



The Effects of Triazine Herbicides on Critical Peroxide Metabolism Gene Expression in MCF-7 and MCF-10A Cells

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Authors' contributions

This work was carried out in collaboration among all authors. Author SJD designed and performed the RNA analysis study. Author NBD designed and performed the colorimetric analysis of oxidation. Authors SJD and NBD prepared the initial drafts of the manuscript. Author JRS managed the statistical analysis, manuscript revisions, and procurement of funding. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To identify the oxidative stress impacts of chloro-s-triazine herbicides on human mammary epithelial cell lines.

Study Design: MCF-7 mammary epithelial carcinoma and MCF-10A mammary epithelial cells were treated with levels of three triazine herbicides in concentrations flanking the US FDA safe levels.

Place and Duration of Study: Department of Biology, Millikin University, in January 2015 through December 2015 and January 2019 through May 2020.

Methodology: We examined the oxidative effects of two triazine herbicides, atrazine and simazine, on estrogen-dependent MCF-7 mammary epithelial carcinoma cells using three different bioluminescent assay techniques. We then utilized real time PCR to analyze gene expression through RT-PCR analysis, in both MCF-7 cells and a non-cancerous cell line, MCF-10A, for both of these triazine herbicides plus the related cyanazine.

Results: At all concentrations of atrazine and simazine, no statistical differences were found in the levels of oxidized glutathione or total oxidized and reduced nicotinamide adenine dinucleotides

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phosphates. In stark contrast, levels of hydrogen peroxide were found to be statistically different from the control at all concentrations of atrazine and simazine tested. Using an Analysis of Variance (ANOVA) we determined that within the enzymatic portion of the hydrogen peroxide pathway there were statistically significant differences in the expression of Peroxiredoxin 1 (PRDX1), Sulfiredoxin (SRXN1), and Thioredoxin (TXN).

Conclusion: Exposure to triazines alters the hydrogen peroxide pathway, which in turn can greatly affect the stability of the cell milieu.

Keywords: Oxidative stress; breast cancer; atrazine; simazine; cyanazine; MCF-7; MCF-10A.

1. INTRODUCTION

Herbicides are widely used on crops to prevent the growth of algae, deep rooted weeds, and other vegetation that would impede the growth of commercial crops. Of the major herbicides, the chloro-s-triazine herbicides are widely used throughout the United States [1], and specifically in the Midwest for cornfields [2]. Atrazine, 2-chloro-4-ethylamino-6-isopropylamino-s-triazine, is one of the most commonly used herbicides in the US with over 70 million pounds applied annually. Simazine, 6-chloro-2-N,4-N-diethyl-1,3,5-triazine-2,4-diamine, is another structurally similar herbicide of the same class. Cyanazine, 2-chloro-4-(1-cyano-1-methylethyl-amino)-6-ethylamine-1,3,5-triazine, is a related herbicide which was banned for agricultural use in 2003 due to its high toxicity. Contact with these herbicides can be through physical contact through the dermis of the skin, but not through inhalation in areas near its application [3]. These herbicides have also been found in increasing amounts in both surface and groundwater [4, 5]. In a nation-wide study of domestic drinking water wells, atrazine was detected in 24% of wells tested and simazine was detected in 9% of wells tested [6]. The European Union banned both of these herbicides from use in 2004 [7]. Conversely, the US EPA has only gone so far as to restrict their use and set an average maximum contamination level of drinking water. The US EPA allows average contamination levels of atrazine up to 3 ppb and simazine up to 4 ppb [6].

The contamination of drinking water is of utmost concern because both atrazine and simazine have been indicated as potential endocrine and reproductive disrupting compounds. induces mammary tumors, decreases birth weights, and delays the onset of puberty and mammary gland development in rats [8]. In humans, population-based epidemiological studies evaluating the risk of breast cancer associated with atrazine and simazine contaminated drinking water have had

contradictory results [1, 9]. Even so, atrazine and simazine have been indicated to increase MCF-7 breast cancer cell viability potentially leading to increased cancerous cell growth [10]. These herbicides have been demonstrated to impact the expression of several genes, such as *GPR30* in MCF-7 cells, as well as potentially impact [11].

Hydrogen peroxide (H_2O_2) is an important molecule in cell signaling and is a product of mitochondrial processes during cell growth and division [12]. Increased oxidative stress has been linked to cancer initiation and progression [13]. Additionally, oxidative stress has been shown to cause tumor stroma to release energy nutrients that fuel cancer cells [14]. During periods of rapid cell proliferation, as in cancerous tumors, the levels of H_2O_2 become cytotoxic and send cells into the apoptotic cascade, or activate H_2O_2 metabolic pathways that can save the cell from apoptosis [15,16]. Hydrogen peroxide metabolism is a necessary process for continued cell health. Multiple enzymatic pathways are present throughout and within specific cell compartments to prevent any cellular or DNA damage from these reactive oxygen species (ROS).

The nuclear factor-erythroid 2 p45-related factor 2 (*NRF2*) pathway is responsible for H_2O_2 metabolism. Once activated, *NRF2* enters the nucleus and serves as a transcription factor for several genes coding for four necessary enzymes [12]. Two of these enzymes contribute to H_2O_2 reduction by binding it as a substrate and protecting the cell from interactions with the ROS. The other coenzymes recycle the primary enzymes when they become inactivated by their interactions with the ROS species.

The first H_2O_2 -binding enzyme is Peroxiredoxin 1 (*PRDX1*), an enzyme that can be found throughout the cell. It is one of four "typical 2 cysteine" peroxiredoxins and serves as an H_2O_2 "sink" within the cell [17]. Although *PRDX1* handles approximately 90% of all ROS

prevention within the cell [18], transcription of *PRDX1* alone would result in a temporary solution to an overabundance of ROS. The role of Sulfiredoxin (*SRXN1*) is to engage with the enzyme-substrate complex and dissociate the two, creating water from the H_2O_2 and reactivating *PRDX1* [19, 20]. Sulfiredoxin serves as an equalizer within the cell, ensuring a balance between chaperone and antioxidant functions of *PRDX1* [21]. Thus, under controlled conditions it is expected that *SRXN1* and *PRDX1* expression would be similar.

The second major H_2O_2 enzyme is Thioredoxin 1 (*TXN*) and its associated enzyme Thioredoxin Reductase 1 (*TXNRD1*). The Thioredoxin–Thioredoxin Reductase pathway is similar in type of relationship between enzymes to that of *PRDX* and *SRXN1*. *TXNRD1* is necessary for reacting with NADPH to reduce *TXN*'s bound and inactive form as a disulfide into its active reduced form [22, 23]. The reduction potential of *TXN* allows the enzyme to continue to interact with H_2O_2 and protect against ROS damages [24, 25].

The presence of these enzymes within the cell is necessary for cell survival. Activation of an enzyme but not the reductase would result in reduced efficacy of ROS protection and can result in death of healthy cells, or protection of cancerous cells. The purpose of our study was to identify any differences in ROS production after atrazine or simazine treatment, followed by examination of the expression of major enzymes in relation to herbicide exposure, and if expression changed in a dose-dependent manner. Additionally, the purpose of this research was to determine if there were differences in expression between cancerous and non-cancerous cell lines.

We hypothesized that the oxidative stress of MCF-7 human breast adenocarcinoma cells would increase as the cells were exposed to increasing concentrations of atrazine and simazine. We further hypothesized that atrazine would have a greater impact than simazine, especially on *PRDX1* [26, 27]. We further hypothesize that the increase in gene expression will be greatest in the non-cancerous cell lines due to the already increased levels in cancerous cells.

For comparison of expression levels, we used the housekeeping gene 36B4, also known as ribosomal protein lateral stalk subunit P0 (*RPLP0*). *RPLP0* codes for a 60S ribosomal subunit, a necessary organelle within the cell for

which consistent transcription is necessary. Its usefulness as a control gene in RT-PCR has been demonstrated in cervical cancer research, and by utilizing it as our control, we ensured normalization between our cancerous and non-cancerous cell lines due to the consistency of its expression throughout the body [28, 29].

2. MATERIALS AND METHODS

2.1 Cell Maintenance and Culture

Human epithelial mammary adenocarcinoma (MCF-7) cells were acquired from the lab of Dr. Ann Nardulli (University of Illinois at Urbana-Champaign, Urbana, IL), subcultured from stocks originally obtained from ATCC (Manassas, VA). Cells were maintained in Modified Eagle's Media supplemented with 5% calf serum and antibiotics in a 5% CO_2 humidified environment. MCF-10A cells were obtained directly from were maintained ATCC (Manassas, VA), and were maintained in DMEM/F-12 media supplemented with 5% horse serum, 0.1 μ g/mL cholera toxin, and MEGS supplement. All cell treatment experiments were performed at least in triplicate. Atrazine was added at 0.3, 3.0, and 30 ppb (μ g/L; 0.64-64 μ M) and simazine was added at 0.4, 4.0, and 40 ppb (μ g/L; 0.92-92 μ M) at concentrations 0.1x, 1.0x, and 10x the EPA maximum concentrations, or DMSO control (1% final concentration).

2.2 Oxidative Stress Assays

Upon reaching 80% confluence, MCF-7 cells were plated into a 96-well plate. After twenty-four hours, the cells were treated with varying concentrations of atrazine or simazine, or with DMSO vehicle as a control (Table 1). After the cells were treated for forty-eight hours, one of three Promega luminescence-based oxidative stress assays was performed (GSH/GSSG-Glo, NADP+ /NADPH-Glo SIMPLE, or ROS-Glo H2O2) per manufacturer's instructions. The GSH/GSSG-Glo and ROS-Glo H2O2 assays were performed in triplicate, while the NADP+/NADPH-Glo SIMPLE assay was performed in quintuplicate. The contents of all wells (~100 μ L) were transferred to plastic Starstedt tubes to measure their luminescence using a luminometer. Due to variation in the amount of luminescence in each replicate, experiments from different days were normalized so the control expression was set to 1.00. Univariate analysis of variance (ANOVA) was

performed using SPSS (IBM SPSS Statistics 21, IBM Corp., Aramok, NY, USA), with $p < 0.05$ indicating a significant variation of treated cells from the controls.

2.3 RNA Isolation and Purification

Upon reaching 80% confluence, cells were plated into 6-well plates and allowed to adhere overnight, followed by treatment with atrazine, cyanazine, or simazine, or DMSO control for 24 hours. The media was then removed from each well, and RNAzol reagent was added to each well and allowed to sit for 5 minutes. The cells were lysed by pipetting up and down, and transferred to an Eppendorf tube. The RNAzol/cell mixture was then frozen at -70°C until purification. To purify RNA, the mixture was thawed and chloroform added to each tube followed by vortexing to mix and extract nucleic acids. After centrifugation, the aqueous phase was placed in a new tube, nucleic acids precipitated with isopropyl alcohol and pelleted by centrifugation, and rinsed with 75% ethanol. After briefly air drying, the pellet was suspended in RNase-free water, and treated with RQ1 RNase-Free DNase (Promega, Madison, WI) to remove DNA contaminants per manufacturer directions. The RNA was then briefly air dried for 5-10 minutes and suspended in 24 μL of RNase-free H_2O . The quantity of RNA was calculated via spectrophotometry at a wavelength of 260 and 280 nm. cDNA was synthesized using Random Primers and M-MLV Reverse Transcriptase (Promega, Madison, WI) per manufacturer directions, and stored at -70°C until needed.

2.4 Real Time PCR

Real-time PCR was conducted on each set of cDNA in duplicate in 20 μL final volume, using iTaq Universal SYBR Green (BioRad, Hercules, CA). Primers for real-time PCR were obtained from IDT DNA Technologies (Coralville, IA), with sequences as shown in Table 2. The plate was then placed in the PCR machine and ran for 60 cycles at the appropriate annealing and extension temperature and length for the specific gene (Table 2).

Values for the individual wells per treatment were averaged. The Ct of a reaction is the cycle number at which PCR products have crossed a threshold. The ΔCt value was determined by

subtracting the Ct mean of our control gene, *RPLP0*, from the Ct mean of our test genes. The $\Delta\Delta\text{Ct}$ value was determined by subtracting the ΔCt value of the treated cells from the control ΔCt of each run. The fold change was determined by raising 2 to the power of $\Delta\Delta\text{Ct}$. Univariate analysis of variance (ANOVA) was performed using SPSS (IBM SPSS Statistics 21, IBM Corp., Aramok, NY, USA), with $p < 0.05$ indicating a significant variation of treated cells from the controls.

3. RESULTS AND DISCUSSION

3.1 Oxidative Stress Assays

MCF-7 mammary epithelial cells were used to examine the oxidative stress response of breast cancer cells exposed to triazine class herbicides, specifically atrazine and simazine. These MCF-7 cells were treated with environmentally relevant concentrations at 0.1x, 1x, and 10x the EPA maximum contamination levels and a DMSO control (Table 1).

The Promega GSH/GSSG-Glo assay detects and quantifies total glutathione (GSH + GSSG), GSSG and GSH/GSSG ratios in cultured cells. The produced luminescence is proportional to the amount of GSH present. The levels of reduced glutathione were found to have no statistical difference from the control at all concentrations of atrazine and simazine tested (Fig. 1A). The levels of oxidized glutathione were also found to have no statistical difference from the control at all concentrations of atrazine and simazine tested (Fig. 1B). There was also no difference in the GSH/GSSG ratio amongst any of the treatments (Fig. 1C).

The Promega NADP+ /NADPH-Glo SIMPLE assay detects total oxidized and reduced nicotinamide adenine dinucleotides phosphates (NADP+ and NADPH, respectively) and determines their ratios. The produced luminescence was proportional to the ratio of NADP+ to NADPH. The levels of NADP+/NADPH were found to have no statistical difference from the control at all concentrations of atrazine tested: 0.3 ppb, 3 ppb, and 30 ppb (Fig. 2). Also, the levels of NADP+/NADPH were found to have no statistical difference from the control at all concentrations of simazine tested: 0.4 ppb, 4 ppb, and 40 ppb (Fig. 2).

Table 1. Herbicide treatments utilized for oxidative stress studies in MCF-7 cells. Values are based on the US EPA maximum contamination levels (MCL) for drinking water

	MCL (µg/L)	0.1x (µg/L)	1x (µg/L)	10x (µg/L)
Atrazine	3	0.3	3	30
Cyanazine	1	0.1	1	10
Simazine	4	0.4	4	40

Table 2. RT-PCR primer design with protocol specific temperatures, primer source and NCBI accession numbers

Gene	Primer sequence	Annealing temperature (°C)	Extension temperature (°C)	Source	NCBI accession #
RPLP0	Forward: CAGCAAGTGGGA AGGTGTAATCC Reverse: CCCATTCTATCAT CAACGGGTACAA	60	72	Cawthon, 2002 [30]	NM_001002.3 NM_053275.3
PRDX1	Forward: TTTGGTATCAGAC CCGAAGC Reverse: TCCCCATGTTTGT CAGTGAA	59	72	Cha, 2009 [31]	NM_002574.3 NM_181696.2 NM_181697.2 NM_001202431.1
SRXN1	Forward: AAGGTGCAGAGC CTCGTGG Reverse: GCTACTGCAAGT CTGGTGTGGA	60	70	Lan, 2017 [20]	NM_080725.2
TXN	Forward: CTGCTTTTCAGG AAGCCTTG Reverse: TGTTGGCATGCA TTTGACTT	60	72	Cha, 2009 [31]	NM_003329.3 NM_001244938.1
TXNRD1	Forward: TCCTATGTCGCTT TGGAGTGC Reverse: GGACCTAACCAT AACAGTGACGC	60	72	Yang, 2014 [32]	NM_182729.2 NM_182742.2 NM_182743.2 NM_003330.3 NM_001261445.1 NM_001261446.1 NM_001093771.2

The ROS-Glo H₂O₂ assay measures the level of hydrogen peroxide. The produced luminescence was proportional to the amount of hydrogen peroxide produced by the cells. The levels of hydrogen peroxide were found to be statistically different from the control at all concentrations tested of atrazine: 0.3 ppb, 3 ppb, and 30 ppb (Fig. 3). In addition, the levels of hydrogen peroxide were found to be statistically different from the control at all concentrations tested of simazine: 0.4 ppb, 4 ppb, and 40 ppb (Fig. 3).

Specifically, hydrogen peroxide was shown to have approximately a 50% reduction at all concentrations of both atrazine and simazine.

3.2 Gene Expression

Due to the statistically significant differences in hydrogen peroxide production following herbicide treatment, we wanted to examine any modifications in gene expression in the peroxide pathway. MCF-7 cells, as well as the non-

cancerous immortalized cell line MCF-10A, were treated with the same concentrations of atrazine or simazine as used in our oxidative stress assays (Table 1), however for gene expression the cells were harvested and RNA levels

examined after twenty-four hours. We also included cyanazine for comparison, as it has known toxicity [33-35]. We selected several genes within the peroxide pathways for examination.

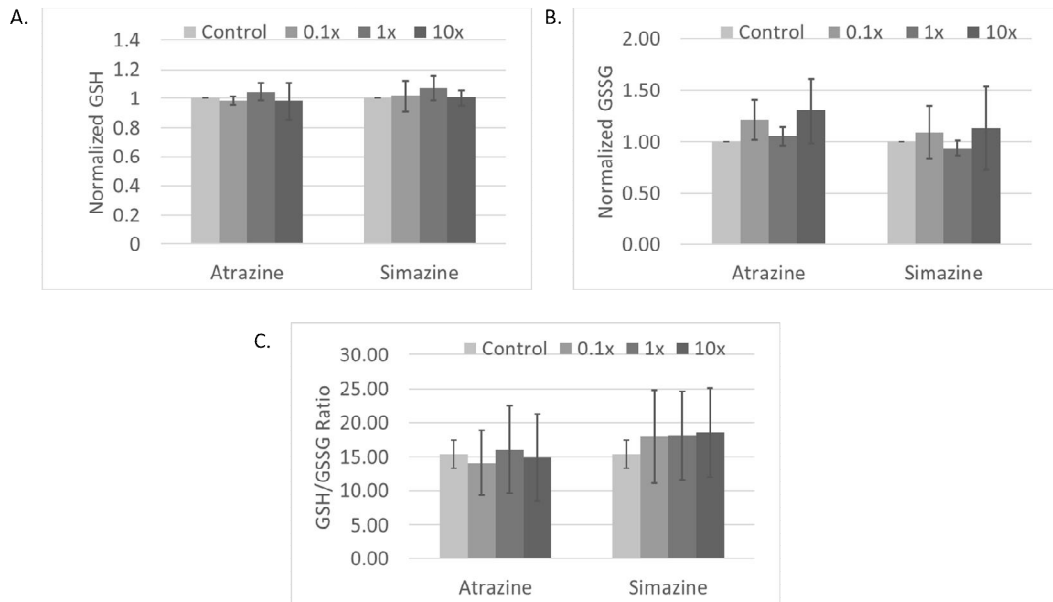


Fig. 1. Levels of reduced and oxidized glutathione measured in MCF-7 cells exposed to atrazine and simazine

MCF-7 cells were treated with 0.3 to 30 ppb atrazine or 0.4 to 40 ppb simazine (0.1x to 10x the EPA MCL) or DMSO control for 48 hours, followed by luminescence-based assaying total or oxidized glutathione. Normalized values for GSH (A) or GSSG (B) are shown; ratios of raw results for GSH and GSSG are shown in C. Results are presented as the average \pm SEM and are representative of 3 assays performed in duplicate. There were no statistically significant differences from the control ($p < 0.05$)

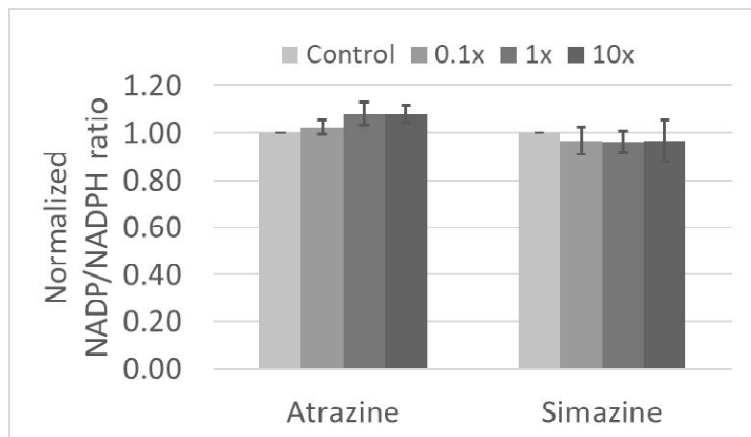


Fig. 2. Levels of total oxidized and reduced nicotinamide adenine dinucleotide phosphates measured in MCF-7 cells exposed to atrazine and simazine

MCF-7 cells were treated with 0.3 to 30ppb atrazine or 0.4 to 40ppb simazine (0.1x to 10x the EPA MCL) or DMSO control for 48 hours, followed by luminescence-based assaying for NADP/NADPH. Results are presented as the average \pm SEM and are representative of 5 assays performed in duplicate. There were no statistically significant differences from the control ($p < 0.05$)

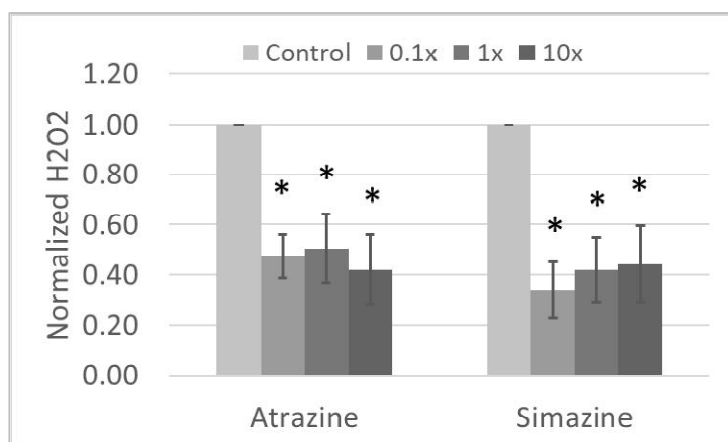


Fig. 3. Levels of hydrogen peroxide measured in MCF-7 cells exposed to atrazine and simazine

MCF-7 cells were treated with 0.3 to 30 ppb atrazine or 0.4 to 40ppb simazine (0.1x to 10x the EPA MCL) or DMSO control for 48 hours, followed by luminescence-based assaying for hydrogen peroxide. Results are presented as the average \pm SEM and are representative of 3 assays performed in duplicate. Statistically significant differences from the control ($p < 0.05$) are indicated (*)

There was a statistically significant difference in PRDX1 expression fold between cell lines ($p = 0.000$) demonstrated by "a" and "b" (Fig. 4). There were also trends towards significance with regard to herbicide ($p = 0.091$), cell type and herbicide ($p = 0.072$), and herbicide and concentration ($p = 0.099$). There was an additional trend with regards to the 1.0x concentration between cell lines ($p = 0.062$). Within MCF-7

cells, there was no statistical difference in expression fold change with regards to either herbicide used or concentration. Within MCF-10A cells there was a statistical difference in expression fold change between herbicides used ($p = 0.033$), and specifically between cyanazine and simazine ($p = 0.025$), as demonstrated by the (#) and (*), respectively.

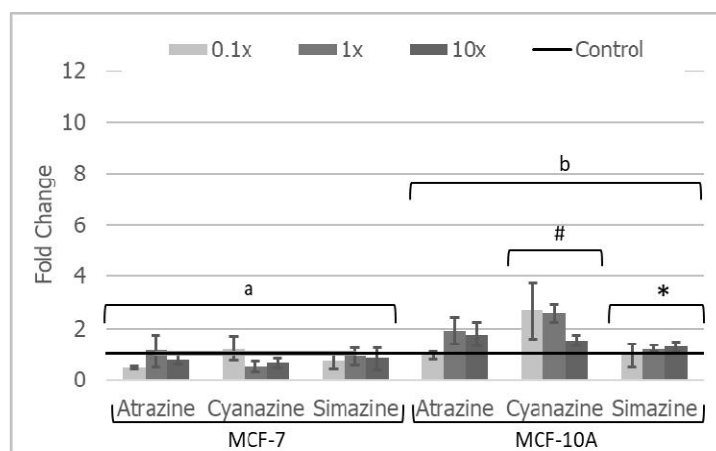


Fig. 4. Expression fold change of PRDX1 mRNA in MCF-7 and MCF-10A cells exposed to atrazine and simazine

Cells were treated with 0.3 to 30 ppb atrazine, 0.1 to 10 ppb cyanazine, or 0.4 to 40 ppb simazine (0.1x to 10x the EPA MCL) or DMSO control for 24 hours, followed by RNA extraction and purification. cDNA was synthesized, and real-time PCR was performed with primers to PRDX1 and the control gene RPLP0. Results shown are relative fold change and represent 3 independent experiments performed in duplicate, \pm SEM. Expression changes between the two cell lines are indicated (a and b, $p = 0.003$). Differences between cyanazine and simazine expression is indicated (# and *, $p = 0.025$). There were no individual statistically different changes from the control

There was a statistically significant difference in *SRXN1* expression fold change between cell lines ($p=0.001$), demonstrated by “a” and “b” (Fig. 5). There were no statistically significant results within the MCF-7 cell line with regard to either herbicide used or concentration. Within the MCF-10A cell line, there was a statistically significant result at the 1.0x concentration compared to the control when compiled between treatments ($p=0.033$, represented by the #).

Between cell lines there was a statistically significant difference in *TXN* expression fold change ($p=0.005$) demonstrated by “a” and “b” respectively (Fig. 6). There were no statistically significant results or trends in *TXN* expression within MCF-7 cells or MCF-10A cell lines.

There was not a statistically significant difference in *TNFRD1* expression between MCF-7 and MCF-10A cell lines (Fig. 7). There were also no statistically significant results or trends within the MCF-7 cell line. Within MCF-10A cells there were trends towards significance with regards to both herbicide and concentration ($p=0.070$ and $p=0.053$ respectively). Furthermore, at the 10x concentration across all herbicides in the MCF-10A cells there was a statistical significance in expression fold change ($p=0.033$, as demonstrated by the *).

Our findings did not support the hypothesis that as MCF-7 human breast adenocarcinoma cells

are exposed to increasing amounts of atrazine and simazine, oxidative stress will be increased. Instead, the results indicate that levels of GSH, GSSG, and NADP+/NADPH were unaffected by exposure to atrazine and simazine at all concentrations tested. Furthermore, results indicate that oxidative stress of MCF-7 cells is reduced in relation to H_2O_2 at all concentrations tested of atrazine and simazine.

Likewise, the results do not support our hypothesis that atrazine would result in the largest significant differences in gene expression of *PRDX1* across both cell lines. In all cases, cyanazine was responsible the largest changes in expression fold. Our initial hypothesis was based on available research, a majority of which is focused on atrazine exposure effects. Cyanazine has been banned in the United States since 2003 [2], and has been demonstrated to have detrimental effects in other mammals such as enlargement of the liver and kidney [36]. These come as a result of higher than acceptable levels of cyanazine that are present in water sources during periods of usage in agriculture, and during portions of the year when cyanazine is not in current usage, it is rarely found to be below its allowed concentration. The lack of research concerned with cyanazine exposure makes this project all the more pressing in its findings, and will be a central part of our future research.

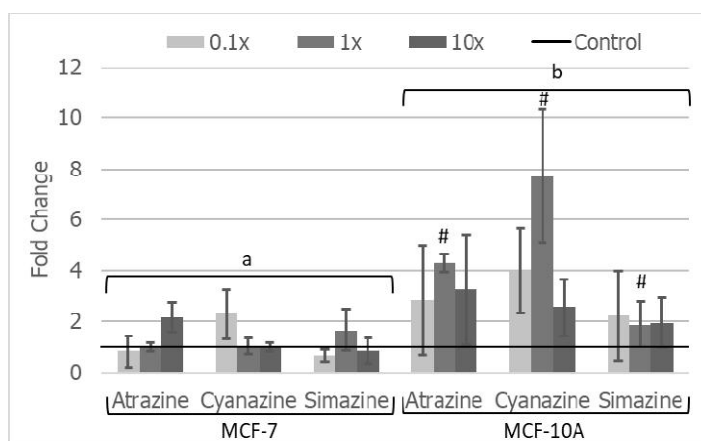


Fig. 5. Expression fold change of *SRXN1* mRNA in MCF-7 and MCF-10A cells exposed to atrazine and simazine

Cells were treated with 0.3 to 30 ppb atrazine, 0.1 to 10 ppb cyanazine, or 0.4 to 40 ppb simazine (0.1x to 10x the EPA MCL) or DMSO control for 24 hours, followed by RNA extraction and purification. cDNA was synthesized, and real-time PCR was performed with primers to *SRXN1* and the control gene *RPLP0*. Results shown are relative fold change and represent 3 independent experiments performed in duplicate, \pm SEM. Expression changes between the two cell lines are indicated (a and b, $p=0.001$). Differences between the 1x and control treatments in MCF-10A cells is indicated (# and *, $p=0.033$). There were no individual statistically different changes from the control

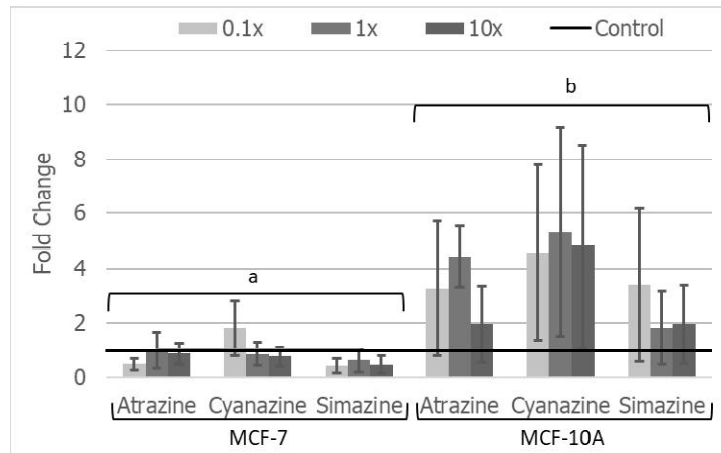


Fig. 6. Expression fold change of TXN mRNA in MCF-7 and MCF-10A cells exposed to atrazine and simazine

Cells were treated with 0.3 to 30 ppb atrazine, 0.1 to 10 ppb cyanazine, or 0.4 to 40 ppb simazine (0.1x to 10x the EPA MCL) or DMSO control for 24 hours, followed by RNA extraction and purification. cDNA was synthesized, and real-time PCR was performed with primers to TXN and the control gene RPLP0. Results shown are relative fold change and represent 3 independent experiments performed in duplicate, \pm SEM. Expression changes between the two cell lines are indicated (a and b, $p=0.003$). Differences between cyanazine and simazine expression is indicated (# and *, $p=0.025$). There were no individual statistically different changes from the control

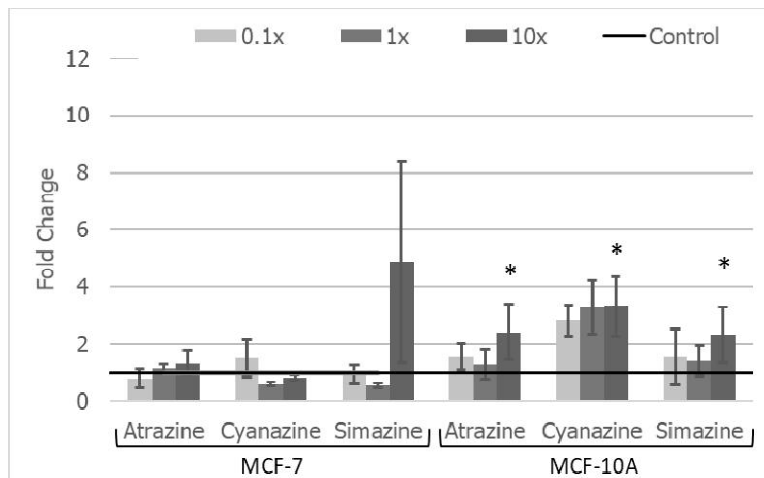


Fig. 7. Expression fold change of TXNRD1 mRNA in MCF-7 and MCF-10A cells exposed to atrazine and simazine

Cells were treated with 0.3 to 30 ppb atrazine, 0.1 to 10 ppb cyanazine, or 0.4 to 40 ppb simazine (0.1x to 10x the EPA MCL) or DMSO control for 24 hours, followed by RNA extraction and purification. cDNA was synthesized, and real-time PCR was performed with primers to TXNRD1 and the control gene RPLP0. Results shown are relative fold change and represent 3 independent experiments performed in duplicate, \pm SEM. Differences between the 10x and control treatments in MCF-10A cells is indicated *, $p=0.033$.

Though *PRDX1* and *SRXN1* did increase in expression as expected, *TXN* and *TXNRD1* also increased, contradicting what we had postulated. The similarity in trends and significant differences in expression between the coenzyme pairs is not ignorable, specifically in the

concentrations of specific herbicides with respect to *PRDX1* and *SRXN1*. This further argues that these herbicides may not have specific enzymatic effects, and that the overall ability to regulate transcription has been compromised [24].

Our final hypothesis was supported. In all cases, the only significant differences in expression fold were found within the MCF-10 A cell line. Though these cells are immortalized, their representation of more normal cell lines demonstrates that these herbicides may only be able to induce effects on healthy cell machinery. The already elevated levels of these antioxidants within cancerous cells may not be able to be induced higher than their mutations already permit [37].

In healthy cells, reactive oxygen species such as hydrogen peroxide are meant to be at a low steady state. Excess in ROS could lead to cell damage or even cell death. Low levels of ROS have been shown to activate cellular proliferation and survival signaling pathways [38]. This could indicate that exposure to atrazine and simazine, even at the US EPA safe levels, actually makes the MCF-7 cells more robust and less susceptible to apoptosis. This supports the findings of Rich, Gabriel, and Schultz-Norton in 2012 that MCF-7 breast cancer cells exposed to atrazine and simazine had increased cell viability [10]. It is also interesting to note that most conventional chemotherapy and radiotherapy drugs work to rid the body from cancer by stimulating an increase in ROS to induce apoptosis whereas these herbicides are causing the exact opposite effect [39].

The expression of these selected enzymes was collected at the 24 hours, and does not encapsulate the full course of time, analyzing for changes in expression before or after this time stamp. There is also concern for the degradation of the mRNA within the cell. Following transcription, rapid mRNA degradation can occur, and could potentially skew results. Additionally, if protein levels were already increased within the cell, this could lead to reduced mRNA transcription as a countermeasure of overexpression of these enzymes. Further analysis of protein levels in concert with mRNA expression could potentially reveal new interactions between the herbicides and the H₂O₂ pathway.

Due to the scope of this project, only four enzymes from the peroxide pathway were evaluated for their expression pattern following exposure to these herbicides. Our focus was on the portion of the H₂O₂ pathway directly involved in the reduction of levels of H₂O₂. One such area that is of great interest are glutathione peroxidases, a closely connected set of enzymes responsible for additional ROS protection in the

cell [40]. The enzymes responsible from protecting DNA and proteins from interactions with H₂O₂ are also transcribed by NRF2 via *ARE* genes [41]. If transcription regulation is at the heart of herbicide effects, we would expect to see elevated levels of these enzymes as well.

4. CONCLUSION

At all concentrations of atrazine and simazine, no statistical differences were found in the levels of oxidized glutathione or total oxidized and reduced nicotinamide adenine dinucleotides phosphates. In stark contrast, levels of hydrogen peroxide were found to be statistically different from the control at all concentrations of atrazine and simazine tested. Within the enzymatic portion of the hydrogen peroxide pathway, there were statistically significant differences in the expression of Peroxiredoxin 1 (PRDX1), Sulfiredoxin (SRXN1), and Thioredoxin (TXN). Thus, exposure to environmentally relevant concentrations of triazines alters the hydrogen peroxide pathway, which in turn may affect the stability of the cell milieu.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENTS

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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