Arch. Environ. Contam. Toxicol. 46, 438–444 (2004)
DOI: 10.1007/s00244-003-3087-5

ARCHIVES OF
Environmental
Contamination
and Toxicology
© 2004 Springer-Verlag New York LLC

Low-Level Atrazine Exposure Decreases Cell Proliferation in Human Fibroblasts

Michelle K. Manske,[1] Lisa A. Beltz,[2] Kavita R. Dhanwada[2]

[1] Environmental Programs, University of Northern Iowa, Cedar Falls, Iowa 50614, USA
[2] Department of Biology, University of Northern Iowa, Cedar Falls, Iowa 50614, USA

Received: 6 June 2003/Accepted: 15 November 2003

Abstract. There is continuing concern that release of certain chemicals into the environment leads to human exposure to toxins, particularly through contaminated ground and surface waters. The triazine herbicide atrazine (2-chloro-4-ethylamine-6-isopropylamino-*S*-triazine) is routinely found as a contaminant in many surface and groundwaters. Little is known of the long-term effects of atrazine exposure on human cells. The objective of this research was to examine whether low-level exposure of atrazine to normal human fibroblast cells in culture had measurable deleterious effects. We show that low-level, short-term exposure of atrazine to normal fibroblasts results in decreased cell proliferation. In two separate assays to measure cell proliferation, either a 1.5-fold decrease in the cell number occurred (saturation density analysis) or a 2-fold decrease in cell proliferation was found (MTT analysis). Experiments to measure apoptosis in cells exposed to low levels of atrazine did not show DNA fragmentation or increased caspase activity. Thus, this analysis shows that short-term exposure to environmentally relevant, low levels of atrazine can be assessed by a decrease in cellular proliferation of normal human cells.

Past and present use of herbicides in many agriculture-rich states is causing continuing environmental health concerns of potential human exposure through contaminated natural resources such as soil and surface water. Triazine herbicides are moderately soluble in water and have relatively low soil-sorption coefficients (Wauchope *et al.* 1992). Thus, when these herbicides are applied to cropland, they may be readily transported to shallow groundwater by infiltration or to surface waters by storm water runoff. Herbicides that run off into streams present concerns because many municipal water supplies in the Midwest are obtained from surface waters or from shallow aquifers in which infiltration from surface waters can affect groundwater quality (Boyd 2000).

The most commonly used herbicide today is atrazine (2-chloro-4-ethylamine-6-isopropylamino-*S*-triazine), belonging to a family of herbicides called chlorotriazines. Atrazine is used to control broadleaf and grassy weeds in corn, sorghum, and conifers as well as other crops (Kamrin 1997). Atrazine acts by irreversibly binding to the photosystem II complex on thylakoid membranes in chloroplasts (Solomon *et al.* 1996). The U.S. Environmental Protection Agency's (USEPA) maximal contaminant level (MCL) for atrazine in drinking water is 3 parts per billion (ppb) or 3 μg/L (USEPA 1996). The presence of atrazine in water supplies is a major concern in the United States because atrazine may be a potential carcinogen. Atrazine is detected 10 to 20 times more frequently during water quality monitoring studies than the next most commonly detected herbicide (Wolf and Nowak 1996).

To understand fully the effects of atrazine on human and animal models, it is important to consider actual levels of contamination. Atrazine is found at relatively low levels in the environment, usually less than 2 μg/L in groundwater sources, but has been found at levels as high as 21 μg/L in groundwater, 42 μg/L in surface waters, 102 μg/L in river basins in agricultural areas, and up to 224 μg/L in Midwestern streams (Kolpin *et al.* 1997). However, the majority of research conducted on atrazine to understand deleterious effects on organisms is based on exposure to high levels of atrazine, rather than environmentally relevant levels. Only few studies have examined effects of low-level atrazine exposure. One recent study by Hayes and colleagues (2002) exposed amphibian larvae to low, ecologically relevant doses of atrazine (0.01–200 μg/L) and found that atrazine levels above 0.1 μg/L induced hermaphroditism (presence of ovaries) and demasculinization (decrease in laryngeal size) of exposed males. In addition, they found that male *Xenopus laevis* had a 10-fold decrease in testosterone levels after exposure to 25 μg/L atrazine. These findings suggest that the exposure of atrazine to whole organisms even at very low levels can lead to altered cellular function.

The majority of research regarding atrazine has demonstrated that high-level exposure to this herbicide can lead to many alterations in a variety of cell types, specifically a high incidence of cellular DNA damage. The effect of commercial-grade atrazine was demonstrated by using erythrocytes from *Rana catesbeiana* (bullfrog) tadpoles that had significant DNA damage in atrazine-treated cells compared to untreated controls (Clements *et al.* 1997). Other recent analyses in human lymphocytes (Ribas *et al.* 1995), yeast cells (Della Croce *et al.* 1996), and rodent cells (Pino *et al.* 1988; Taets *et al.* 1998)

Correspondence to: Kavita R. Dhanwada; *email*: Kavita.Dhanwada@uni.edu

have all shown increased DNA damage in cells exposed to atrazine compared to unexposed controls. Flow cytometric analysis showed that atrazine caused a significant increase in the CV (correlation of variance) of G1 peaks as compared to controls and that it may cause whole-cell clastogenicity at low concentrations in Chinese hamster ovary (CHO) cells (Biradar and Rayburn 1995). Other studies have found associations between atrazine exposure and an increased incidence of cancer in humans (Donna *et al.* 1989; Van Leeuwen *et al.* 1989; Weisenburger 1990; Mills 1998) and other animal models, specifically rodents (Donna *et al.* 1986; Pinter *et al.* 1990; Stevens *et al.* 1994).

One other study that did examine low-level, long-term atrazine exposure showed that a decrease in body weight occurred in female Wistar rats after low-level atrazine exposure in the diet for 6 and 12 months (Cantemir *et al.* 1997). In addition, in a retrospective epidemiological study, a potential association between levels of herbicides in Iowa drinking water and the incidence of intrauterine growth retardation (birth weight below the tenth percentile for gestational age) in humans was suggested by Munger and colleagues (1997). The Iowa Statewide Rural Well Water Survey indicated that water supplied by the Rathbun Rural Water System (RRWS) in southern Iowa had elevated levels of atrazine contamination. The RRWS had the highest levels of atrazine (2.2 μg/L) compared to other Iowa surface water suppliers (0.7 μg/L) and groundwater sources (undetectable). Through epidemiological analysis, researchers found that the area served by the RRWS (with the elevated atrazine levels) had a greater risk of intrauterine growth retardation than other southern Iowa communities (Munger *et al.* 1997). It is important to remember that the levels of atrazine at RRWS were below the MCL levels set by the EPA.

While extensive research has been done using high doses of atrazine, few experiments have been conducted to determine the effects of low-dose, short-term exposure to atrazine on human cells. Thus, the purpose of this study was to examine the specific effects of low-level atrazine exposure on human cells by assessing cell proliferation and apoptosis.

Materials and Methods

Cells and Media. Normal human fibroblasts, DET 551 (CCL-100; American Type Culture Collection [ATCC], Manassas, VA), and human peripheral blood mononuclear cells (PBMCs) were used in this study. Fresh cultures of DET 551 cells were reconstituted from frozen stocks and maintained in a humidified incubator at 37°C with 5% CO_2. The adherent DET 551 cells were passed every 4 to 7 days. Minimal essential medium (MEM; Gibco-BRL, Gaithersburg, MD) was prepared as directed and 1 mmol of sodium pyruvate (Gibco-BRL) was added. The growth medium was supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and antibiotics, 50 U/mL penicillin and 50 μg/ml streptomycin (Gibco-BRL). The PBMCs, composed of lymphocytes and monocytes, were prepared from venous blood of normal healthy volunteers 18–55 years old from among the students and faculty/staff of the University of Northern Iowa as approved by the University's Human Participants Review Committee. Blood was diluted twofold with phosphate-buffered saline (PBS; Fisher Scientific, Hanover Park, IL), layered over Ficoll–Hypaque (specific density = 1.077; Sigma Chemical Co., St. Louis, MO) in 50-ml tubes and centrifuged at 350*g* for 45 min at 20°C. The interface containing PBMCs was then removed and washed twice with PBS by centrifugation for 7 min at 4°C. Cells were counted in a hemocytometer. Cell viability was determined by trypan blue exclusion and was >99% at culture onset. Cells were resuspended in RPMI 1640 tissue culture medium with HEPES (Fisher Scientific) containing a mixture of penicillin G (10 U/ml), streptomycin (10 μg/ml), and L-glutamine (2.92 mg/ml) (Gibco-BRL) with 5% FBS (HyClone Laboratories).

Test Compounds. Atrazine (98% purity) was obtained from Chem Service (West Chester, PA) and was dissolved in dimethyl sulfoxide (DMSO; 99.9% purity; Sigma). Test chemicals were added directly to the medium before the medium was added to cell cultures. The concentration of DMSO in the media varied from 0.03% for 3 μg/L atrazine in DMSO to 0.94% for 300 μg/L atrazine in DMSO.

Saturation Density Analysis. The first method used to measure cell proliferation was saturation density analysis. In this assay, DET 551 cells (passages 17–20) were plated at 2×10^5 cells/100-mm dish in MEM with 0.5% FBS (low serum medium). Cells were placed in a 37°C incubator with 5% CO_2 for 48 h in the low serum medium to synchronize cells. After 48 h, the medium was removed and complete medium with 10% FBS containing increasing concentrations of atrazine or DMSO (0, 3, 10, 50, 100, 200, and 300 μg/L) was added. Dilutions of atrazine were made from an atrazine stock concentration of 32 mg/L dissolved in DMSO. Cells were grown under treatment conditions for 5 days at 37°C and 5% CO_2. Cells were harvested and individual cells were counted by the trypan blue exclusion method. Viability of cells in all treatment groups and controls was over 99%. The actual number of cells treated with increasing concentrations of atrazine or solvent control was compared to the number of control cells. Cells were counted using a hemocytometer. All experiments were done at least three separate times. The saturation density analysis had three replicates per sample.

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] Assay. To confirm the results for cell proliferation, an alternate method was used to measure cell proliferation. This method, in contrast to saturation density analysis, does not rely on individual cells being counted; rather it is a calorimetric assay. DET 551 cells (passages 15–17) were plated in flat-bottom 96-well tissue culture-treated plates at 2000 cells/well in low serum medium and incubated for 48 h. After 48 h, medium was removed and complete medium with 10% FBS and increasing concentrations of atrazine or DMSO (0, 3, 10, 50, 100, and 200 μg/L) were added for an additional 48 or 72 h. The stock concentration for atrazine was the same as before, 32 mg/L in DMSO. For MTT analysis, 20 μl of 5 mg/ml MTT (Sigma) in PBS (2.2 m*M* Na_2PO_4, 8.1 m*M* NaH_2PO_4, 154 m*M* NaCl) was added to cells and plates were incubated for 4 h at 37°C. HCl (0.04 *N*) in isopropanol was added to each well, cells were vigorously resuspended, and optical density (OD) was read at 570 nm on a UV/VIS spectrophotometer (Molecular Devices Corp., Sunnyvale, CA). A similar procedure was followed for human PBMCs. One million PBMCs were plated in 24-well tissue culture plates in RPMI with 5% FBS containing increasing concentrations of either atrazine or DMSO (40, 100, or 200 μg/ml). Cells were incubated for 96 h at 37°C with 5% CO_2. At that time, 150 μl of MTT solution (5 mg/ml) was added and cells were incubated for an additional 4 h at 37°C. HCl (0.4 *N*) in isopropanol was then added, cells were vigorously mixed, and OD was read at 570 nm. The increase in OD had a positive correlation with viable cell number and indicated enhanced cell growth compared to control cells. All experiments were performed at least three separate times with each experiment having eight replicates (wells) per treatment group.

Immunohistochemical Analysis. Apoptosis was examined using the Apoptosis Detection Kit (R&D Systems, Minneapolis, MN). Apoptotic cells are detected with a biotin–streptavidin system complexed with

horseradish peroxidase (HRP) and a diaminobenzidine (DAB)-based stain. The manufacturer's suggested protocol was followed. Monolayers of DET 551 cells (passages 15–18) were plated on electrostatically treated slides (Fisher Scientific) in low serum medium and incubated for 48 h at 37°C. After 48 h, the medium was removed and complete medium containing atrazine or DMSO (0, 3, 50, or 200 μg/L) was added. After an additional 48 h, cells were fixed with formaldehyde, permeabilized with proteinase K, and quenched with 10% H_2O_2 in methanol to reduce background staining. Biotinylated nucleotides were incorporated into the 3′-OH ends of the DNA fragments by terminal deoxynucleotidyl transferase (TdT). Detection of apoptotic cells occurred by exposing slides to a streptavidin–HRP detection solution followed by a working solution of DAB substrate. Methyl green was used as counterstain for 5 min. Slides were washed in butanol and mounted using 1 drop of mounting medium per sample. A positive control, cells treated with TACS nuclease after permeabilization, was also included.

Caspase Assay. To measure apoptosis enzymatically, caspase-3 activity in cell extracts was measured using the CaspASE Colormetric Assay System protocol (Promega, Madison, WI). DET 551 cells (passages 16–18) were plated on 100 × 20-mm tissue culture dishes at 5.0×10^5 cells/dish in low serum medium for 48 h at 37°C and 5% CO_2. After 48 h, medium was removed and complete medium with 10% FBS and increasing concentrations of atrazine or DMSO (0, 3, 100, 200, and 500 μg/L) were added for 48 h. Lysates made from cells treated with 1.25 μ*M* staurosporine (Aldrich, St. Louis, MO) in medium for 4 h served as a positive control for the induction of caspase activity. Lysates made from cells treated with 50 μ*M* Z-VAD-FMK in 10% FBS complete growth medium for 24 h served as a negative control. Z-VAD-FMK is a cell-permeable caspase inhibitor that irreversibly binds to the catalytic site of caspases to inhibit induction of apoptosis (Abu-Qare *et al.* 2001). The manufacturer's suggested protocol was followed to make cell lysates. Five microliters of the lysate was used for protein concentration determination using the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA). The cell extracts (50 μg protein/well) were added to a 96-well plate containing caspase assay buffer, DMSO, 100 m*M* dithiothreitol (DTT; Sigma), and DEVD-pNA colorimetric substrate and incubated at 37°C for 4 h. The OD was then read at 405 nm in a UV/VIS spectrophotometer (Molecular Devices) and compared to a standard curve generated using purified *p*-nitroanaline (pNA). This compound is released from the substrate upon cleavage by DEVDase. Free pNA produces a yellow color after cleavage that is proportional to the amount of DEVDase activity present in the sample. The increase in OD positively correlates with the amount of caspase-3 activity. All experiments were done at least three times. The caspase assay had two replicates per sample.

Statistical Analysis. One-way ANOVA analysis using Tukey's Studentized range test was performed to determine if a statistical difference between means was present. Mean comparisons were made between the medium control and atrazine-treated samples, the medium control and DMSO-treated samples (served as solvent control), and atrazine and DMSO-treated samples. Differences were determined to be statistically significant at $p < 0.05$. All programs were run using SAS statistical software.

Results

Effects of Atrazine on Cell Proliferation

Saturation density and MTT cell proliferation analysis were both performed to investigate growth of cells in the presence of the herbicide atrazine. DET 551 cells exposed to increasing concentrations of atrazine showed a decrease in cell growth compared to control cells (Table 1) by saturation density analysis. Cells that had been exposed to 50, 200, and 300 μg/L atrazine had a statistically significant, 1.5-fold decrease in cell number compared to control cells. However, it is noted that the highest concentration of DMSO (300 μg/L) alone may have had slight toxicity by itself since the percentage DMSO in these cells is higher than in the other samples (0.94%) and these cells were exposed to the chemical for 5 days, 2 days longer than in the MTT analysis. MTT analysis, an additional method used to measure cell proliferation, was also performed to reiterate the saturation density analysis. MTT is a tetrazolium salt that is pale yellow and produces a dark-blue formazan product when incubated with living mammalian cells. The mitochondrial enzymes of the cell cleave MTT to form a formazan salt (Mosmann 1983). Figure 1A and Table 2 show that with a 48-h exposure to atrazine a statistically significant decrease in DET 551 cell proliferation was once again detected. Statistical analysis showed that cells treated with 10, 50, and 200 μg/L atrazine differed significantly in their ability to proliferate from the DMSO solvent-treated cells. The differences between the DMSO solvent control and the medium-exposed cells were not statistically significant. Similar data were found with a 72-h exposure to atrazine (data not shown). However, the decrease in cell growth that occurred in DET 551 cells seems to be specific for these cells and not all normal human cells. When PBMCs were treated with increasing concentrations of atrazine, there was no decrease in cell growth (Fig. 1B) compared to control cells.

Table 1. Saturation density analysis of DET 551 fibroblasts

Atrazine concentration (μg/L)	Atr treated cells (cells/ml)	DMSO control (cells/ml)
0	$22 \times 10^5 \pm 0.92$	$22 \times 10^5 \pm 0.92$
10	$19 \times 10^5 \pm 0.57$	$21 \times 10^5 \pm 0.28$
50	$17 \times 10^5 \pm 0.53$*	$21 \times 10^5 \pm 1.45$
100	$15 \times 10^5 \pm 0.31$	$21 \times 10^5 \pm 1.29$
200	$12 \times 10^5 \pm 0.42$*	$17 \times 10^5 \pm 0.72$
300	$10 \times 10^5 \pm 0.46$*	$15 \times 10^5 \pm 0.61$

Note. Cell number after a 5-day exposure to atrazine or DMSO solvent control ± standard error. The original number of cells plated was 2×10^5 cells/plate.
* Statistically significant difference from control treatment, $p < 0.05$.

Determination of Apoptosis in Atrazine-Treated Cells

To study the underlying causes of the decrease in cell proliferation with atrazine-treated cells, experiments were done to determine if the decrease in cell number was due to apoptosis of the treated cells. Two assays, a qualitative immunohistochemical analysis and a quantitative enzymatic analysis, were performed. In the immunohistochemical analysis, DET 551 cells were exposed to either 3 or 200 μg/L atrazine or DMSO for 48 h before the assay was performed. Atrazine and DMSO-treated samples were compared quali-

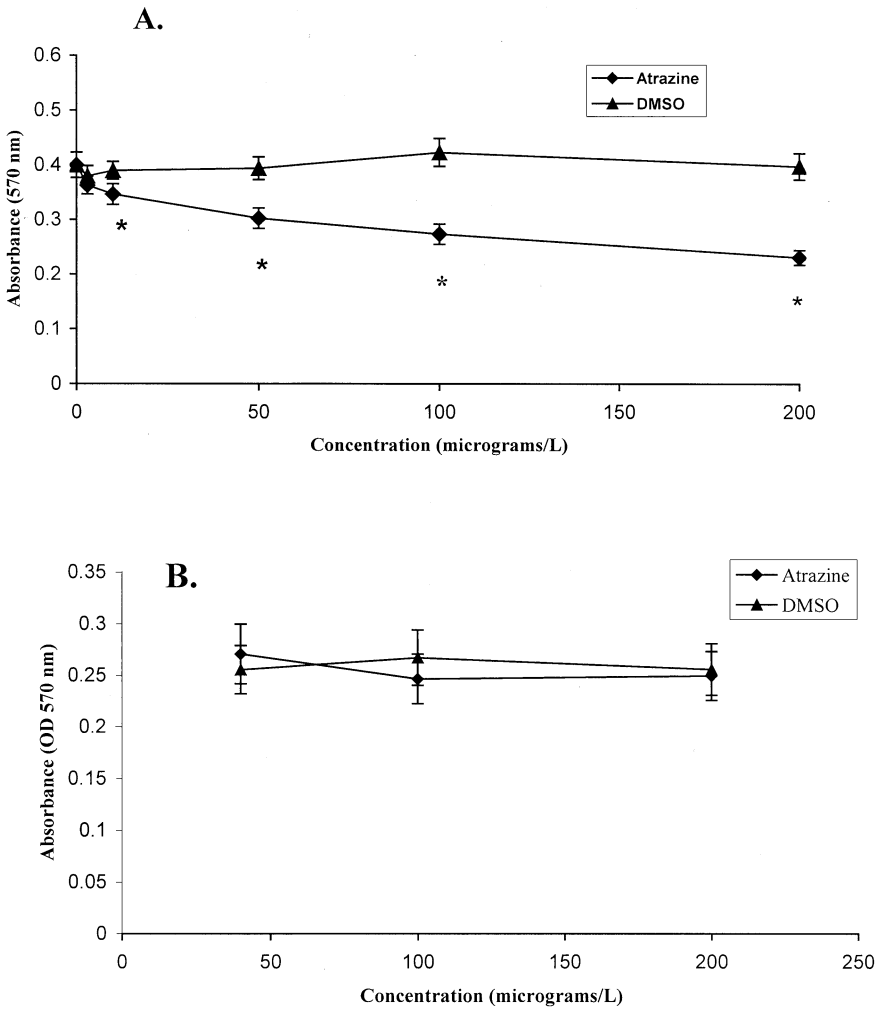

Fig. 1. MTT analysis of (A) DET 551 human fibroblasts exposed to increasing concentrations of atrazine after a 48-h exposure or (B) human PBMCs exposed to atrazine after a 96-h exposure. *Statistically significant difference compared to control treatment ($p < 0.05$). Error bars indicate one standard error above and below the mean

Table 2. MTT analysis of DET 551 cells after a 48-h exposure to atrazine (Atr) or DMSO

Atr concentration (μg/L)	Absorbance at 570 nm	
	Atr-treated cells	DMSO control
0	0.400 ± 0.070	0.400 ± 0.070
10	0.363 ± 0.050*	0.379 ± 0.057
50	0.302 ± 0.058*	0.394 ± 0.049
100	0.274 ± 0.057*	0.423 ± 0.078
200	0.231 ± 0.042*	0.398 ± 0.074

Note. Absorbance values of cells at 570 nm after a 48-h exposure to atrazine or DMSO solvent control ± standard error.
* Statistically significant difference from control treatment, $p < 0.05$.

tatively to a TACS nuclease-positive control (Fig. 2). TACS nuclease generates DNA breaks in every cell so that with this treatment the majority of cells should have brown nuclear staining. When cells treated with 200 μg/L atrazine or DMSO were compared to control cells, there was a slight increase in the number of brown-staining cells (Fig. 2). The level of apoptosis in atrazine-treated cells was low compared to the positive control. However, this was a qualitative assay and did not give specific numbers, although the data were similar in three separate experiments. Thus, to determine if this small increase in the presence of apoptotic cells was significant, a quantitative assay to measure apoptosis was performed.

An enzymatic assay that measured caspase activity was used to detect apoptosis in atrazine-treated cells. Cytosolic thiol proteases called caspases are key enzymes involved in apoptosis (Yue *et al.* 1998). These enzymes are the effectors of the apoptotic signal in a proteolytic cascade. The CaspACE Assay System (Promega), a colorimetric assay that measures the activity of caspase-3, was used. The activation of this enzyme occurs earlier in the apoptosis process than the degradation of DNA, which was demonstrated with the immunohistochemical assay. Figure 3 shows that caspase activity in atrazine-treated samples was quite low, with values ranging from 7.31 to 9.53 pmol/μl. These values were similar to the value of the negative control (cells that had been treated with Z-VAD-FMK), however, they were two to three times less than the value of the positive control cells treated with staurosporine, which had an average of 23.5 pmol/μl of caspase-3 activity. The value for caspase-3 activity for DMSO solvent control cells was similar to that for medium-treated cells (Fig. 3).

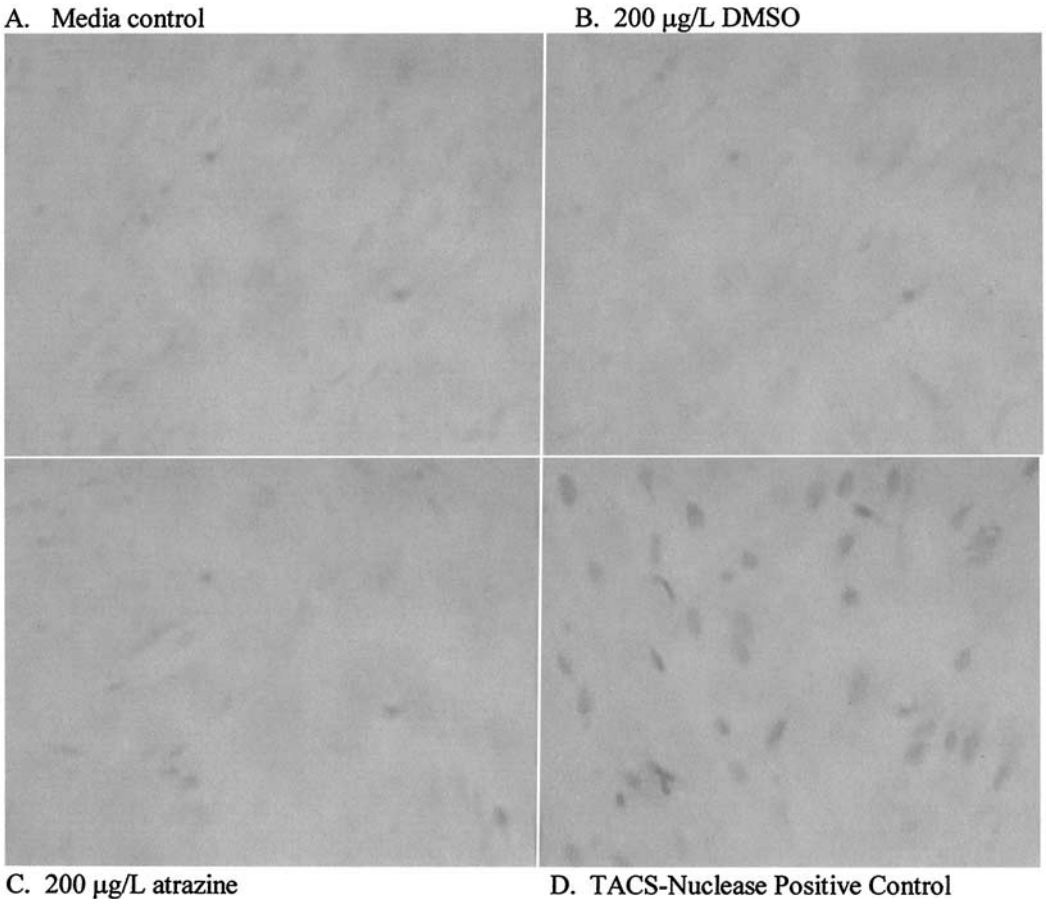

Fig. 2. Immunohistochemical analysis of DET 551 human fibroblasts after a 48-h exposure to atrazine compared to control. Monolayers of DET 551 fibroblasts were plated on slides grown in (A) medium alone, (B) 200 µg/L DMSO as solvent control, or (C) 200 µg/L atrazine or (D) were treated with TACS nuclease (positive control). Atrazine was dissolved in DMSO. Solvent concentrations did not exceed 0.63% DMSO

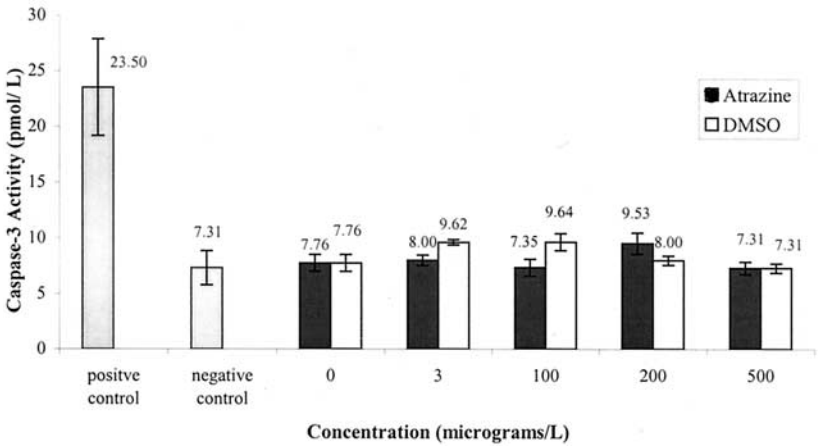

Fig. 3. Caspase-3 activity in DET 551 cells after exposure to increasing concentrations of atrazine. *Statistically significant difference from control treatment ($p < 0.05$). Error bars indicate one standard error above and below the mean

Discussion

This study examined the effects of low-level, short-term exposure to atrazine on normal human cells. Two assays that measured cell proliferation, saturation density and MTT analysis, showed that normal fibroblasts exposed to increasing levels of the herbicide atrazine had a decreased level of growth after 2–5 days. These cells had a dose-dependent, statistically significant decrease in cell proliferation in both assays, whereas no statistically significant decrease in proliferation occurred in solvent control-treated cells (Tables 1 and 2, Fig. 1A). In the saturation density analysis the highest concentration of DMSO (300 µg/L) may have had an effect on cell growth by itself since there was large a decrease in cell number compared to control cells, however, it was not statistically different from the control as was its atrazine counterpart. This may have occurred for a couple of reasons: (1) The concentration of solvent was nearly 1% in these cells, and (2) the cells were exposed to the chemical for 5 days, 2 days longer than the other assay used. No such solvent effect was seen in the MTT proliferation assay

(Table 2, Fig. 1A). The inhibition of growth was not seen in all normal human cells since atrazine had no effect on the growth of PBMCs (Fig. 1B). These cells grew to levels that were similar to control cells with no inhibition of cell growth in the presence of atrazine. Interestingly, our previous work has shown that these two cell types also behave differently from one another when exposed to either nitrate or nitrite. DET 551 cells were not inhibited in their growth by either nitrate or nitrate, while lymphocytes were significantly so (Ustyugova *et al.* 2002), suggesting that cell type might play an important role in sensitivity to environmental contaminants. Thus, based on our current data, a statistically significant specific decrease in cell proliferation of normal human fibroblasts is seen after low-level, short-term atrazine exposure.

The decrease in cell proliferation could have occurred from necrosis (accidental cell death) due to atrazine toxicity or could have occurred due to an increase in apoptosis (programmed cell death). If atrazine exposure was toxic to cells and resulted in death, it would have been detected in cell viability studies. However, viability of cells after atrazine exposure was similar to control cells and the low levels of atrazine being used in this analysis have not been shown to cause cell necrosis in previous studies. There are many other potential routes of toxicity that would lead to inhibition of cell growth. One possible explanation for the decrease in cell number could be that atrazine exposure was leading to DNA damage in cells and apoptosis was the result, thus fewer cells were found after a specific time period.

Two methods were employed to evaluate the effects of atrazine on apoptosis in human fibroblasts. This phenomenon could explain the decrease in cell proliferation seen after a 48-h exposure to atrazine and can easily be measured. In the qualitative immunohistochemical analysis that was used to visualize DNA fragmentation, only a slight increase in nuclear staining of atrazine-treated cells (Fig. 2) was detected. To increase sensitivity and detect any low-level apoptosis that may have occurred, an enzymatic assay that measured caspase-3 activity was performed. The results showed that all atrazine-treated (3–500 μg/L) samples had caspase activity similar to the negative control (Fig. 3). No significant differences were detected between the control treatments and atrazine-treated cells. This implies that short-term atrazine exposure (48 h) and concentrations up to 500 μg/L may not be enough to cause a significant increase in caspase-3 activity leading to apoptosis.

Results from our study correlate well with other studies that have shown that atrazine exposure was associated with a significant reduction in the number of cells. Anterior pituitary cells of rats exposed to increasing atrazine concentrations, 5–50 mg/L or ppm, also showed decreased viability of these cells within 72 h after atrazine exposure (Zechner-Krapan *et al.* 1998), however, the researchers used much higher concentrations of atrazine (up to 100–1000X higher) in their study. We saw a decrease in cell proliferation of human fibroblasts after a 48-h exposure (vs. a 72-h exposure) to 50–200 μg/L atrazine, much lower doses than those used with the anterior pituitary rat cells. A statistically significant decrease in body weight was also shown in female Wistar rats after exposure to atrazine for 6 and 12 months (Cantemir *et al.* 1997). The decrease in body weight would suggest a decrease in cell proliferation. Additionally, the epidemiological study by Munger *et al.* (1997) found an increased risk associated with low-level exposure (2.2 μg/L) to pesticide-contaminated water from the RRWS in southern Iowa among pregnant mothers to an increased incidence of intrauterine growth retardation. If a line of reasoning similar to that in our study is applied, then the decrease in body weight of the fetuses in the study by Munger and colleagues (1997) may be attributed to a decrease in cellular proliferation from atrazine exposure in the mothers.

We have shown that levels as low as 10 μg/L of atrazine were able to cause a significant decrease in cellular proliferation of human fibroblasts. While the MCL for atrazine is 3 μg/L, this level is usually exceeded manyfold during crop growing seasons. Thus far, this decrease in cellular proliferation has not been attributed to increases in either apoptosis or necrosis. Further studies need to be conducted to examine different components of the cell cycle to determine if atrazine-treated cells are being halted in their progression through the cell cycle and thus taking longer for a cell to complete one cycle. To address the decrease in cellular proliferation, flow cytometric analysis will be performed in future experiments to quantitate cells in each stage of the cell cycle. If atrazine is involved in slowing cells down in their progression through the cell cycle, our research can perhaps provide evidence at the cellular level for an increased incidence of intrauterine growth retardation seen in communities exposed to atrazine.

Acknowledgments. This study was funded by Environmental Programs at the University of Northern Iowa as well as a GRASP award funded by the College of Natural Sciences at the University of Northern Iowa. The current work, done primarily by M.M., is in partial fulfillment of the requirements for an M.S. degree. The authors wish to thank Drs. Ed Brown, Laura Jackson, and Sue Joslyn for a critical reading of the manuscript.

References

Abu-Qare AW, Abou-Donia MB (2001) Biomarkers of apoptosis: Release of cytochrome c, activation of caspase-3, induction of 8-hydroxy-2′-deoxyguanosine, increased 3-nitrotyrosine, and alteration of p53 gene. J Toxicol Environ Health 4:313–332

Biradar DP, Rayburn AL (1995) Flow cytogenic analysis of whole cell clastogenicity of herbicides found in groundwater. Arch Environ Contam Toxicol 28:13–17

Boyd RA (2000) Herbicides and herbicide degradates in shallow groundwater and the Cedar River near a municipal well field, Cedar Rapids, Iowa. Sci Total Environ 248:241–253

Cantemir C, Cozmei C, Scutaru B, Nicoara S, Carasevici E (1997) p53 protein expression in peripheral lymphocytes from atrazine chronically intoxicated rats. Toxicol Lett 93:87–94

Della Croce C, Morichetti E, Intorre L, Soldani G, Bertini S, Bronzetti G (1996) Biochemical and genetic interactions of two commercial herbicides with the monooxygenase system and chlorophyllin. J Environ Pathol Toxicol Oncol 15:21–28

Clements C, Ralph S, Petra M (1997) Genotoxicity of select herbicides in *Rana catesbeiana* using the alkaline single-cell DNA electrophoresis (comet) assay. Environ Mol Mutagen 29:277–288

Donna A, Betta PG, Robutti F, Bellingeri D (1986) Carcinogenicity testing of atrazine: Preliminary report on a 13-month study on male Swiss albino mice treated by intraperitoneal administration. Giorn Ital Med Lavoro 8:119–121

Donna A, Crosignani P, Robutti F, Betta PG, Bocca R, Mariani N,

Ferrario F, Fissi R, Berrino F (1989) Triazine herbicides and ovarian epithelial neoplasms. Scand J Work Environ Health 15: 47–53

Hayes TB, Collins A, Lee M, Mendoza M, Noriega N, Stuart AA, Vonk A (2002) Hermaphorditic, demasculinized frogs after exposure to the herbicide atrazine at low ecological relevant doses. Proc Natl Acad Sci USA 99:5476–5480

Kamrin P (1997) Herbicide profiles: Toxicity, environmental impact and fate. CRC Press, Boca Raton, FL

Kolpin DW, Sneck-Fahrer DA, Hallberg GR, Libra RD (1996) Temporal trends of selected agricultural chemicals in Iowa's groundwater, 1982–95: Are things getting better? J Environ Qual 26: 1007–1017

Mills PK (1998) Correlation analysis of pesticide use data and cancer incidence rates in California counties. Arch Environ Health 53: 410–411

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63

Munger R, Isacson P, Hu S, Burns T, Hanson J, Lynch CF, Cherryholmes K, Van Dorpe P, Hausler WJ (1997) Intrauterine growth retardation in Iowa communities with herbicide-contamination drinking water supplies. Environ Health Perspect 105:308–314

Pino A, Maura A, Grillo P (1988) DNA damage in stomach, kidney, liver and lung of rats treated with atrazine. Mutat Res 209:145–147

Pinter A, Torok G, Borzsonyi M, Surjan A, Csik M, Kelecsenyi Z, Kocsis Z (1990) Long-term carcinogenicity bioassay of the herbicide atrazine in F344 rats. Neoplasma 37:533–544

Ribas G, Frenzilli G, Barale R, Marcos R (1995) Herbicide-induced DNA damage in human lymphocytes evaluated by the single-cell gel electrophoresis (SCGE) assay. Mutat Res 344:41–54

Soloman KR, Baker DB, Richards RP, Dixon KR, Klaine SJ, La Point TW, Kendall RJ, Weisskopf CP, Giddings JM, Giesy JP, Hall LW, Williams WM (1996) Ecological risk assessment of atrazine in North American surface waters. Environ Toxicol Chem 15:31–76

Stevens JT, Brekenridge CB, Wetzel LT, Gillis JH, Luempert III LG, Eldridge JC (1994) Hypothesis for mammary tumorigenesis in Sprague-Dawley rats exposed to certain triazine herbicides. J Toxicol Environ Health 43:139–153

Taets C, Aref S, Rayburn AL (1998) The clastogenic potential of triazine herbicide combinations found in potable water supplies. Environ Health Perspect 106:197–201

USEPA (1996) Drinking water regulations and health advisories EPA 822-B-96-002. Office of Water, US Environmental Protection Agency, Washington, DC

Ustyugova IV, Zeman C, Dhanwada KR, Beltz LA (2002) Nitrates/nitrites alter lymphocyte proliferation and cytokine production. Arch Environ Contam Toxicol 43:270–276

Van Leeuwen JA, Waltner-Toews D, Abernathy T, Smit B, Shoukri M (1999) Associations between stomach cancer incidence and drinking water contamination with atrazine and nitrate in Ontario (Canada) agroecosystems, 1987–1991. Int J Epidemiol 28:836–840

Wauchope RD, Buttler TM, Hornsby AG, Augustijn-Beckers PWM, Burt JP (1992) SCS/ARS/CES Pesticide properties database for environmental decisionmaking. Rev Environ Contam Toxicol 123:1–157

Weisenburger DD (1990) Environmental epidemiology of non-Hodgkin's lymphoma in eastern Nebraska. Am J Ind Med 18: 303–305

Wolf SA, Nowak, PJ (1996) A regulatory approach to atrazine management: Evaluation of Wisconsin's groundwater protection strategy. J Soil Water Conserv 51:94–97

Yue TL, Wang C, Romanic AM, Kikly K, Keller P, DeWolf WE Jr, Hart TK, Thomas HC, Storer B, Gu JL, Wang X, Feuerstein GZ (1998) Staurosporine-induced apoptosis in cardiomyocytes: A potential role of caspase-3. J Mol Cell Cardiol 30:495–507

Zechner-Krpan V, Kniewald J, Kniewald K (1998) Application of anterior pituitary cell culture in toxicological research. Arch Hig Rada Toksikol 49:219–229