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Cell Cycle Analysis of Atrazine-Treated Human Fibroblast Cells Using Flow Cytometry

Andrea L. Austin
University of Northern Iowa

CELL CYCLE ANALYSIS OF ATRAZINE-TREATED HUMAN FIBROBLAST CELLS USING FLOW CYTOMETRY

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Andrea L. Austin

University of Northern Iowa

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ABSTRACT

Atrazine is the most frequently used triazine herbicide in the United States. Studies have shown an association between herbicide exposure and increased levels of DNA damage, reproductive and endocrine problems, and an increased risk for certain cancers. The United States Environmental Protection Agency (USEPA) has set a maximum allowable limit at 3 parts per billion (ppb). Previous work from our lab has shown decreased cell proliferation of normal human fibroblasts after low-level atrazine exposure for 72 hours at 0.8-100 ppb without a corresponding increase in apoptosis or necrosis.

The objective of the current study is to determine a mechanism for the observed decrease in cell number after atrazine exposure. We used flow cytometric analysis to see if treated cells progressed through the cell cycle differently than control cells and resulted in fewer cells after exposure. We hypothesized treated cells would take longer to cycle, so fewer cells would be present after a specific amount of time. Synchronized and unsynchronized normal human fibroblasts were exposed to increasing concentrations of atrazine (0-300 ppb) for 24, 32, or 48 hours and flow cytometric analysis was performed.

Results suggest a G1 block in atrazine-treated cells after 24, 32, and 48 hour exposure in both synchronized and unsynchronized cells. This block would increase the length of the cell cycle, thus supporting our hypothesis. Additionally, the number of apoptotic cells after treatment was comparable to control, again supporting previous growth study results.

This study by: Andrea L. Austin

Entitled: Cell cycle analysis of atrazine-treated human fibroblast cells using flow cytometry

has been approved as meeting the thesis requirement for the Designation University Honors.

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Date

Dr. Kavita R. Dhanwada, Honors Thesis Advisor

5/2/07
Date

Jessica Moon, Director, University Honors Program

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Date

Dr. Kavita R. Dhanwada, Chair, Thesis Committee

4/30/07
Date

Dr. Lisa Beltz, Member, Thesis Committee

5/1/07
Date

Dr. Barbara Hetrick, Head, Biology Department

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Background Review

In 2001, United States agricultural producers applied 553 million pounds of herbicides, with atrazine being the second most common (Kiely, 2004). Figure 1 shows a visual representation of the distribution of atrazine use in the United States.

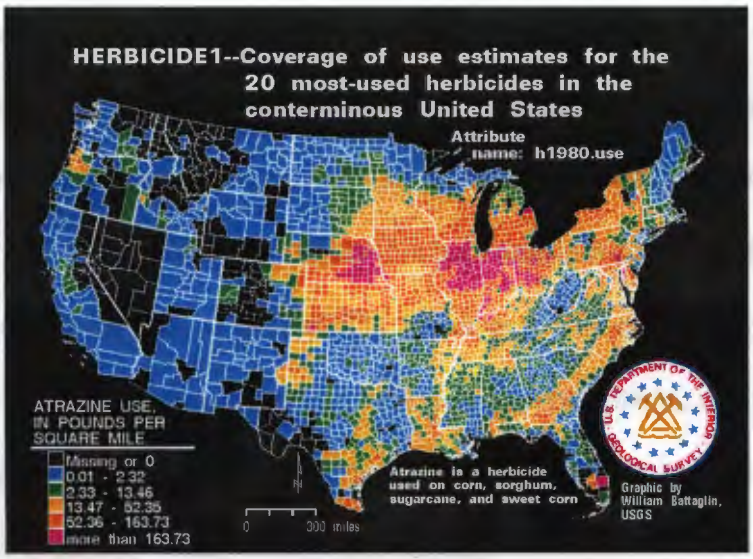


Figure 1: Atrazine use across the United States. (http://water.usgs.gov, 2007)

Atrazine (2-chloro-4-ethylamine-6isopropylamino-s-triazine) is classified as a Restricted Use Pesticide (Restricted use, 2003). It is a member of the chlorotriazine family, distinguished by the presence of a six-member ring containing three nitrogen atoms and three carbon atoms (Figure 2).

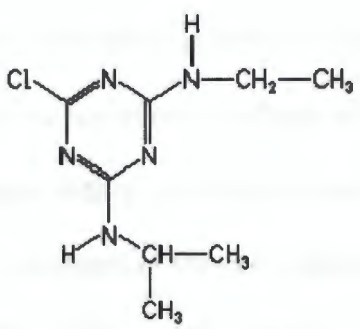


Figure 2: Chemical structure of atrazine. (http://plantandsoil.unl.edu, 2007)

Properties and target effects of atrazine

Atrazine has a half-life of approximately 35-125 days (Villanueva et al., 2005). Many factors influence the persistence and the breakdown of this herbicide including precipitation, temperature, soil pH, and soil microbial activity (Uhler, 1992). Atrazine affects target plants, broadleaf weeds, by inhibiting photosynthesis; it blocks the electron transport chain of photosystem II (Cherifi et al., 2001). It is used heavily on maize, sorghum, sugarcane, wheat, guava, and macadamia nut crops (Atrazine, 2003). However, these plants are resistant to atrazine due to the presence of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and enzymatic action glutathione S-transferase (GST), which promotes degradation of this herbicide (Cherifi et al., 2001). Although atrazine is very effective on target plants, it is unfortunately associated with many non-target effects in animals and humans, due to its mobility in soil and its stability in surface and ground water (Weber et al., 2006).

Non-target effects of atrazine

Several epidemiological studies have linked atrazine exposure with cancer, reproductive problems, and birth anomalies. Farm workers are frequently exposed to many herbicides, including atrazine. The state of California maintains a population-based cancer registry and the Department of Pesticide Regulation maintains records on the pounds of herbicide/pesticide applied annually in each county. A study by Mills (1998) showed that there was a positive correlation between leukemia, brain and testicular cancer and atrazine exposure among Hispanic males. Additionally, atrazine exposure positively correlated with prostate cancer in African American men (Mills, 1998). Women with previous exposure to triazine herbicides (including atrazine) showed a two-to threefold increase in the risk of developing epithelial ovarian cancer compared to unexposed women (Donna et al., 1989). An increased risk for stomach cancer was

seen when atrazine-containing drinking water was consumed at levels corresponding to 0.05-0.649 ppb (Van Leewen et al., 1999). An increase of one case of stomach cancer per 100,000 for each increase of 0.05 ppb of atrazine was seen. The atrazine levels in this study were *well below* the acceptable limits of Ontario, Canada of 60 ppb (Van Leewen et al., 1999).

Increased birth anomalies among pesticide applicators and also the general population were observed in areas of high pesticide application (Garry et al., 1996). All pesticides correlated with anomalies were registered as restricted-use pesticides, and included: alachlor, atrazine, bentazon, bromoxynil, cyanazine, dicamba, EPTC, imazethapyr, MCPA, metolachlor, and trifluralin. Birth anomalies were identified by the body system affected and included circulatory/respiratory, urogenital, and musculoskeletal/integumental anomalies. Births conceived from 1989-1992 were matched with the names of registered pesticide appliers, and required five identifying pieces of information (first, middle, last, birth date and county) matched to ensure that the father in the birth records matched a pesticide applicator. In regions with high pesticide use, there was a risk of 30/1,000 birth anomalies per live births of children born to pesticide appliers versus 23.7/1,000 for the general population of the same region. In non-crop regions, there was 23.7/1,000 for pesticide appliers' children and 18.3/1000 among the general population in the same region. The risk for birth anomalies was highest when conception occurred in the spring, coinciding with herbicide application. This study found a significant increase for all birth defects with atrazine use (Garry et al., 1996). When birth anomalies were limited to the central nervous system, circulatory/respiratory, urogenital, and musculoskeletal/integumental systems, the male/female sex ratio was 2.8 for appliers compared to 1.5 for the general population in areas of high chlorophenoxy herbicide/fungicide use, compared to 1.04-1.07 for normal births (Garry et al., 1996).

An increased risk of giving birth to babies with intrauterine growth retardation (IUGR) was found when drinking water was consumed from the Rathbun rural water system near Ottumwa, Iowa (Munger et al., 1997). IUGR is typically defined as birth weight below the 10^{th} percentile (Munger et al., 1997). Neonatal death is 40 times greater among those born with IUGR and is also associated with cerebral palsy, seizures, mental retardation, and lower respiratory infections (Kiely et al., 2005). The Rathbun reservoir, the source of drinking water, contained atrazine and other pesticide contaminants. It was found that the risk of IUGR was 1.8 times greater in communities with water supplied from Rathbun compared to the 38 communities within the same counties that obtained water from other sources (Munger et al., 1997). Prematurity was "marginally" less in the Rathbun communities than the control communities (Munger et al., 1997). Interestingly, the level of atrazine in the Rathbun rural water system was at a mean level of 2.1 ppb, below the Environmental Protection Agency's (EPA's) limit of 3 parts per billion (ppb). It also contained many other contaminants, but all were within allowable limits. This study controlled for confounding factors including maternal smoking and socioeconomic variables, factors that can also increase the risk for IUGR.

In a more recent study by Villanueva and colleagues (2005), it was shown that there was a slightly increased risk of small for gestational age (SGA) infants born to women that consumed atrazine-contaminated drinking water. Small for gestational age (SGA) is an equivalent term for IUGR. This increased risk occurred among infants whose gestational period was within any or all of the third trimester during the peak levels of atrazine, from May to September. Additionally, there was also "borderline" significance of prematurity when the first trimester occurred from May to September (Villaneuva et al., 2005). The geometric mean of the atrazine concentration in raw water for this peak period for this study was 0.052 ppb or micrograms/liter (μ/l) versus a

median value of 2.1 ppb or µg/l reported by Munger and colleagues (1997). However, this study had not controlled for tobacco smoke and socioeconomic variables as had been done in Munger's (1997) study. The premature condition of babies and SGA did not exhibit a dose-response to atrazine. Also, the authors caution that other factors, which require further research to identify, may produce a seasonal increase in prematurity and SGA.

Cellular effects

While epidemiological studies offer descriptive information, they do not explain the specific cellular effects. There have been several studies that have demonstrated changes in *in vivo* and *in vitro* models post-atrazine exposure. S-triazine compounds, including atrazine, were found to act as antiandrogens in *in vivo* and *in vitro* studies of the rat prostate (Kniewald et al., 1995). S-triazines (atrazine, prometryne and deethylatrazine) resulted in the inhibition of enzymatic activities necessary for the production of testosterone (Kniewald et al., 1995). Atrazine also acts as an endocrine disruptor by inducing aromatase production (Sanderson et al, 2001). Aromatase converts androgens to estrogen. A 2-to 2.5-fold increase in aromatase production was observed in an adrenocortical carcinoma cell line (H295R) after a 24 hour exposure of 30 microM (6,471 ppb) of the triazines, including atrazine (Sanderson et al, 2001). A study by Hayes and colleagues (2002) showed that *Xenopus laevis* larvae exposed to greater than or equal to 0.1 ppb of atrazine induced hermaphroditism and caused demasculinization of the larynges in exposed males. Testosterone levels were also 10 times lower in sexually mature *X. laevis* males exposed to 25 ppb of atrazine. The authors suggest that the changes in sex characteristics due to atrazine exposure may be partially responsible for the global decline in amphibian species (Hayes et al., 2002).

Another study found that rats fed atrazine at 100 and 200 mg/kg of body weight per day showed significant reduction in serum testosterone levels, luteinizing hormone levels, and the weight of androgen-dependent organs (Trentacoste et al., 2001). They also observed that the experimental group had a 9% decrease in body weight compared to control. However, when the experimental group was fed food to diminish the gap, the effects of food deprivation and atrazine exposure were similar. Thus, food deprivation in the experimental group may have contributed to the observed reproductive and endocrine effects (Trentacoste et al., 2001).

Another study also suggested that atrazine inhibits testosterone production in male rats (Friedmann, 2002). This study examined the effects of atrazine in both in *in vivo* and *in vitro* systems. Juvenile rats (22-48 days old) were exposed to 50 mg of atrazine/kg of body weight for either 3 or 27 days to measure the effects of acute versus chronic exposure, respectively. Both the acute and chronic exposure groups showed statistically significant reductions of approximately 50% in the serum and intratesticular testosterone levels compared to control. The reduction of the serum and intratesticular testosterone observed was greater than what would be expected by a reduction due to body weight alone as postulated by Trentacoste and colleagues (2001). For the *in vitro* experiments, Leydig cells were cultured and treated with 50 mg/l (50 part per million, ppm) of atrazine for 3 hours and a 35% reduction in testosterone production was detected compared to control (Friedmann, 2002). These cells are found in the interstitial tissue of the male testes and are primarily responsible for testosterone production.

Along with the endocrine effects, atrazine has been associated with increased levels of DNA damage. Flow karyotype analysis showed increased chromosome damage after atrazine exposure compared to caffeine (positive control) and control (Rayburn et al., 2001). This increase in damage was measured by a distortion of the largest chromosome peak, which was

statistically significant at 3, 30, and 300 ppb concentrations of atrazine for a 48 hour exposure, although a dose-response trend was not observed. The damage was similar at the three concentrations. However, a microscopic study by Kligerman et al. (2000) failed to show an increase in chromosomal damage in human lymphocytes exposed to atrazine. The flow karyotype analysis by Rayburn and colleagues (2001) examined approximately 2,200 hundred chromosome spreads per replicate, far more than were examined microscopically, thus the flow analysis was able to observe relatively the rare chromosomal breakage events.

Cell cycle changes within mussel haemocytes exposed to the herbicide 2, 4-dichlorophenoxy acetic acid (2, 4-D) were observed by flow cytometric analysis (Miĉić et al., 2004). 2,4-D is a member of the chlorophenoxy family of herbicides. The EPA classifies 2, 4-D as a Class D compound, meaning there is insufficient evidence for human carcinogenicity (EPA, 1997), however members of this family may cause chromosomal abnormalities and cell cycle delays. This *in vivo* study of mussels showed that harvested haemocytes underwent a G1 arrest after a 4 day exposure to 20 μg/g of 2, 4-D. In addition, sister chromatid exchanges (SCEs) were observed in cells exposed to 100 μg of 2,4-D/gram of body weight compared to an absence of SCEs in control samples, suggesting a mechanism of cellular DNA damage that could lead to changes in cell cycle patterns.

Many of the published studies of the cellular effects of atrazine exposure used high levels of atrazine, levels not usually found in the environment. Previous studies within our lab have shown that atrazine levels as low as 0.8 ppb result in decreased cell proliferation compared to control cells with no corresponding apoptosis or necrosis (Manske et al., 2004). This phenomenon of decreased cell proliferation may suggest an explanation for intrauterine growth retardation observed in the study of the women drinking water from the Rathbun Reservoir

(Munger et al., 1997). Importantly, the Dhanwada (2003) study used atrazine levels below the EPA limit of 3 ppb, as found in the Rathbun Reservoir. The goal of current study was to determine the biological mechanism underlying the decreased cell proliferation. Flow cytometric analysis was used to determine whether atrazine-treated cells progressed through the cell cycle differently than control cells.

Flow cytometry

Flow cytometry has many uses and can be most widely defined as the flow of particles (in our case, cells) in liquid through a beam of light. Flow cytometers have a light source within an optical bench to stabilize the light beam. The cytometer uses a laser to emit light at a specified wavelength. The laser intersects perpendicularly with the liquid stream. This light illuminates the stained particles and emits fluorescent light, which is then converted into an electrical impulse (Givan, 1992). This impulse is then quantitated and analyzed by computer analysis.

Photodetectors surround the stream and are at right angles to the light beam. The angle of these photodetectors is significant, because if nothing deflects the light, no signal will be produced. The side scatter (SSC) detector is positioned at a right angle to the light beam. The intensity of light scattered by the SSC detector indicates cellular granularity (Givan, 1992). The forward scatter (FSC) detector beam provides information on cell size and is positioned directly across from the illumination.

There are many applications of flow cytometric analysis. Examining the DNA content of cells is useful for determining the cell cycle progression of cellular populations. Cells go through a growth cycle that consists of the phases G_1, S, G_2 and M. During the G_1 phase, the cells have a DNA content referred to as 2C DNA. During the S phase, the cell replicates its DNA; the DNA content is referred to as having between 2C-4C. DNA replication is completed by the beginning

of G_2 phase, therefore the cell has 4C DNA content, double the DNA content in the G_1 phase. Cell division occurs during mitosis (M phase) and when completed, two cells are produced each once again containing 2C DNA.

The most commonly used dye to stain DNA is propidium iodide. This dye intercalates between stacked bases in DNA and the double-stranded regions RNA. Thus, RNase must be added to cells prior to staining them to measure DNA content. The cells are permeabilized with either ethanol or a detergent that allows the propidium iodide to enter into the cell. This dye absorbs light at 488 nm and emits fluorescence above 600 nm. The intensity of fluorescence is dependent on the amount of DNA that it binds (Givan, 1992).

Cells have several checkpoints within the cell cycle, in which they can pause to repair DNA damage or wait for proper signals before transitioning to the next phase (Figure 3).

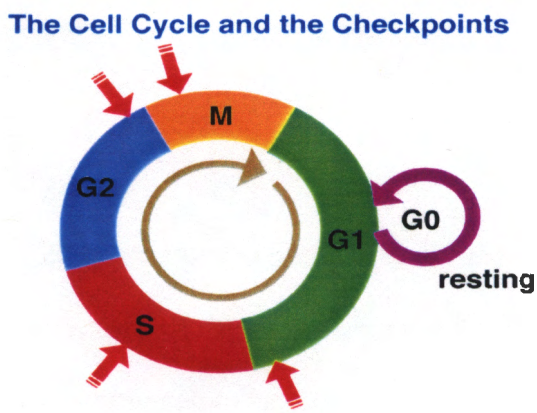


Figure 3: The cell cycle
homepage.mac.com/enognog/checkpoint.htm (2006).

Of these checkpoints, the G_1 checkpoint is the most important within eukaryotic cells (Alberts et al., 1994). Once cells progress beyond this point, they have committed substantial resources to dividing.

We were interested in determining the biological mechanism underlying decreased cell proliferation in atrazine-treated cells. Manske and colleagues (2004) showed that there was no increased DNA fragmentation or increased caspase activity, two indicators of apoptosis, in atrazine-exposed cells compared to control. This suggested that decreased cell proliferation observed in atrazine-treated cells was likely due to the cells progressing more slowly or pausing in the cell cycle. We hypothesized that cells treated with low to moderate levels of atrazine undergo a G_1 block, resulting in decreased cell proliferation compared to control cells. Flow cytometric analysis will allow us to determine a mechanism for this decrease, evident by an accumulation of cells in a particular phase of the cell cycle in atrazine-treated cells compared to control. Additionally, flow cytometric analysis should be more sensitive to apoptosis than the previously used microscopic examination for DNA fragmentation and measurements of caspase activity.

Materials and Methods

Cells

Normal human fibroblasts, DET 551 (American Type Culture Collection) were grown in Eagle's Minimum Essential medium (EMEM) with Earle's Balanced Salt Solution (EBSS) and 2 mM L-glutamine (Gibco) supplemented with 1.0 mM sodium pyruvate (Gibco), 0.1 mM nonessential amino acids (Gibco), 1.5 g/L sodium bicarbonate, 10% fetal bovine serum (FBS) (Hyclone), and 1% penicillin-streptomycin, pH 7.3 (Gibco). Cells were maintained at 37°C with 5% CO_2. Cells were passed every 4-7 days when they were 80-90% confluent. Prior to splitting, medium was aspirated and cells were washed with calcium-magnesium-free 1X phosphate buffered saline (CMF-PBS) (Bio Whittaker). DET 551 cells are an adherent cell line, and required Trypsin-EDTA (O.25%) (Gibco) to dislodge the cells from the plate. Once the cells were dislodged, 10% serum-containing medium was added to stop the trypsin action. Cells were resuspended by pipetting. A 1:4 dilution of the cell suspension was removed and added to new 100 mm tissue culture plates.

Atrazine exposure of DET 551 cells

Approximately 1 x 10^6 cells were plated per experimental 100 mm dish. Within 24 hours of passage, the medium was aspirated and atrazine-containing medium was added. The atrazine medium was made from a stock solution and appropriate dilutions were made and added to each dish. The dilutions used were 0 parts per billion (ppb)- the control, 3 ppb, 100 ppb, and 300 ppb. The dishes were returned to the 37°C with 5% CO_2 incubator for 24, 32, 48, or 96 hours.

Atrazine quantitation

Atrazine (Chem Services, Westchester, PA) was dissolved in serum-and-antibiotic free media. The stock solution was quantitated by liquid chromatography-mass spectroscopy (LC-MS) (Agilent 1100 Series). Atrazine standards were dissolved in acetonitrile and quantitated on the LC-MS with a C8 column (60% acetonitrile and 40% water). Atrazine standards of 2.5, 5, 7.5, 10, 12.5, 15, and 20 part per million (ppm or mg/L) were used to generate a standard curve . A linear regression line was generated and used to determine the concentration of the experimental atrazine solution.

Cell synchronization

To determine the effects of cell synchronization prior to atrazine exposure, DET 551, cells were serum starved for 48 hours prior to the addition of atrazine. Low serum media deprives the cells of needed growth signals, thus they remain in the G_0 sub-phase of the cell cycle. Both 1% and 0.5% serum-containing media were evaluated for cell synchronization. After 48 hours, atrazine containing media was added for 24, 32, 48, and 72 hours.

Harvesting of cells

To harvest cells, media were aspirated, followed by a PBS wash. One ml of trypsin was added to each plate. Plates were incubated at room temperature for 3-5 minutes. Once cells were dislodged, 3 ml of 10% serum-containing media were added to stop the action of the trypsin. Cells were resuspended and contents were transferred to conical tubes and centrifuged at 2000 rpm for seven minutes. Media were aspirated, leaving the cell pellet. One milliliter of PBS was added to resuspend cells. Cells were centrifuged at 500 x g for 5 minutes. PBS was removed carefully to avoid disturbing the pellet. The Cycle Test kit (BD Biosciences) was used subsequently with one minor change: 0.5 ml of the buffer was used to wash cells instead of 1 mL

and 0.5 mL of buffer was used to resuspend the cells prior to counting. Cell concentrations of at least 500,000 cells/ml were obtained. Samples were then analyzed or stored at -80°C.

An alternative procedure was also tested (Flemington, 2006) that involved ethanol permealization. In this method, cells were dislodged and centrifuged in the same manner as previously described. After the serum containing media was aspirated, cells were resuspended in 500 µl of 1X PBS containing 0.1% glucose. Five milliliters of cold 70% ETOH were immediately added and sample was vortexed. Cells were stored at 4°C for 1 hour to one week to fix cells prior to flow cytometric analysis (Flemington, 2006).

Sample preparation.

Samples stored in CycleTest Buffer solution were prepared exactly according to the protocol described in the CycleTEST Plus kit (BD Biosciences).

For cells permeabilized with ethanol, an alternative preparation method was used. Two to three hours prior to flow cytometric analysis, cells were centrifuged at 400 x g for 5 minutes and the liquid was removed. Cells were resuspended in 5 ml of 1X PBS and centrifuged for 5 minutes at 400 x g. The PBS was removed and cells were centrifuged for an additional 2 minutes at 400 x g to remove any residual PBS. Twenty microliters RNase (10 mg/ml) was added to the cells and samples were incubated at 37°C for 15 minutes. Five hundred microliters of propidium iodide (PI) solution (69 µM PI in 38 mM sodium citrate, pH 7.4) was added and the sample was incubated for an additional 30 minutes at 37°C.

For both procedures, samples were filtered with 700 µm nylon mesh (Spectrum Laboratories), light-protected, and stored on ice prior to flow cytometric analysis. Samples were run within 3 hours of the addition of PI.

Flow cytometric analysis.

Quality control of the flow cytometer was performed with DNA QC Particles (BD Biosciences) each time prior to analysis. Three sets of particles are used for quality control. Chicken erythrocyte nuclei (CEN) are ethanol-fixed nuclei from chicken blood. Chick erythrocytes have nuclei, while human erythrocytes do not. This preparation produces single nuclei, doublets, triplets, and larger aggregates. The particles allow for examination of instrument linearity and resolution. This allows for setting the instrument photomultiplier tube (PMT) voltages and amplifier gains. When stained with propidium iodide, the doublet, triplet peaks should have channel values 2 and 3 times greater, respectively, than the singlet peak. The settings obtained were used to run the samples.

Calf thymocyte nuclei (CTN) are fixed in formaldehyde. CTN provide a control for normal DNA levels for all stages of the cell cycle. Most of the nuclei are in the G_0/G_1 phase, and fewer are in the S and G_2 and M phases. There are also doublets of the G_0/G_1 nuclei and these can be distinguished from the cells in the G_2 and M singlets by pulse processing which allows for better cell cycle estimates of the percentage of cells in the G_2 and M phases.

Lastly, 2 μm sized beads were used to verify cytometer alignment. They naturally emit fluorescence (without the addition of exogenous PI) in both FL1 and FL2 channels. This allows for verification independent of the stain or sample preparation. BD Biosciences recommended use of the beads if appropriate settings weren not attainable for CEN and CTN samples.

The following settings were after following quality control analysis for all experiments (Table 1).

Table 1: Flow cytometer detector and amps settings

Parameter	Detector	Voltage	AmpGain	Mode
P1	FSC	E00	1.00	Lin
P2	SSC	350	1.00	Lin
P3	FL1	600	1.00	Lin
P4	FL2	448-450	1.00	Lin
P5	FL3	650	1.00	Lin
P6	FL2-A		1.00	Lin
P7	FL2-W		4.00-4.45	Lin

The threshold parameter was always the forward scatter (FSC) with a value of 52. The Compensation Value was 0.0% for all detectors. 20,000 events were analyzed per sample and analyzed with ModFit DNA Analysis software (Verity Software House). Automatic linearity function was enabled.

Statistical Analysis

Data obtained from with the ModFit DNA Analysis software was entered into a Microsoft Excel spreadsheet. The averages and standard deviations were calculated for each test condition. Each experiment was repeated at least 3 times and averages were calculated. Standard deviation, standard error, and student T-Test analyses were determined from each set of cell cycle experiments. The student T-Test was two-tailed and compared samples of varying atrazine levels to control. Samples with p values less than 0.05 were found to be statistically significantly different from control.

Results

To determine if cell cycle alterations occurred in atrazine exposed cells compared to unexposed cells, flow cytometric analysis was performed. We attempted to determine if there was an accumulation of cells in a specific phase of the cell cycle in atrazine-treated cells compared to control cells.

Atrazine quantitation

LC-MS chromatography was performed with prepared concentrations of pure atrazine. A linear regression curve was determined (Figure 4) and the concentration of stock atrazine solution was determined to be 6.96 ppm. This process was repeated for later experiments and the stock concentration was determined to be 6.31 ppm (Figure 5). These stock solutions were used to make all dilutions used during the experiments.

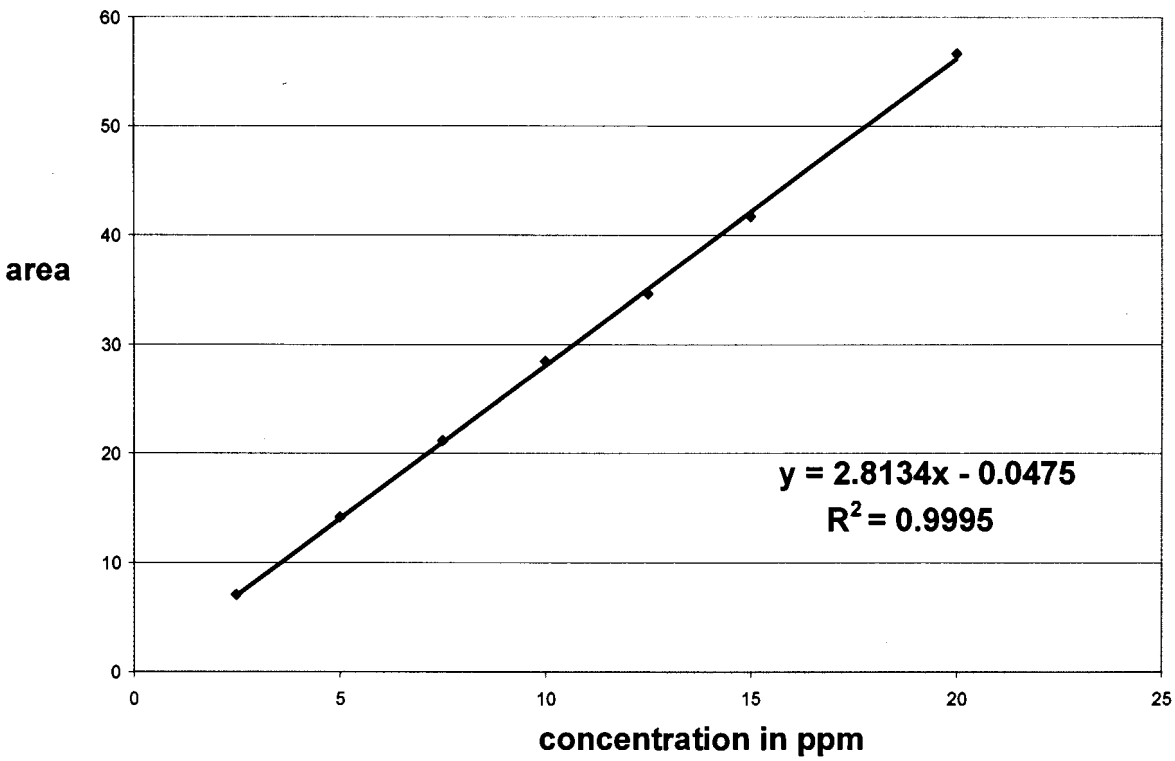


Figure 4: Atrazine standard curve. The concentration of atrazine was found to be 6.96 ppm.

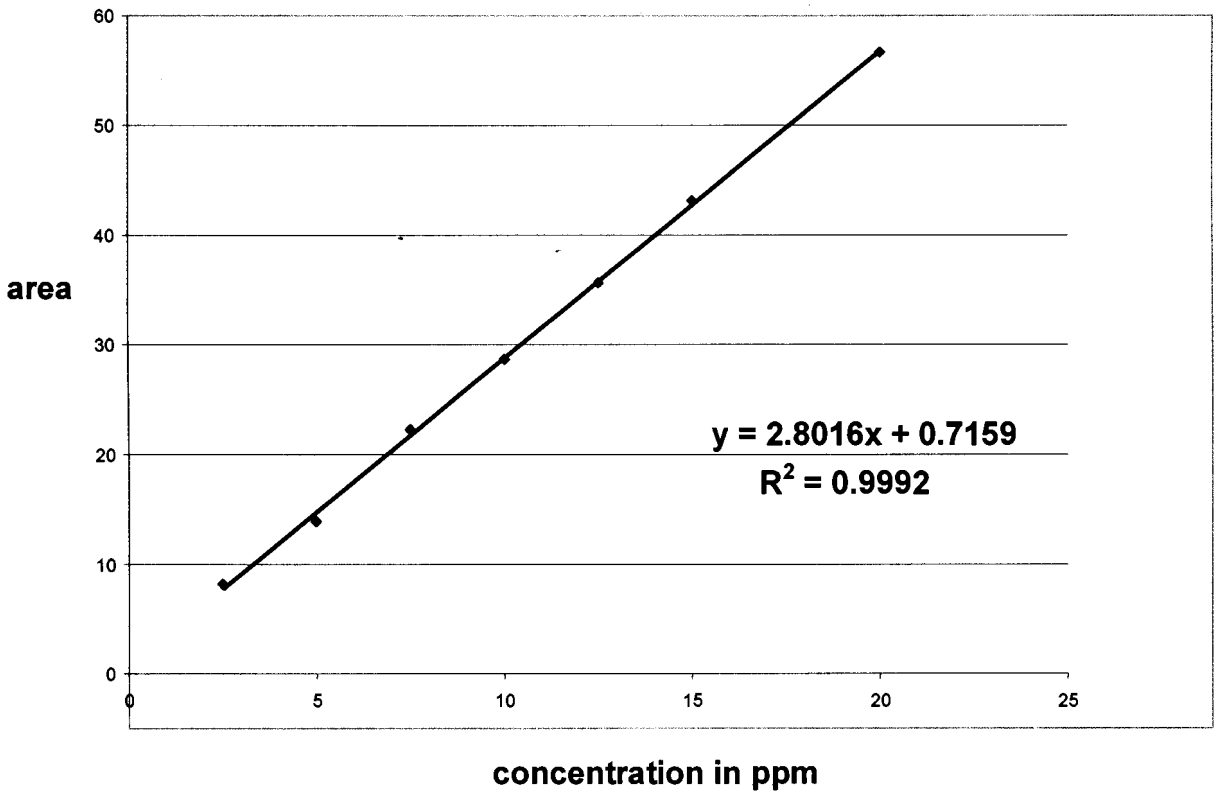
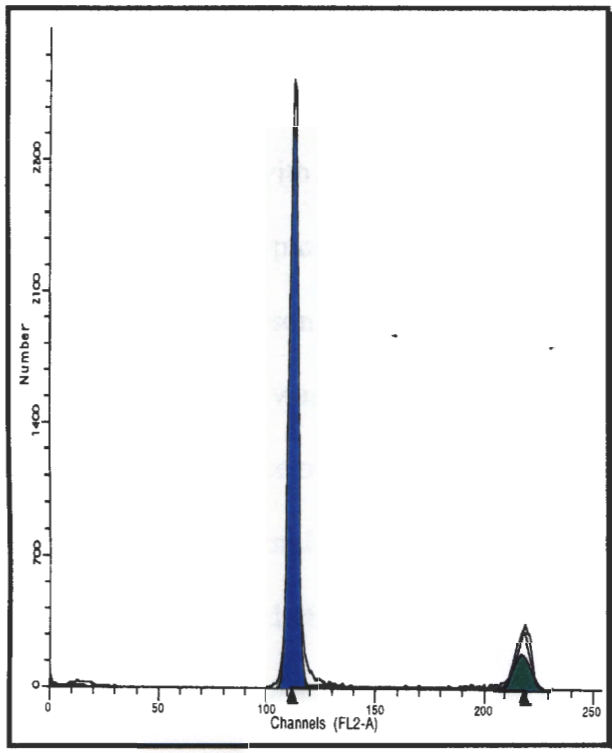


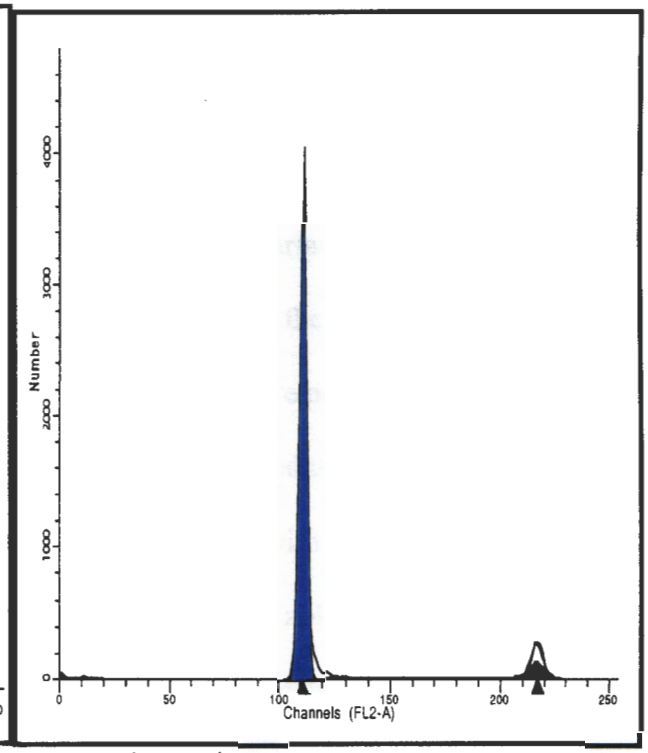
Figure 5: Atrazine standard curve. The concentration was found to be 6.31 ppm.

Representative ModFit histograms of flow cytometric analysis

To determine the DNA content within cells, cells were stained with propidium iodide. Flow cytometric analysis was performed and histograms were further analyzed with the ModFit DNA analysis software. Representative flow cytometric histograms are below (Figure 6).



6A: 0 ppb (control)



6B: 3 ppb atrazine

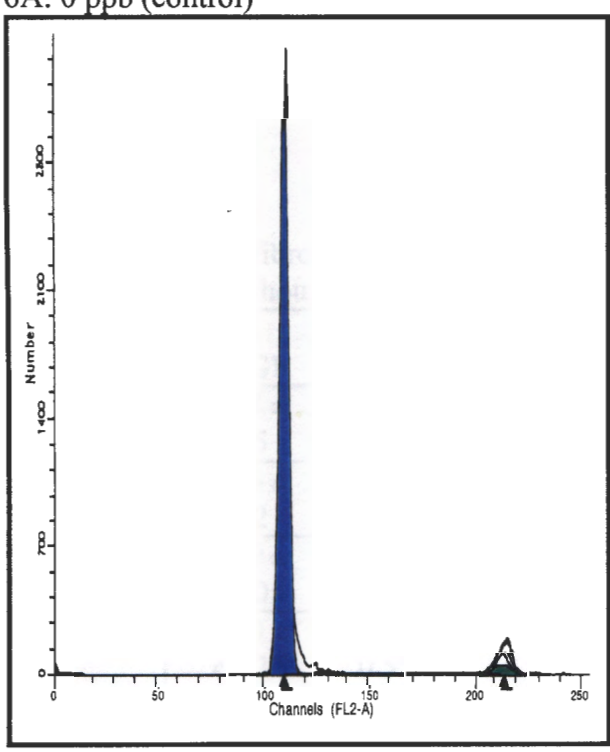
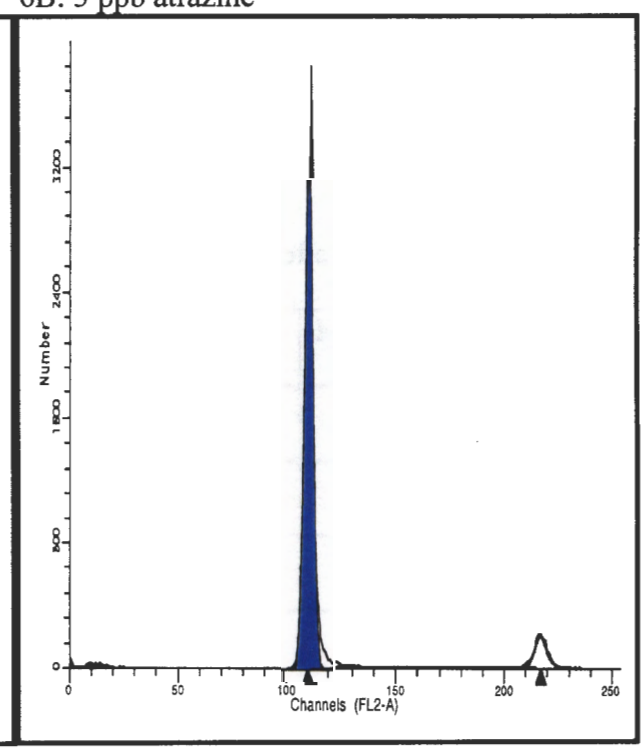


Figure 6C: 100 ppb atrazine



6D: 300 ppb atrazine

Figure 6: Histograms from ModFit DNA Analysis of unsynchronized cells exposed to atrazine for 48 hours. Representative histograms of control (A) and atrazine treated cells (B-D). Blue and green peaks correspond to the G0/G1 and G2/M portions of the cell cycle respectively. S phase have an intermittent amount of DNA and are found in between the G0/G1 and G2/M peaks.

Cell cycle analysis of cells without synchronization

The percentage of cells in each phase of the cell cycle (G_0/G_1, G_2/M, and S) was determined. Along with percentages of cells in specific phases of the cell cycle, the percent of cells undergoing apoptosis was also examined. The coefficient of variation was found for each sample. For comparison between samples, the ratio of cells in G_1 of the experimental samples compared to control was also calculated. Lastly, Student T-tests were performed to determine statistical significance of results. Values less than 0.05 are considered to be statistically significant. An accumulation of cells in the G_0/ G_1 phase was seen with increasing concentration of atrazine for 24 and 48 hours with no prior cell synchronization (Tables 2 and 3). Atrazine concentrations of 100 and 300 ppb showed statistically significant accumulation of cells in the G_1 phase compared to control at 24 hours (Table 2), while all three concentrations of atrazine gave statistically significant accumulations of cells in G_1 after a 48 hour atrazine exposure (Table 3).

Table 2: DET 551 fibroblasts exposed to increasing concentrations of atrazine for 24 hours without synchronization. n=3.

Atrazine conc.	% G0/G1	% G2 & M	% S	% apoptosis	% CV	Ratio of cells in G1 to control	T-test
0 ppb	50.63 ± 5.35	20.09 ± 3.27	29.28 ± 2.17	0.17 ± 0.22	2.34 ± 0.42	1.00	
3 ppb	58.14 ± 4.72	15.90 ± 1.63	25.96± 3.22	0.58 ± 0.65	2.29 ± 0.23	1.16 ± 0.19	0.22
100 ppb	61.19 ± 3.70	14.50 ± 0.35	24.30 ± 3.50	0.73± 0.46	2.23 ± 0.38	1.21 ± 0.097	0.018
300 ppb	66.70 ± 4.02	13.22± 0.59	20.09 ± 3.63	0.47 ± 0.44	2.11 ± 0.35	1.32 ± 0.064	0.00096

Table 3: DET 551 fibroblasts exposed to increasing concentrations of atrazine for 48 hours without synchronization. n=3

Atrazine conc.	% G0/G1	% G2 & M	% S	% apoptosis	% CV	Ratio of cells in G1 to control	T-test
0 ppb	78.97 ± 9.94	9.82 ± 4.51	11.21 ± 6.04	2.88 ± 2.12	1.82 ±0.36	1.00	
3 ppb	83.08 ± 10.25	8.52 ± 3.80	8.40 ± 6.46	0.62 ± 0.62	1.69 ± 0.42	1.053 ± 0.029	0.033
100 ppb	85.27 ± 11.32	6.50 ± 5.28	8.23 ± 6.03	0.038 ± 0.35	1.95 ± 0.15	1.079 ± 0.0026	0.00060
300 ppb	87.35 ± 13.44	4.83 ± 7.83	7.82 ± 5.61	1.35 ± 1.10	1.81 ± 0.29	1.103 ± 0.044	0.015

Cell cycle analysis of cells with synchronization

To determine the serum concentration necessary for synchronization of DET 551 cells, cells were analyzed after 48 hours of growth in varying levels of serum-containing media. Medium containing 0.5% serum resulted in the highest level of synchronization, with over 90% of the cells in G_0/G_1 (Table 4). Thus, in subsequent experiments involving cell synchronization, cells were plated in 0.5% serum containing media for 48 hours.

Table 4: Cell cycle analysis of cells grown in varying concentrations of serum, n=1

Serum concentration (no atrazine)	% G0/G1	% G2 & M	% S	% CV
10%	44.44	22.45	33.11	1.66
1%	72.97	10.26	16.77	1.82
0.5%	90.39	4.79	4.82	4.9

After cell synchronization, cells were exposed to increasing levels of atrazine for 24 or 48 hours. The accumulation of cells in G_0/G_1 phase was observed in the *48 hours* of exposure post cell synchronization and was statistically significant for the 100 and 300 ppb treatments (Table 5).

Table 5: DET 551 fibroblasts exposed to increasing concentrations of atrazine for 48 hours after cell synchronization. n=3.

Atrazine conc.	% G0/G1	% G2 & M	% S	% apoptosis	% CV	Ratio of cells in G1 to control	T-test
0 ppb	72.77 ± 2.75	8.60 ± 0.97	18.63 ± 1.80	3.50± 3.45	3.13 ± 0.84	1.00	
3 ppb	76.42 ± 0.83	7.29 ± 0.48	16.30 ± 0.37	4.27 ± 3.14	2.82 ± 0.52	1.051 ± 0.039	0.088
100 ppb	78.79 ± 0.31	6.93± 0.74	14.27 ± 1.00	3.57 ± 2.90	2.65 ± 0.35	1.084 ± 0.046	0.034
300 ppb	77.82 ± 3.08	6.63 ± 1.04	15.54 ± 2.05	6.03 ± 3.37	2.70 ± 0.23	1.0697 ± 0.026	0.0098

No trend in an accumulation of cells in the G_0/G_1 phase was observed in cells exposed to atrazine for 24 hours post-synchronization. The ratio of cells in G_1 was essentially the same as control (Table 6).

Table 6: DET 551 fibroblasts exposed to increasing concentrations of atrazine for 24 hours after cell synchronization. n=3.

Atrazine conc.	% G0/G1	% G2 & M	% S	% apoptosis	% CV	Ratio of cells in G1 to control	T-test
0 ppb	60.29 ± 2.67	17.31 ± 1.98	22.41 ± 0.74	1.31 ± 2.26	2.47 ± 0.45	1.00	
3 ppb	54.15 ± 10.0	21.98 ± 7.12	23.87 ± 2.88	1.65 ± 1.02	2.35 ± 0.48	0.90 ± 0.15	0.31
100 ppb	59.95 ± 3.41	18.24 ± 1.79	21.81 ± 1.88	0.75± 0.73	2.55 ± 0.09	1.00 ± 0.08	0.94
300 ppb	60.57 ± 2.95	16.07 ± 1.94	23.37 ± 2.42	0.69 ± 0.78	2.40 ± 0.10	1.01 ± 0.06	0.85

With no increase in the number of cells in G_0/G_1 after a 24 hour atrazine exposure post-synchronization, cells were exposed for 32 hours post-synchronization to allow 8 hours for cells to acclimate to 10% serum-containing medium once again. Table 7 shows that an increasing number of cells were observed in the G_0/G_1 phase with atrazine concentrations of 3 and 100 ppb. While there was a drop in the percentage of accumulated cells in G_1 from 100 ppb to 300 pbb,

the number of accumulated cells in G_0/G_1 for 300 ppb is statistically indistinguishable from control (Table 7).

Table 7: DET 551 fibroblasts exposed to increasing concentrations of atrazine for 32 hours after cell synchronization. n=1.

Atrazine conc.	% G0/G1	% G2 & M	% S	% apoptosis	% CV	Ratio of cells in G1 to control
0 ppb	61.84	10.41	27.75	3.19	3.23	1.00
3 ppb	67.53	5.49	26.98	7.18	2.35	1.09
100 ppb	70.43	9	20.57	5.78	2.19	1.14
300 ppb	62.31	12.3	25.39	3.82	2.19	1.01

The effects of increased cell age and atrazine exposure longer than 48 hours

Many initial experiments utilized passage 19 cells. There was no accumulation of cells in the G_0/G_1 phase when passage 19 cells were exposed to atrazine for 48 hours (Table 8).

Table 8: Passage 19 DET 551 fibroblasts exposed to increasing concentrations of atrazine for 48 hours without cell synchronization. n=2

Atrazine conc.	% G0/G1	% G2 & M	% S	% apoptosis	% CV	Ratio of cells in G1 to control	T-test
0 ppb	85.97 ± 1.39	3.20 ± 3.52	10.83 ± 2.14	1.18 ± 0.21	1.75 ± 0.099	1.00	
3 ppb	86.17 ± 1.82	2.26 ± 3.20	11.57 ± 1.37	0 ± 0	1.81 ± 0.31	1.00 ± 0.0049	0.55
100 ppb	83.77 ± 2.55	2.77 ± 2.62	13.48 ± 0.078	0.025 ± 0.05	2.17 ± 0.21	0.97 ± 0.014	0.12

The accumulation of cells in G_0/G_1 showed no difference in cells exposed to atrazine for 96 hours compared to control. Also, there was a slight decrease in the accumulation from 3 ppb to 100 ppb, but the number of cells in G_0/G_1 was around 90% even in control (Table 9).

Table 9: Passage 17 DET 551 fibroblasts exposed to increasing concentrations of atrazine for 96 hours without cell synchronization.n=1

Atrazine conc.	% G0/G1	% G2 & M	% S	% apoptosis	% CV	Ratio of cells in G1 to control
0 ppb	91.42	4.89	3.69	0.48	1.56	1.00
3 ppb	93.51	2.33	4.16	0.26	1.39	1.02
100 ppb	92.97	3.2	3.83	0.02	1.69	1.02

Ethanol permeabilization protocol

In an alternative protocol for cell fixation, cells were permeabilized with ethanol. There was no observed trend in the accumulation of cells in the G_0/G_1 phase for cells exposed to atrazine for 24 and 48 hours without prior synchronization with the ethanol permeabilization (Table 10 and 11). The aggregates were substantially higher with this protocol than with the Cycle Test kit. The average percent of aggregates for all of the Cycle Test trials was 2.868 ± 2.046 (n=84) (data not shown) compared to an average of 15.268 ± 6.21 (n=15) for all of the ethanol permeabilized trials (Table 10 and 11). The data was statistically significant at 300 ppb for the 24 hour exposure.

Table 10: DET 551 fibroblasts exposed to increasing concentrations of atrazine for 24 hours without synchronization, ethanol permeabilization. n=2

Atrazine conc.	% G0/G1	% G2 & M	% S	% apoptosis	% CV	Ratio of cells in G1 to control	T-test	Aggregates
0 ppb	62.85 ± 7.33	16.05 ± 5.89	21.11 ± 1.44	1.06 ± 0.47	6.185 ± 1.56			18.23 ± 2.90
3 ppb	62.23 ± 7.25	13.27 ± 4.35	24.51 ± 2.91	0.0005 ± 0.0071	5.18 ± 2.08	0.99 ± 0	0.00	17.65 ± 2.57
300 ppb	66.96 ± 7.93	11.36 ± 2.93	21.69 ± 5.00	0.22 ± 0.24	6.11 ± 0.86	1.07 ± 0.0071	0.006	18.88 ± 7.20

Table 11: DET 551 fibroblasts exposed to increasing concentrations of atrazine for 48 hours without synchronization, ethanol permeabilization. n=2

Atrazine conc.	% G0/G1	% G2 & M	% S	% apoptosis	% CV	Ratio of cells in G1 to control	T-test	Aggregates
0 ppb	80.39 ± 2.20	4.46 ± 0.45	15.16 ± 1.75	0 ± 0	4.65 ± 0.29	1.00		10.58 ± 3.84
3 ppb	79.97 ± 1.39	5.49 ± 0.21	14.54 ± 1.60	0 ± 0	5.55 ± 0.42	1.00 ± 0.0071	0.42	9.16 ± 4.24
100 ppb	82.24 ± 1.47	4.4 ± 2.58	13.37 ± 1.10	0 ± 0	5.94 ± 0.28	1.025 ± 0.49	0.55	14.11 ± 10.57
300 ppb	77.98 ± 6.47	6.06 ± 0.91	15.96 ± 5.56	0.14 ± 0.20	4.84 ± 0.19	0.97 ± 0.057	0.53	14.32 ± 9.36

Discussion

Many studies have demonstrated negative, non-target effects of herbicides, including atrazine, on human health. Atrazine is the second most widely applied herbicide in the United States, and is frequently found as a ground water contaminant (Kiely, 2004). The goal of this study was to determine if atrazine-exposed DET 551 cells progress through the cell cycle differently than control cells.

Previous studies from our lab showed that DET 551 cells exposed to low doses of atrazine showed decreased cell proliferation compared to control cells. No DNA fragmentation or increased caspase activity was observed in atrazine-treated cells compared to control (Manske et al., 2004). The current study attempted to determine if the observed decrease in cell proliferation in atrazine-treated cells was due to cells progressing more slowly through the cell cycle.

Results showed that there was an accumulation of cells in the G_0/G_1 phase of the cell cycle in cells exposed to atrazine for 24 hour-unsynchronized (Table 2) and 48 hour-unsynchronized and synchronized (Table 3 and 5) and 32 hour-synchronized (Table 7) at 3, 100, and 300 ppb compared to control cells not exposed to atrazine. This accumulation was statistically significant at 100 ppb and 300 ppb in the 24 hour (unsynchronized) and the 48 hour (synchronized and unsynchronized) exposures. The 3 ppb treatment also produced a statistically significant accumulation in the 48 hour unsynchronized exposure. Passage 19 cells did not have an accumulation of cells in any phase of the cell cycle. They were not used in further studies since they had very high levels of cells in the G_0/G_1 phase, even in the control cells (Table 8). Nor was an accumulation of cells observed in samples prepared via ethanol permeabilization.

Additionally, the percent of aggregates in the ethanol permeabilized samples was very high (Table 10 and 11).

Thus, results showed an accumulation of cells in the G_0/G_1 phase with increasing atrazine concentration, suggesting a G_1 block of the cell cycle. This block seemed to occur irregardless of cells being exposed to atrazine for 48 hours (Tables 2 and 3) or 24 hours (Table 1); a general increase in cell number in the G_0/G_1 phase was seen. We had hypothesized that the effects of the atrazine would be more evident if the majority of cells started at the same point of the cell cycle. To determine if synchronization was important, cells were first incubated in low serum (0.5% FBS) medium for 48 hours prior to atrazine exposure. As seen in Table 5, cells accumulate in the G_0/G_1 phase, but with less variation compared to unsynchronized conditions (Table 2). However, the greatest accumulation of cells in the G_0/G_1 phase occurred when unsynchronized cells were exposed to atrazine for 24 hours (Table 2). There was a 6% increase in G_0/G_1 cells after a 3 ppb atrazine exposure and more accumulation in G_0/G_1 with increasing concentrations of atrazine, suggesting a dose-response effect. Student T-tests were performed and $p < 0.05$ represented statistically significant results. We observed that the 3 ppb atrazine treatment, the current EPA limit, produced statistically significant results in the 48 hour unsynchronized experiment with a p value of 0.033.The higher concentrations of atrazine also provided statistically significant results for atrazine exposures of 24 and 48 hours in unsynchronized cells. We expect increasing the number of experiments performed, especially at the lower doses of atrazine exposure, would produce statistically significant results at these levels of atrazine.

The deviation between samples was highest among the 48 hour unsynchronized cells. We hypothesize that after 48 hours, there is a build-up of individual cells within the population with varied cell cycle progression compared to the majority of cells. Results show 24 hour

synchronized cells produced inconsistent results in their ability to cause a G_1 block (Table 6). It may be that the cells had insufficient time to emerge from the starvation period. Thus, we extended the exposure time to 32 hours to allow cells time to emerge from the starvation-induced G_0 block and acclimate to the media, and a G_0/G_1 accumulation occurred (Table 7). Alternatively, post-synchronization, we could replace media with 10% serum-containing media, and then replace with atrazine-containing media for 24 hours.

Atrazine exposure has been correlated with cancer (Mills, 1998, Donna et al., 1989, Van Leewen et al., 1999). However, in our studies, we did not have the conditions that have been used by many scientists in the cancer studies. The study by Mills (1998) and also the study by Donna and colleagues (1989) found increased numbers of cancers in pesticide applicators and these individuals were likely exposed to higher doses of herbicides than the low and moderate levels used in our studies. Accelerated growth of cells often occurs in several types of cancer, but this was not observed in these experiments. In fact, cells progressed more slowly through the cell cycle, resulting in fewer cells than control. Additionally, we looked at the short-term effects of atrazine for 24-48 hours, and it may be that long-term exposure of atrazine results in DNA damage that causes mutations, leading to cancer.

This current study suggests a mechanism for the decreased cell proliferation of atrazine-exposed cells. This mechanism may be the biological mechanism responsible for the increased risk of IUGR in babies born to mothers that consumed herbicide-contaminated water (Munger et al., 1997). Fetal growth is due to rapidly dividing cells, and if there was a slow down in cellular division, it would be magnified during intense growth period of fetal development.

It is not known how atrazine affects cells to cause a G_1 block in DET 551 cells. Rayburn and colleagues (2001) showed increased DNA damage in atrazine-exposed cells, including at 3

ppb. We would like to measure DNA damage in the DET 551 cells to determine if increased DNA damage, perhaps measured by the Comet assay, correlates with alterations in cell cycle progression.

Initially, passage 16 cells were often thawed, and cells were in passage 19 when ready for an experiment. DET 551 cells are normal human fibroblast cells. Normal cells have a finite life span. In our lab, the DET 551 cells were typically not used for experiments past passage 22 because the cells were not dividing, which could be observed by microscopic examination. The flow cytometric analysis of the passage 19 cells showed that the control samples had an average of nearly 86% of cells in the G_0/G_1 phase (Table 8). Excluding the 96 hour (Table 9) and ethanol permeabilization experiments (Table 10 & 11), the percent of cells in G_0/G_1 phase for control ranged from 50.63-78.87%, much lower than 86%. This suggests that the passage 19 cells may already be approaching senescence, and thus not ideal for observing cell cycle changes in normal, dividing cells. This also highlights that flow cytometric analysis shows a phenomenon, cellular senescence, which had not been observed microscopically.

Cells that were exposed to atrazine for 96 hours had over 91% of the control cells in the G_0/G_1 phase (Table 9). While cell age was not triggering senescence, the high confluence triggered quiescence due to contact inhibition. This experiment highlights the importance of plating the proper number of cells to avoid high confluence, which will obscure cell cycle changes.

The Cycle Test kit utilizes a trypsin-containing solution to permeate the cell's membrane, allowing the propidium iodide to stain the DNA. Ethanol permeabilization is less expensive than the Cycle Test kit. This method was attempted, but resulted in roughly five times the level of aggregates, or clumping, compared to samples prepared with the Cycle Test kit (Tables 10 &

11). The accumulation of cells in the G_0/G_1 phase was not observed in samples prepared with the ethanol permeabilization. This was most likely due to aggregates obscuring the actual results.

Future work

Our study suggests that low to moderate atrazine exposure causes a G_0/G_1 block in normal, human fibroblast cells. Statistically significant reductions in growth occurred in cells exposed to atrazine at 3 ppb for 48 hours without synchronization. Further studies should evaluate if cells exposed to levels below the EPA limit also produce a reduction in cell growth. In our experimental system, cells were exposed to atrazine for 24 - 48 hours. When people are exposed to atrazine through drinking water, the exposure is long-term. Thus, the effects of atrazine, such as decreased cell growth and slower cell cycle progression, are likely to be magnified with long-term exposure. It may be quite important to study the long-term effects of atrazine as well as concentrations below the EPA limit of 3 ppb.

We would like to determine how the G_1 block is induced. It may be that atrazine exposed cells arrest to repair DNA damage caused by the atrazine. Alternatively, the atrazine may impair the DNA repair mechanisms, which is a reversible effect. To test this, cells would be exposed to atrazine and then placed in atrazine free medium for the same amount of time. If the cells emerge from the G_1, it would suggest that atrazine may affect DNA repair processes reversibly. Additionally, Western blots could be performed to examine if the levels of the tumor protein 53, a protein involved in cell cycle regulation, are up or down-regulated in atrazine-exposed cells compared to control cells.

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