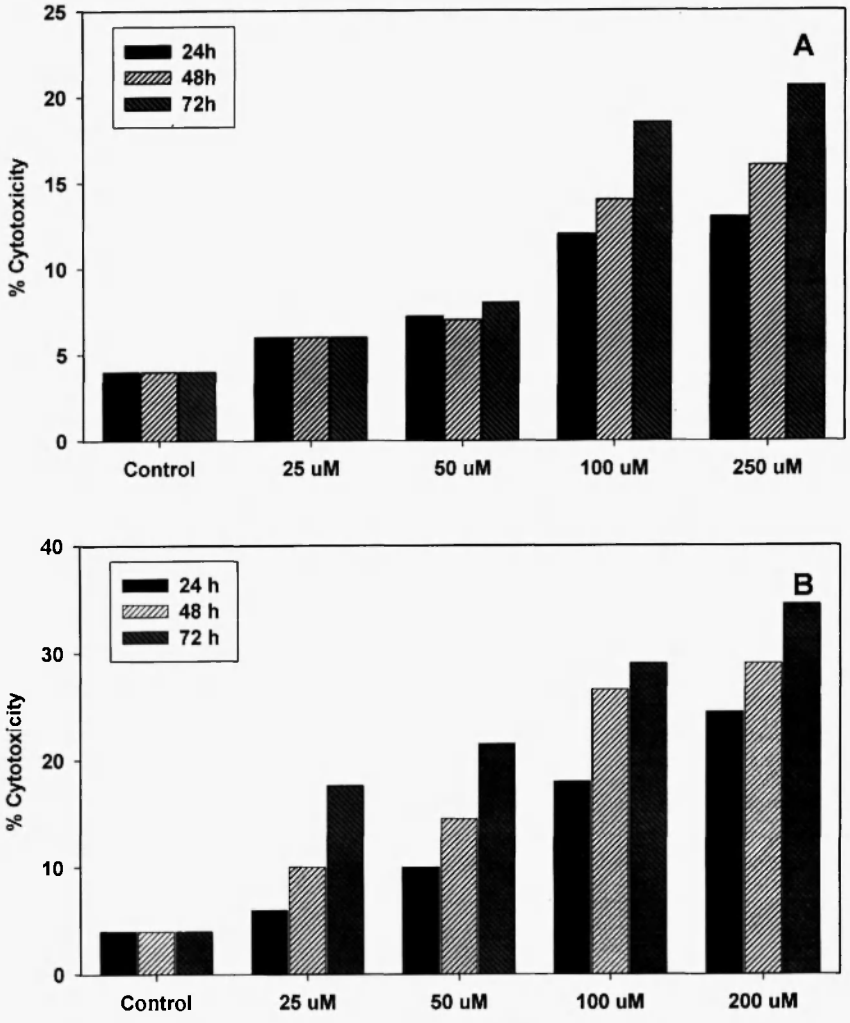
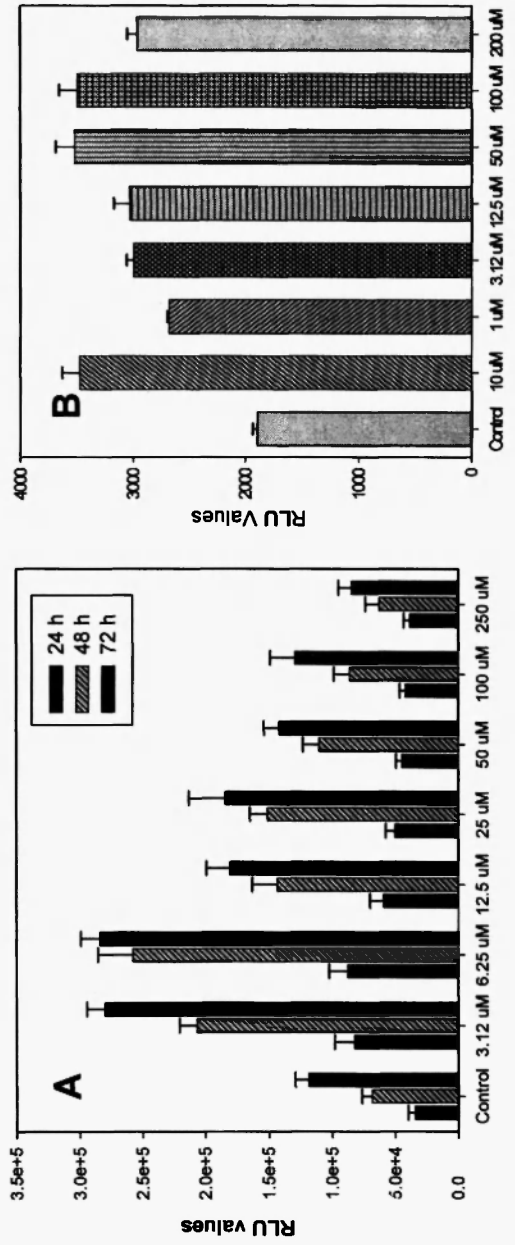


Fig. 7: Dose- and time-dependent effect of DEET (**A**) or chlorpyrifos (**B**) on human hepatoma HepG2 cell viability. Cultures of HepG2 cells were exposed to increasing concentrations of DEET (0, 25, 50, 100 and 250 μM) and chlorpyrifos (0, 25, 50, 100 and 200 μM) for 24, 48 and 72 h and cell viability was semi-quantitatively assessed by the trypan blue exclusion assay and expressed as % cytotoxicity. Each bar represents the value derived from counting >100 and up to 200 cells in each treated sample.



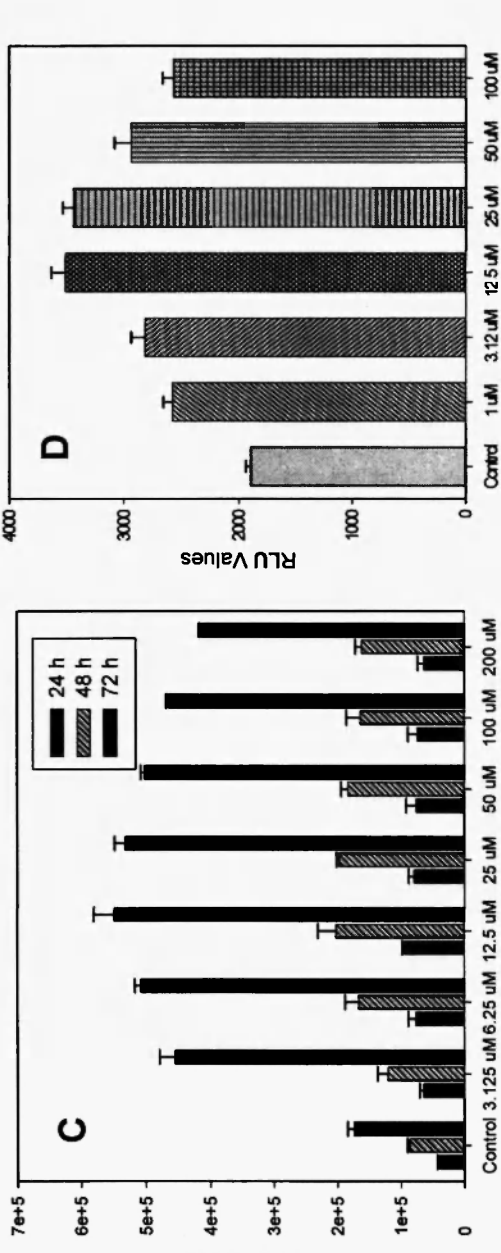


Fig. 8: Dose- and time-dependent effects of DEET (**A** and **B**) and chlorpyrifos (**C** and **D**) on caspase-3/7 activity in human hepatoma HepG2 cells and in primary human hepatocytes, respectively. Cultured HepG2 cells and primary human hepatocytes were exposed to various concentrations (0, 1, 3.12, 6.25, 12.5, 25, 50, 100 and 250 μM) of DEET or chlorpyrifos (0, 1, 3.12, 6.25, 12.5, 25, 50, 100 and 200 μM) along with (not shown) β-naphthoflavone (20 μM), rifampicin (10 μM) and actinomycin D (1 μM), inducers of caspase-3/7, and the caspase-3/7 specific inhibitor Z-DEVD-FMK (0.05 μM) for 24, 48 and 72 h. Caspase-3/7 activity was quantitatively measured by Caspase-Glo® 3/7 assay kit. Each bar represents the mean of relative luminescence unit (RLU) value of 2-3 independent experiments for HepG2 cells and 2 individuals for primary human hepatocytes. Three to 4 well determinations per group per experiment were performed and error bars are the standard error of the mean.

recent study indicated that CYP induction may not have a mechanistic relationship with insecticide cytotoxicy /39/. In the present study, DEET significantly induced mRNA expression of CYP3A, CYP2B, CYP2A and CYP1A2; while chlorpyrifos induced CYP1A1, CYP1A2, CYP3A4, and to a lesser extent CYP1B and CYP2B, in primary human hepatocytes. Since CYP3A and CYP2B may be co-regulated as a result of activation of the human pregnane X receptor (hPXR) and CYP1A is regulated by the aromatic hydrocarbon receptor (AhR), variations in CYP gene expression may be due to the interactions of pesticides and the regulatory elements involved in induction of these CYP isoforms /47-49/.

CYP3A4 is the major enzyme among human CYPs which, along with CYP2B6 and CYP1A1, plays a predominant role in the metabolism of clinical drugs, and numerous other xenobiotics and endogenous substrates /30,50,51/. Chlorpyrifos and DEET mediate mRNA expression of CYP isoforms, particularly CYP3A4, CYP2B6 and CYP1A1, and this has been supported by the demonstration of their translation into functional proteins as shown by the data on CYP3A-specific protein expression, testosterone metabolism and CYP1A1-specific activity assays. Previous evidence has indicated that pesticide mediated CYP induction has significant impact on drug/pesticide, hormone/pesticide and pesticide/pesticide interactions /30, 39,42/, suggesting that DEET and/or chlorpyrifos exposure may disrupt drug, pesticide or hormone metabolism and thus cause concern for human health.

Chlorpyrifos is a potent acetylcholinesterase inhibitor well known for its neurotoxicity in various rodent neuronal cell systems *in vitro* and *in vivo*. This has led to increasing concern and restricted use /52,53/. Chlorpyrifos induced apoptotic cell death in human monocytes *in vitro* at somewhat higher doses than those used in the present study /54/. Although DEET is considered a relatively benign chemical, anecdotal reports involving heavy and excessive exposure indicate a variety of possible toxic side effects /1,5/. The mode of action of DEET cytotoxicity is mostly unknown. The cellular signaling mechanisms of DEET and chlorpyrifos mediated cytotoxicity potential have not been characterized in human hepatocytes, the primary site of metabolism. In the present study, DEET at moderately high doses and chlorpyrifos at much lower doses induced adenylate kinase release, an indicator of cytotoxicity. The release of adenylate kinase was lower at

higher doses of DEET and chlorpyrifos. At the highest doses (200-250 μM) of DEET and chlorpyrifos the levels of cytotoxicity were 15-20% and 25-30%, respectively; and this was semi-quantitatively substantiated by the trypan blue exclusion assay data. Both DEET and chlorpyrifos are capable of potentially inducing major metabolic enzymes at the lower doses while causing cytotoxic effects at higher doses, thus these pesticides may have an impact on human health.

Previous studies indicated that concurrent application of DEET and permethrin induce urinary excretion of 3-nitrotyrosine and 8-hydroxy-2′-deoxyguanosine, markers of DNA damage and oxidative stress, and mitochondrial cytochrome c release in the rat /55/. Initiation of programmed cell death (apoptosis) is femonstrated by the release of mitochondrial cytochrome *c* activity, activation of caspases, elevation of 8-hydroxy-2-deoxyguanosine levels, increased levels of 3-nitrotyrosine, and alterations of p53 gene expression /56/. Genotoxic effects of DEET were reported in primary human nasal mucosal cells /57/. Chlorpyrifos mediated apoptosis was indicated by staining with Annexin-V, activation of caspase-3 and DNA fragmentation in the human monocyte U937 cell line /54/. In the present study, DEET is a mild, while chlorpyrifos is a relatively potent, inducer of adenylate kinase and caspase-3/7, indicators of apoptotic cell death. Thus, in rodents and humans, both DEET and chlorpyrifos induced apoptotic cell death in hepatocytes.

Like chlorpyrifos, DEET can induce mRNA and functional protein of the major and important CYP isoforms in human hepatocytes. Whether this functional CYP induction has any correlation with the induction of cytotoxic effects cannot be ascertained from this study. Both DEET and chlorpyrifos at higher dose levels induce functional CYPs and exert cytotoxic effects in human hepatocytes. Recent *in vitro* studies have demonstrated the induction profile of CYP mRNA in rat intestine and liver slices by known inducers is very similar when compared to that *in vivo* /58,59/. Therefore, DEET and chlorpyrifos mediated induction of functional CYP along with their cytotoxicity via apoptosis in human hepatocytes suggests that high-level short-term exposure to chlorpyrifos and DEET alone or in combination may have significant health impact in humans, which warrants further risk analysis and assessment.

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