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EFFECTS OF THE PESTICIDES ATRAZINE, METOACHLOR AND DIAZINON AND BINARY MIXTURES ON PROLIFERATION OF HUMAN FIBROBLASTS

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ABSTRACT
The frequent and heavy use of pesticides in agriculture has led to the contamination of surface and ground waters worldwide. Many questions have arisen about the human health effects of exposure to these pesticides and their mixtures. Most of the information about the adverse human health effects due to environmental contaminants comes from studies that focus on exposure to single rather than multiple contaminants since many of the environmental regulations regarding levels of xenobiotic contamination refer only to individual compounds. In this study, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) cell proliferation assays were performed with normal human fibroblasts to test the toxicity of environmentally relevant levels of three of the most heavily used pesticides in the United States: atrazine, metolachlor and diazinon, either alone or in binary mixtures. MTT analysis showed a statistically significant decrease in cell proliferation compared to the control at low levels of each of the three single pesticides tested (> 0.8 ppb (parts per billion) atrazine, > 1.6 ppb metolachlor and > 1.6 ppb diazinon) as well as with binary mixtures of either atrazine and metolachlor or atrazine and diazinon. When experimental results were compared to predictions of toxicity based on the response addition model, the mixture of atrazine and metolachlor was shown to generally be antagonistic, while the mixture of atrazine and diazinon resulted in additive responses. These findings, along with other studies, indicate that current regulatory standards for pesticides in drinking water may not sufficiently protect human health.

ACKNOWLEDGEMENTS
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INTRODUCTION

Pesticides are heavily used in the United States, especially in agricultural areas such as the Midwest. In 1997, approximately 770 million pounds of pesticides were used on farms in the United States; this amount has remained relatively constant since 1975. Currently, pesticides are used on nearly one million U.S. farms and more than 800,000 farmers are certified to apply pesticides (1). The widespread use of synthetic organic pesticides over the past several decades has led to their frequent detection in ground water, surface water, aquatic biota, sediment and the atmosphere (2-5). In a study by the National Water Quality Assessment Program (NAWQA) that investigated surface and ground water in the United States, atrazine, metolachlor and diazinon, the most frequently detected pesticides, were found in 78.66 percent, 68.53 percent and 35.76 percent respectively of 5196 samples (6).

Atrazine (2-chloro-4-ethylamine-6-isopropylamino-s-triazine), the most heavily used pesticide in the United States, is a chlorotriazine herbicide. The concentration of atrazine detected in direct runoff water has been as high as 700 ppb (7). In drinking water, the EPA has established a maximum contaminant level (MCL) established for atrazine of three parts per billion (ppb). However, concentrations of atrazine in Midwestern surface waters can be as high as 108 ppb during and immediately following the peak planting season (8).

Although the EPA has restricted its use and put limits on maximum allowable levels, the continued presence of atrazine in the environment has been shown to be associated with several health risks in humans, such as an increased incidence of DNA damage, endocrine effects, reproductive problems and cancer. Significant DNA damage has been observed in human lymphocytes (9), yeast cells (10), rodent cells (11,12) and in erythrocytes from Rana catesbeiana (bullfrog) tadpoles (13) exposed to low levels of atrazine compared to unexposed controls. A number of endocrine effects are associated with atrazine exposure, such as inhibition of hormone surges of prolactin (14) and luteinizing hormone (15) while increasing levels of progesterone (15).

Although atrazine does not bind to the estrogen receptor, it is considered to be an endocrine disruptor because it increases the activity of aromatase (16), the enzyme that converts testosterone and other androgens to estrogens. In a study measuring aromatase activity in different cell lines exposed to atrazine (65 to 6500 ppb) there was a significant increase in enzymatic activity in cells after exposure to the herbicide (17). Hayes et al. (18) recently demonstrated that amphibian larvae exposed to low levels of atrazine (0.1-200 ppb) exhibited hermaphroditism and demasculinization of exposed males, and a tenfold decrease in testosterone levels was found when Xenopus males were exposed to 25 ppb atrazine.

Epidemiological studies have also indicated an association between atrazine exposure and cancer incidence. Farm workers in the San Joaquin valley of California showed an association between atrazine exposure and several types of cancer, including brain cancer and testicular cancer in Hispanic males and prostate cancer in black males (19). A Canadian study found a positive association in both males and females between the incidence of stomach cancer and atrazine-contaminated drinking water (20). A study of Italian female farm workers to determine the relationship between herbicide exposure...
and ovarian mesothelial tumors found that there was a higher relative risk (RR = 2.7) for women that had definitively been exposed to atrazine (21). Atrazine exposure as well as increased duration of this exposure has also been associated with an increased risk of non-Hodgkin’s lymphoma in males (22).

Reproductive health problems of both males and females have also been associated with exposure to atrazine. Savitz and colleagues (23) surveyed farm couples on their reproductive health and timing of chemical herbicide applications. Results indicated that when farm activities included chemical applications (atrazine, glyphosate, butyric acid and insecticides), there was nearly a two-fold increase in the incidence of miscarriages and pre-term deliveries (23). Munger and colleagues (24) found an increased risk of intrauterine growth retardation (IUGR) in women who were exposed to atrazine-contaminated drinking water in several southeast Iowa communities that used the Rathbun Reservoir as a source of drinking water (24). The communities served by the Rathbun Reservoir had a greater number of positive detections of the herbicides atrazine, metolachlor, alachlor cyanazine and 2,4-D compared to Iowa communities served by other sources of drinking water. Interestingly, however, although the average concentrations of atrazine found at the site (2.2 ppb) were higher than those of the other contaminants measured (except chloroform and bromodichloromethane), these levels were still below the federal drinking water standard of three ppb. Overall, women supplied with drinking water from Rathbun Reservoir had almost double (RR = 1.8) the incidence of IUGR compared to towns with less contaminated water.

While most of the exposure to environmental contaminants is in the form of complex mixtures, the actual effects of exposure to a mixture compared to the effects of exposure to the individual components of the mixture have only recently been studied. Mixtures have been described as having additive, synergistic (greater than additive), or antagonistic (less than additive) interactions. Due to the common use of atrazine in the U.S., many studies have included atrazine in their mixtures, however, no consistent trends are observable.

Several of these studies have tested the toxicity of mixtures of atrazine and different organophosphate (OP) insecticides to invertebrates. In these experiments, atrazine alone did not affect acetylcholinesterase activity; however when the organisms were exposed to atrazine (40 ppb or higher) in combination with some OPs (diazinon, chlorpyrifos and methyl parathion), there was a significant decrease in acetylcholinesterase activity as compared to the OP alone (25-27). In other studies, an additive interaction was found between atrazine and simazine, another triazine herbicide, on the olfactory-mediated endocrine response in male salmon (28). Binary mixtures of atrazine and lindane or methyl parathion responded differently in different organisms. These pesticide combinations showed additive toxicity towards Daphnia magna while an antagonistic effect was seen on the growth of the alga Selenastrum capricornutum (29).

Many studies that analyze the effects of pesticide mixtures on cells use very high, environmentally irrelevant concentrations of pesticides. In this present study, we attempted to understand the types of interactions that could occur between a mixture of two herbicides or a mixture of an herbicide and an insecticide on human cells when both components of the mixture were present at environmentally relevant concentrations. Cell
proliferation was measured after exposure of normal human fibroblasts to atrazine, metolachlor or diazinon or a herbicide:herbicide (atrazine and metolachlor) or herbicide:insecticide (atrazine and diazinon) mixture.

Metolachlor (2-chloro-6'-ethyl-N-(2-methoxy-1-methylethyl)acet-o-toluidide) is a chloroacetanilide herbicide used to control certain broadleaf and annual grassy weeds. Metolachlor is the second most popular herbicide used in the United States and presents many of the same problems as atrazine in terms of contamination of surface and ground water sources (6). Metolachlor, like atrazine, has also been found in rain, ice, fog and seawater (30-32). The U.S. EPA does not currently have a MCL for metolachlor, but it does have a lifetime health advisory level (HAL) of 100 ppb. The mechanism(s) of metolachlor’s effects on mammals are not well known, although 100 ppb metolachlor was shown to have cytotoxic effects in human lymphocytes (33).

Diazinon (O,O-diethyl 0-2-isopropyl-6-methyl(pyrimidine-4-yl) phosphorothioate) is an organophosphate insecticide. In a recent NAWQA report, diazinon was the most frequently detected insecticide in surface and ground water samples (6). The U.S. EPA does not currently have a MCL for diazinon, but it does have a lifetime HAL of 0.6 ppb. As with many other organophosphate insecticides, diazinon toxicity is due to the inhibition of the enzyme acetylcholinesterase. Diazinon has also been found to induce sister chromatid exchange (SCE) in human peripheral blood lymphocytes (34) and the mudminnow Umbra limi (35) but not in Chinese hamster ovary cells (36). Human blood cultures exposed to diazinon (20 ppb to 20 ppm) also showed decreased replicative indices, suggesting that diazinon has genotoxic effects (34).

In vitro methods to determine toxicity are common and widely used for screening and ranking chemical toxicities. These methods have also been taken into account sporadically for risk assessment purposes (37). In many of these methods, information on cell survival and/or proliferation is used in the evaluation of chemical toxicity, as well as to explore the cellular mechanisms of the toxicity response of cells. The purpose of this study was to use normal human cells and in vitro methods to assess the effects of three pesticides, atrazine, metolachlor and diazinon, alone or in combination on the growth of normal human fibroblast cells.

MATERIALS AND METHODS

Cells

DET 551 cells (normal human fibroblasts) were obtained from the American Type Culture Collection (CCL-110, Rockville, MD). Cell cultures were maintained in a humidified incubator at 37°C with 5 percent CO2. The cells were passed every five to seven days. Cell cultures with passage numbers between 14 and 19 were used in this study.

Cell Culture Medium

Minimum Essential Medium (MEM, GIBCO) with 10 percent fetal bovine serum (FBS, HyClone Laboratories), 1 percent antibiotics (50 units/ml penicillin, 50 μg/ml streptomycin, GIBCO), and 1mM sodium pyruvate (GIBCO) was used as regular medium. Starvation medium was the same as regular medium, except for a reduced concentration of FBS (1 percent).

International Journal of Global Health
Pesticides

Atrazine (98 percent purity), metolachlor (96.1 percent purity), and diazinon (98.7 percent purity) were purchased from Chem Service (West Chester, PA). They were directly dissolved in regular medium to make individual stock solutions of 10 ppm atrazine, 100 ppm metolachlor, and 10 ppm diazinon. Stock solutions were stored at 4 °C for no more than three months. Before use, the stock solutions were diluted to test concentrations in regular medium.

The concentration of each stock solution was tested at both the beginning and the end of the three-month storage period. Samples were concentrated by a solid phase extraction (SPE) procedure in which the samples were eluted from Supelclean ENVICarb cartridges (Supelco, Bellefonte, PA) with methanol and methylene chloride. After concentration under N2, the residue was dissolved in methanol and analyzed by reverse phase HPLC with a mobile phase of 40 percent acetonitrile in water. At each time point, the measured pesticide concentrations were within + 9 percent of the nominal dose (data not shown).

MTT Assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was performed as described by Mosmann (38), with some modifications (39). This cell proliferation assay is based on the ability of mitochondrial enzymes in viable cells to metabolize MTT. On the first day of the MTT assay, 2000 cells were added to each well of a 96-well plate. The cells were plated in starvation medium to synchronize cells in the G0 phase of the cell cycle. After 48 hours, the starvation medium was aspirated, and treatment medium was added into each well. A minimum of eight replicates for each different pesticide concentration was plated. Cells were grown in treatment medium for 72 hours at 37°C and 5 percent CO2.

To measure cell proliferation after 72 hours, 20 µl of MTT (98 percent purity, Sigma) solution (5 mg/ml in PBS) was added to each well. After four hours incubation at 37°C and 5 percent CO2, 100 µl of isopropanol with 0.04 M HCl was added to each well to dissolve the formazan product. After mixing the cells in solution, the absorbance was read at 570 nm on a SPECTRAMaxPLUS384 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Percent of cell proliferation compared to control (percent proliferation) was calculated based on the method of van de Loosdrecht et al. (40).

Eight different concentrations of each pesticide were tested (atrazine: 0.4, 0.8, 1.6, 3.2, 6.3, 13, 25, 50 ppb; metolachlor: 0.8, 1.6, 3.2, 6.3, 13, 25, 50, 100 ppb; diazinon: 0.4, 0.8, 1.6, 3.2, 6.3, 13, 25, 50 ppb). Factorial design (4 x 4) was used in studying interactive effects between combinations of two pesticides. The use of factorial design has been suggested by the EPA as one of the most valuable statistical approaches for risk assessment of chemical mixtures (41,42). Four different concentrations of each pesticide were selected according to environmental relevance, and the combinations between four concentrations of atrazine (1.6, 3.2, 13, 25 ppb) and four concentrations of either metolachlor (1.6, 6.3, 25, 50 ppb) or diazinon (0.8, 1.6, 3.2, 13 ppb) were tested. Each experiment was performed three times, with eight to 16 replicates per experiment.
Data Analysis

Student-t tests (p < 0.05) were performed using SAS to determine statistical differences between each treatment and the control (cells treated with regular medium only). Confidence intervals (95 percent) of treatment groups were also calculated in SAS. Regression equations were calculated for each pesticide using the mean percent proliferation and the nominal dose.

Predictions of the combined effects of pesticide mixtures were calculated based on the EPA guidelines for the risk assessment of chemical mixtures (43). This method (response addition) assumes that the individual components of the mixture are toxicologically independent (44). In the response addition model, the expected response to the mixture of two chemicals is calculated as one minus the probability of not responding to either component of the mixture. Therefore, the formula for calculating the effect of the mixture based on the response addition model is:

\[ p_{\text{mix}} = p_1 + p_2 - p_1 \times p_2 \]  

where \( p_1 \) is the probability of responding to component 1 and \( p_2 \) is the probability of responding to component 2. The expected response from equation (1) was then compared with the actual data from the mixture experiments. At each dose, mixture toxicities were considered to be additive if they were not significantly different from the calculated value of \( p_{\text{mix}} \), synergistic if the response was significantly greater than that predicted by the model, or antagonistic if the response was significantly lower than that predicted by the response addition model.

RESULTS

Based on the MTT assay, all of the pesticides examined resulted in a decrease in the proliferation of DET 551 cells compared to the control when tested singly or as part of a binary mixture. With the herbicides atrazine and metolachlor, this effect was observed at concentrations below the regulatory levels (MCL or HAL) set for each pesticide. For diazinon, an insecticide, a decrease in cell proliferation was first observed at 1.6 ppb, at a concentration greater than the EPA lifetime HAL of 0.6 ppb.

Effect of Single Pesticides on Cell Proliferation

Statistically significant decreases in cell proliferation compared to control were seen at low levels of each of the three pesticides tested (> 0.8 ppb atrazine, > 1.6 ppb metolachlor and > 1.6 ppb diazinon). A significant increase in cell proliferation was observed at the lowest tested concentration of diazinon (0.4 ppb). After a threshold at which no decrease in proliferation was observed at the lowest tested doses, the toxicities associated with each pesticide increased in a dose-dependent manner (Figure 1). While the decreases in cell proliferation appear to plateau at the highest tested doses, previous studies have shown that atrazine continues to affect cell proliferation at higher doses (45), suggesting that this observation is an artifact of the range of pesticide concentrations studied.
Figure 1. Concentration-response curves for proliferation of DET 551 human fibroblast cells after a 72 hr exposure to atrazine (A), metolachlor (B) or diazinon (C). Each concentration was tested 24 - 48 times. Y-error bars indicate 95 percent confidence intervals.

*Significantly different from control (regular medium) (two-tailed Student t-test, p < 0.05).

Effects of Pesticide Combinations on Cell Proliferation

When the proliferation of cells treated with a binary mixture of either atrazine and metolachlor or atrazine and diazinon was compared with that of the controls, a significant decrease in cell proliferation was observed (Figure 2). Decreases in cell proliferation, however, were not always dependent on the dose of metolachlor or diazinon. In comparison to the atrazine component of the mixture alone, cell proliferation was significantly decreased in the majority of the mixtures (Figure 2). The few exceptions in which the mixture was equal in toxicity to the atrazine component alone were seen at the lowest tested concentration of one of the mixture components (1.6 ppb atrazine + 1.6 ppb metolachlor, 1.6 ppb atrazine + 6.3 ppb metolachlor, 13 ppb atrazine + 1.6 ppb metolachlor, and 13 ppb atrazine + 0.8 ppb diazinon), and were more commonly observed in the herbicide:herbicide mixture.

When the experimental results were compared to predictions of toxicity based on the response addition model, the mixture of atrazine and metolachlor was shown to generally be antagonistic, while the mixture of atrazine and diazinon resulted in additive responses (Table 1).
Figure 2. Comparison of cell proliferation in DET 551 human fibroblast cells after a 72-hour exposure to combinations of atrazine and metolachlor (A-D) or atrazine and diazinon (E-H). Atrazine is indicated by “A,” metolachlor is “M” and diazinon is “D”. All of the treated cells had significantly lower proliferation than the control (regular medium). Each concentration was tested 24-48 times. Y-error bars indicate 95 percent confidence intervals.

*Significantly different from cells treated with atrazine only (two-tailed Student t-test, p < 0.05).

Figure 2 A-H

A: 1.6 ppb atrazine + metolachlor

B: 3.2 ppb atrazine + metolachlor

C: 13 ppb atrazine + metolachlor

D: 25 ppb atrazine + metolachlor

E: 1.6 ppb atrazine + diazinon

F: 3.2 ppb atrazine + diazinon
DISCUSSION

Pesticide contamination is widely detected in ground and surface water. The effects of pesticides on non-target organisms have become a serious environmental health concern. Humans are also at risk when contaminated ground or surface waters are used as sources of drinking water. Although the U.S. EPA has established drinking water criteria for many pesticides, these regulations do not take into account the effects of complex mixtures of contaminants in the environment. To date, very few studies have investigated the toxicity of low-level pesticides and pesticide combinations.

In this study, we investigated the effects of low-level, short-term exposure of three pesticides (atrazine, metolachlor, diazinon), and their combinations on normal human fibroblasts (DET 551 cells). The toxicity of the pesticides to the cells was examined by measuring cell proliferation relative to controls. The three pesticides tested significantly inhibited the proliferation of human cells in a dose-dependent manner. Previous studies in our laboratory have also shown this to occur with atrazine, however at a higher level of the pesticide (10 ppb, 45). In the current study, we have found that atrazine was also able to induce a significant decrease in cell growth (Figure 1a) at concentrations below the MCL. Low levels of both metolachlor (1.6 ppb) and diazinon (1.6 ppb) were also shown to significantly inhibit cell growth (Figure 1b, c). Thus, we have increased the level of sensitivity in measuring pesticide effects.

The reason for this increased sensitivity may be that, in the current study, the pesticides were all dissolved directly into the treatment medium whereas atrazine was previously dissolved in dimethyl sulfoxide (DMSO) (45). The toxicity of the solvent to the cells may have masked the effects of atrazine exposure. Our current experimental protocol appears to more accurately mimic environmental exposure conditions, since exposure of pesticides usually occurs at low levels and under aqueous conditions.

Our results may provide an explanation for the increased risk (relative risk = 1.8) of intrauterine growth retardation in women from an area in southeastern Iowa in which the drinking water source is contaminated with a mixture of pesticides and other contaminants, all at low levels (24). We suggest that the mechanism of the observed intrauterine growth retardation could be related to the effects of atrazine and metolachlor (two contaminants present in relatively high concentrations in Rathbun
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Abbreviations: ppb, parts per billion; t*StErr, Student t-test statistic; LL, lower limit; UL, upper limit; CI, confidence interval; a, antagonistic; +, additive; s, synergistic.
Reservoir) on rates of cell proliferation. In this case, atrazine and metolachlor, possibly in combination with the other contaminants found in Rathbun Reservoir, inhibited cell growth, thus resulting in decreased cell proliferation in the fetus over the gestational period, leading to the low birth-weight condition of the baby.

To investigate the underlying mechanism of the observed reduction in cell proliferation, we measured the levels of p53, a cell cycle regulatory protein, in the treated cells. In normal cells, p53 is produced and quickly degraded and so is found at relatively low levels. However, when DNA damage occurs, the degradation of p53 is inhibited and thus levels of p53 in the cell increase, resulting in an arrest of cell cycle progression to repair any DNA damage. p53 acts as a transcriptional activator to stimulate the expression of a number of genes involved in DNA repair, cell cycle arrest and apoptosis. Depending on the amount of DNA damage in the cell, the cell can either undergo apoptosis or arrest its cell cycle so that the DNA damage can be repaired. In our previous studies, we found that DET 551 cells exposed to atrazine (3-200 ppb) did not undergo apoptosis (45). Thus, we hypothesized that the observed reduction in cell proliferation was due to an increase in p53 and arrest of the cell cycle. However, when p53 levels were quantitated after pesticide exposure, there was no statistically significant increase in the levels of this protein (data not shown). In contrast to our findings, Cantemir et al. (46) found increased levels of p53 in peripheral lymphocytes of rats chronically treated (six-12 months) with atrazine (2.7–5.4 mg/kg body weight). This difference is most probably due to the differences in the concentrations of atrazine, the duration of exposure or the experimental system used.

In this study, atrazine, metolachlor and diazinon reduced proliferation of DET 551 cells. However, the mechanism does not appear to involve apoptosis or a p53 dependent arrest of the cell cycle at the G1 checkpoint (as indicated by increased p53 concentrations). We are currently investigating the hypothesis that these cells may arrest at the other cell cycle checkpoints. Another possibility under study is that the p53 protein in DET 551 cells may not be the wild-type form of the protein and thus may not function as expected.

We also examined the effects of combinations of atrazine and either metolachlor or diazinon, two other pesticides commonly found in contaminated water. When the pesticides were combined, the mixture of the two herbicides (atrazine and metolachlor) showed an antagonistic response, while atrazine and diazinon responded as predicted by the additive interaction model (Table 1).

Previous studies have reported synergistic toxicity for mixtures containing atrazine. For example, synergistic toxicity has been found for 50:50 mixtures of atrazine and alachlor in amphibians (47), binary mixtures of atrazine and diazinon, parathion, carbofuran or DDT in several species of insects (48), and atrazine and methyl parathion mixtures in Chironomus tentans (49). However, not all studies have demonstrated that atrazine mixtures will cause synergistic toxicity. For example, additive toxicity was found for mixtures between atrazine and carbofuran in C. tentans midges (50), and antagonistic toxicity was found for mixtures between atrazine and methoxychlor (an organochlorine insecticide) in C. tentans midges (49). Thus, the variety of interactions produced by atrazine mixtures indicates that the types of interactions between contaminants depend
on the species, experimental endpoints, contaminants and the concentrations of the contaminants.

The results of this study, along with others, suggest that drinking water standards based on concentrations of single pesticides may not adequately protect human health. In fact, even the EPA MCL of 3 ppb atrazine in drinking water may not be sufficiently protective of human health. Thus, with the appearance of more and more pesticides as contaminants of many ground and surface water sources, environmental regulatory agencies will need to provide guidelines for mixtures of pesticides and other contaminants at environmentally relevant concentrations.

REFERENCES


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