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ARTICLE

Failure of pesticides to alter migration of cancerous and non-cancerous breast cell lines *in vitro*

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Organochlorine pesticides are routinely used in agricultural processes across the United States. Compared to surrounding areas, Illinois ranks as one of the highest users of triazine herbicides due to corn and soybean production. These pesticides have been detected in dietary sources and drinking water, thus leading to risks to human health. With conflicting reports as to whether pesticides play a role in tumor metastasis, we examined the migration rate for cancerous (MCF-7 and MDA-MB-231) and normal (MCF-10A) breast cells after exposure to six different pesticides using an *in vitro* scratch assay. Physiological concentrations of two insecticides (chlorpyrifos and resmethrin) and four herbicides (acetochlor, atrazine, cyanazine, and simazine) were applied to the cells for up to 72 hours and the ability of treated cells to regrow over a wounded area was assessed in 24-hour increments. Interestingly, significant differences in recovery after exposure to these compounds were not observed for any pesticide tested. However, reductions in recovery percentages were observed when comparing pesticide exposure to 17β-estradiol, a known trophic hormone for many breast cancers, in a cell type-dependent manner. Thus, although statistically significant increases in migration could be observed after estrogen exposure, short-term exposure to pesticides did not increase cell migration in this wound assay.

Introduction

Breast cancer is one of the most common forms of cancer in women, and is responsible for thousands of deaths annually [1, 2]. While approximately half of these cancers have unknown etiology, there are several risk factors which can increase the chance of cancer development; these include repeated exposure to hormone replacement therapy and environmental toxins [3-9]. Elevated levels of estrogenic compounds have been strongly correlated with an increased risk of tumorigenesis [10-13].

Environmental chemicals have often been thought to influence cancer initiation and metastasis [5, 14]. Over the last several decades, the role of pesticides in cancer development and progression has drawn increasing attention. While evidence indicates that organochlorine herbicides such as atrazine may cause the development of reproductive cancers [13, 15], conflicting studies lead questions to the toxicity of the compounds and the impact they may have on cancerous cells, especially in the estrogen receptor α (ERα)-positive MCF-7 cell line [16, 17]. However, a definitive separation between the effect upon migration and proliferation is not always seen; Pestana et al highlighted this difference in a comparison of the effect of organochlorines on ERα positive and negative cell lines [17]. One explanation for reported differences in proliferation within the literature could be due to the specific compounds assayed, as not all organochlorine pesticides share the same chemical conformation and may interact with ERα differently [18-20]. This results in a wide variety of proliferation outcomes that is dependent upon the compound and concentration tested, as well as the presence of nuclear hormone receptors such as ERα and androgen receptor (AR) within the cell lines examined [21-24]. These conflicting studies fuel a debate over the mechanism of action for pesticide action. While some studies have indicated potent activities of these compounds on steroid, nuclear, or even G- protein coupled receptors [25-28], others have indicated either no interaction or antagonism instead [29, 30].

Epidemiological evidence based on surveying rural communities has suggested that the increased incidence of reproductive cancers may be due to direct human exposure to these pesticides [31]. Exposure to agricultural pesticides and the potential risks thereto are a concern in central Illinois, where recent assessments of pesticide usage indicated that over twenty-seven million pounds of herbicides and 1.3 million pounds of insecticides are applied annually for corn production [32, 33]. This leads to runoff and detectable levels of these compound in the watershed and soil in application areas, which then in turn contaminate local drinking water sources [34-38]. Although there have been several studies regarding the effects of atrazine [26, 30, 39-41], there are several other compounds that deserve further investigation.

To assess the effect of pesticide exposure on breast cancer cell growth, we performed an *in vitro* wound assay [42]. In this assay, a thin line of cells was removed from a monolayer culture and regrowth through the line was monitored for up to 72 hours. For our study, cells were treated with one of four herbicides or two insecticides commonly used in Illinois [43]. Acetochlor, 2-chloro-N-(ethyoxymethyl)-N-(2-ethyl-6-methylphenyl)acetamide, is a

Notes and references

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component of several commercial herbicides [44] and has been shown to be an endocrine disruptor [45]. Acetochlor has been detected at high levels in the urine of farmers who utilize the compound, especially during times of application [46]. Three related organochlorine compounds were also selected: atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine), cyanazine (2-(4-chloro-6-ethylamino-1,3,5-triazin-2-ylamino)-2-methylpropionitrile, and simazine (6-chloror-N2,N4-diethyl-1,3,5-triazine-2,4-diamine). These compounds have all been shown to exhibit some mutagenicity or carcinogenicity [45, 47, 48]. Cyanazine has previously been applied in excess of 20 million pounds annually, yet was removed from the market by 2002 [49]. Cyanazine is a Group C possible human carcinogen as dietary exposure has increased the incidence of mammary tumors and triggered mutagenesis in murine lymphoma [36]. While banned in the United States, it is still used throughout Africa, Europe, Central Asia, and South America. Two insecticides, chlorpyrifos (*O,O*-diethyl *O*-3,5,6-trichloror-2-pyridyl phosphorothioate) and resmethrin (5-benzyl-2-furylmethyl (1RS)-cis,trans-2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropane-carboxylate), were also chosen as they have been shown to influence gravidity in mammals [45, 50]. Although other studies have focused upon large doses of these organochlorine herbicides [44], occasionally several thousand-fold above the safe level designated by the U.S. Environmental Protection Agency (EPA)[51], we have felt it is pertinent to study physiologically relevant doses that fall within the levels found in the environment.

Experimental

Chemicals The following chemicals were obtained from Sigma-Aldrich (St. Louis, MO): dimethylsulfoxide (DMSO), Minimum Essential Medium Eagle (MEM), cholera toxin, acetochlor (CAS 34256-82-1), atrazine (CAS 1912-24-9), chlorpyriphos (CAS 2921-88-2), cyanazine (CAS 21725-46-2), resmethrin (CAS 10453-86-8), and simazine (CAS 122-34-9). All compounds were analytical grade quality with a minimum purity of 94%. 17*β*-estradiol was purchased from Cayman Chemical Company (Ann Arbor, MI). MEM Richter's Modification was obtained from Hyclone (Logan, UT). HBSS, Leibovitz's L-15 Medium, MEGS supplement, epidermal growth factor, horse serum, and DMEM-F12 media were purchased from Life Technologies (Grand Island, NY). Calf serum and fetal bovine serum were purchased from PAA Laboratories (Dartmouth, MA). Penicillin-Streptomycin solution and MEM without phenol red were purchased from Cellgro (Manassas, VA). Gentamycin sulfate was obtained from Teknova (Hollister, CA). MCF-7, MDA-MB-231, and MCF-10A cell stocks (ATCC, Manassas, VA) were obtained from current cultures in the lab of Dr. Ann M. Nardulli (University of Illinois, Urbana, IL).

Cell culture MCF-7 cells were maintained in a closed flask at 37°C in phenol red-containing Modified Eagle's Media (MEM) supplemented with 5% calf serum and antibiotics (50 IU/mL penicillin, 50 µg/mL streptomycin, and 5 µg/mL gentamycin sulfate). Forty-eight hours prior to plating, cells were changed to phenol red-containing MEM supplemented with 5% calf serum and antibiotics and in a humidified, 5% CO_2 environment at 37°C. Twenty-four hours prior to plating, cells were transferred to phenol red-free MEM supplemented with 5% charcoal-dextran stripped calf serum and antibiotics in a humidified, 5% CO_2 environment at 37°C. MDA-MB-231 cells were maintained in a closed flask at 37°C in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum and antibiotics. Cells were transferred to phenol red-containing MEM and phenol red-free MEM at forty-eight and twenty- four hours prior to plating, as described above for MCF-7 cells. MCF-10A cells were maintained in DMEM/F12 media supplemented with 5% horse serum, MEGS supplement, and antibiotics in a humidified, 5% CO_2 environment at 37°C.

Wound assay The wound assay was based on that published by Liang, et al [42]. When cells reached ninety percent confluency, they were plated evenly into 12-well plates in maintenance (MCF-10A cells) or phenol-red free MEM (MCF-7 and MDA-MB-231 cells). Cells were allowed to adhere for twenty-four hours prior to the initiation of the assay. Adhered cells were artificially wounded by removing the cells in a single line from the bottom of the well using the end of a 200 µl disposable pipet tip. Dislodged cells were removed with an HBSS wash and were treated in maintenance (MCF-10A cells) or phenol red-free MEM (MCF-7 and MDA-MB-231 cells) containing 50 nM pesticide (atrazine, acetochlor, chlorpyrifos, cyanazine, resmethrin, or simazine), 50 nM 17β-estradiol, or DMSO vehicle control at 37°C in a humidified, 5% CO_2 environment. To prevent depletion of nutrients, media including estradiol, DMSO or the pesticides were replaced after 48 hours. Two images of the wounded area were taken per well in non-overlapping areas every twenty-four hours for three days using a Panasonic Lumix DMC-LZ5 camera (Panasonic, Secaucus, NJ). Acquired images were analyzed using ImageJ software v1.45s (NIH, Bethesda, MD). Data from four independent experiments with treatments repeated in triplicate internally were compiled, and statistical analysis was performed using a repeated measures analysis of variance through SPSS v21.0 software (IBM, Armonk, NY).

Results and discussion

To assess cell migration of cancerous and non-cancerous breast cells, an *in vitro* wound assay was performed. This method is based on the observation that after creation of an artificial gap on a confluent monolayer of cells, the cells on the edge of that gap will migrate until new cell-cell contacts are established. Thus, a monolayer of cells was subjected to an artificial wound by mechanical removal of the cells. Following wound induction, cells were exposed to pesticide, and regrowth into the wounded area was monitored for up to 72 hours. In previous work, we examined the effects of between 10 and 10000 nM of each of these compounds, and found that there was little effect on cell viability [52]; thus, we felt that any effect we observed would be due to changes in migration rather than changes in cell number.

During the 72 hours post-wound induction, the cells began to migrate across the cleared area (Fig. 1). The primary difference between cell-populated areas and the wound is texture. The wounded area is clear while the edges of the wound are speckled with cells (Fig. 1). As a control for growth, cells were also exposed to 17β-estradiol. This compound typically results in increased growth of ER-containing cell lines, such as MCF-7 which is ERα positive, yet do not affect the growth of MDA-MB-231 or MCF-10A cells which are considered ER-negative [52-55]. The amount of clear area was calculated, and the area for the 0 hour time point was normalized to 100% open, or 0% recovered, within each independent experiment. Recovery percentages were subjected to repeated measures ANOVA. Based upon initial between-subjects tests, cell-dependent differences were observed and cell lines were re-analyzed separately for statistical differences in recovery.

Subsequent repeated measures ANOVA of the MCF-7 data indicated a significant effect of compound by Mauchly's Test of Sphericity ($p < 0.000$), thus one-way ANOVA was performed for each compound at each time point. Post-hoc effects indicated a significant decrease

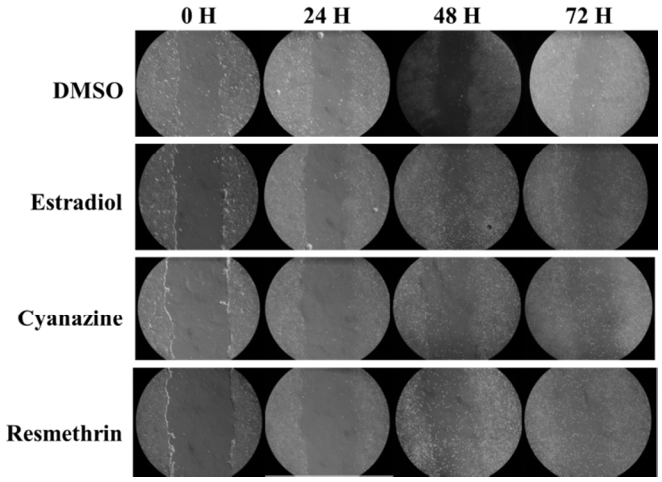

Fig 1. Images of MCF-7 cells from time-lapse pictures acquired at 0, 24, 48, and 72 h after artificial wound induction. MCF-7 cells were removed from a portion of the well, and were then treated with 50nM of pesticide, 50 nM 17β-estradiol, or DMSO vehicle control. Early images (0 and 24 h) show a wide gap where a lane of cells was removed by the wound assay. Later images (48 and 72 h) show the effect of progressive narrowing of this lane where the cells migrated to fill the wound.

in the amount of recovered area only after resmethrin and simazine treatment, compared to the DMSO vehicle control at 24 hours (Fig. 2). Atrazine, simazine, and resmethrin all showed lower levels of recovery than 17β-estradiol within the first 24 hours of exposure. However, by 48 hours, the recovery rates had altered. Only 17β-estradiol showed a significant increase in recovery compared to the DMSO control, while all three triazines (Fig. 2A) and resmethrin (Fig. 2B) showed significantly less recovery than estradiol yet no difference from DMSO. These same trends were observed again at 72 hours post-wound.

Analysis of the MDA-MB-231 cells through repeated measures ANOVA also indicated an effect of compound by Mauchly's Test of Sphericity ($p = 0.019$); thus, one-way ANOVA was performed for each compound and time point. At 24 hours, only simazine showed a lower recovery compared to the negative DMSO control, yet atrazine, resmethrin, and simazine-treated cells all exhibited less recovery than 17β-estradiol (Fig. 3). After 48 hours, the only statistically significant difference in recovery was an increase for acetochlor compared to DMSO. In the final observation at 72 hours of treatment, almost no differences in recovery percentages were seen, with only a slightly lower recovery for simazine compared to 17β-estradiol.

Unlike the two cancerous cell lines, a repeated measures test of the recovery percentages in the MCF-10A cells indicated that there was no effect of compound (Mauchly's Test of Sphericity result of $p = 0.684$, Fig. 4). Growth rates at each time point showed no differences for pesticide-treated cells compared to the controls. Full recovery was observed by 72 hours post-wound induction.

This assessment of cell migration between cancerous and non-cancerous cells highlights that there are very few differences in how these cells behave when exposed to pesticides. In all three cell lines tested, regardless of cellular milieu, the pesticides did not show large differences in cell recovery rates compared to the DMSO controls. In the MCF-7 cells, some of the pesticides showed small decreases in recovery percentages compared to the positive control for growth, 17β-estradiol. Resmethrin and simazine elicited the most significant

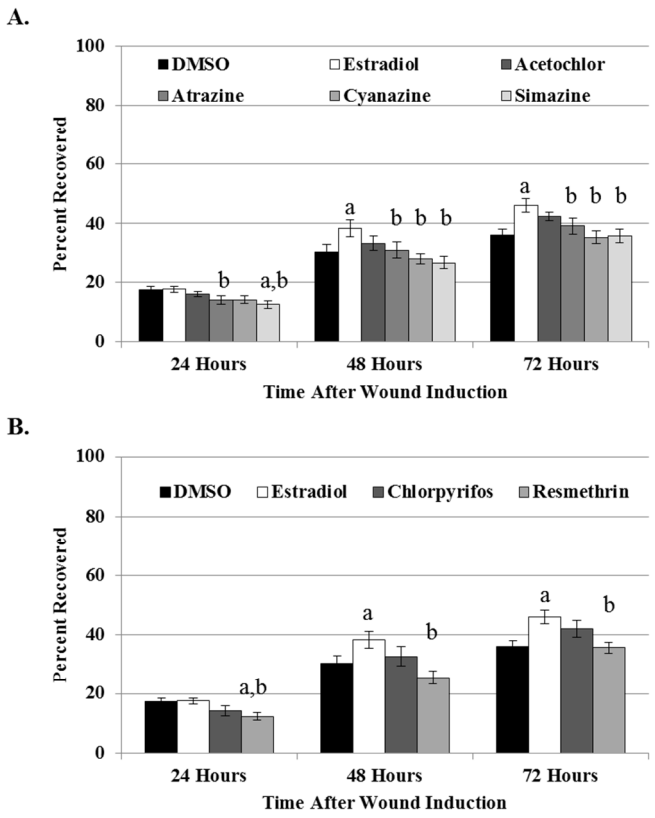

Fig 2. Percent wound recovery in herbicide-treated cells compared to controls. MCF-7 breast cancer cells were plated, a wound was induced, and cells were treated with 50 nM herbicide (A) or insecticide (B) or 17β-estradiol, or DMSO vehicle control in phenol red-free media with 5% CDCS. Media was replaced at 48 hours. Wound gaps were photographed twice per well in non-overlapping areas in 24-hour intervals, and open area was measured using ImageJ software (NIH, Bethesda, MD). Results represent four independent experiments performed in triplicate, and are presented as average percent recovery from the initial wound ± SEM. Significant differences ($p < 0.05$) from the DMSO (a) or 17β-estradiol (b) controls are indicated.

changes in recovery rate compared to the 17β-estradiol control; however this was only a small difference in recovery rate and was within five percent of the DMSO value at twenty-four hours. The difference was eliminated by 72 hours of treatment. In the MDA-MB-231 cells, simazine and resmethrin showed a delayed recovery, with significantly less of the area recovered at 24 hours yet no difference by 72 hours. In the MCF-10A cells, no statistically different changes in migration were detected.

Initially, we expected that due to the differences in structures and modes of action, that the insecticides and herbicides chosen might alter cell migration [25]. We had previously observed almost no changes in cell viability for these compounds in these same three cell lines over a wide range of concentrations [52]. Only simazine showed a slight, albeit not always significant, increase in cell viability compared to the DMSO control. This was observed in all cell lines and thus may not be an ER-specific response. However, in our current study, rather than observing a more rapid recovery, simazine did not increase migration but rather delayed it at the 24 hour time point in both cancerous cell lines. Simazine has previously been shown to induce mammary tumors in rats and is classified by U.S. EPA as a possible human carcinogen [56-58]. Thus, while a compound may illicit carcinogenicity, it may not necessarily lead to migration

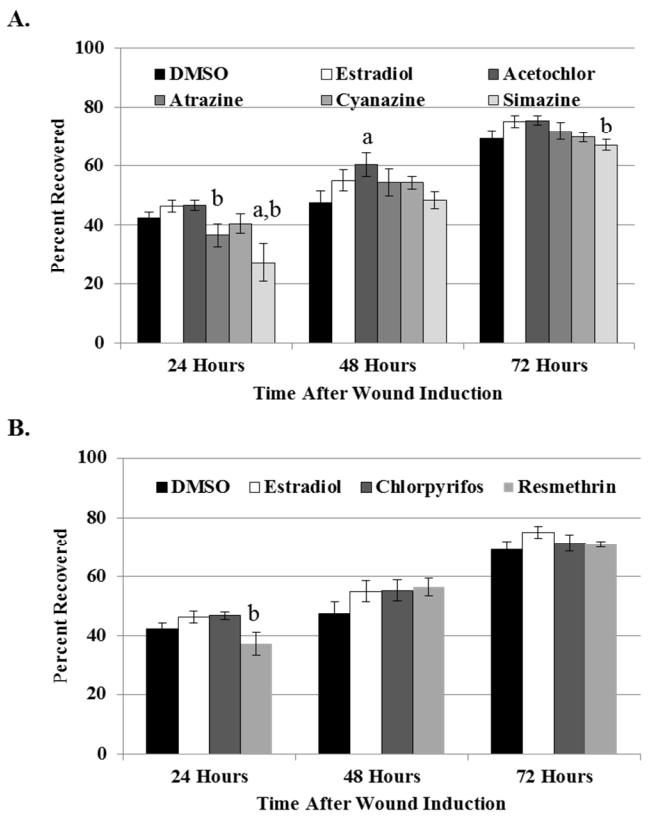

Fig 3. Percent wound recovery in herbicide-treated cells compared to controls. MDA-MB-231 breast cancer cells were plated, a wound was induced, and cells were treated with 50 nM herbicide (A) or insecticide (B) or 17β-estradiol, or DMSO vehicle control in phenol red-free media with 5% CDCS. Media was replaced at 48 hours. Wound gaps were photographed twice per well in non-overlapping areas in 24-hour intervals, and open area was measured using ImageJ software (NIH, Bethesda, MD). Results represent four independent experiments performed in triplicate, and are presented as average percent recovery from the initial wound ± SEM. Significant differences ($p < 0.05$) from the DMSO (a) or 17β-estradiol (b) controls are indicated.

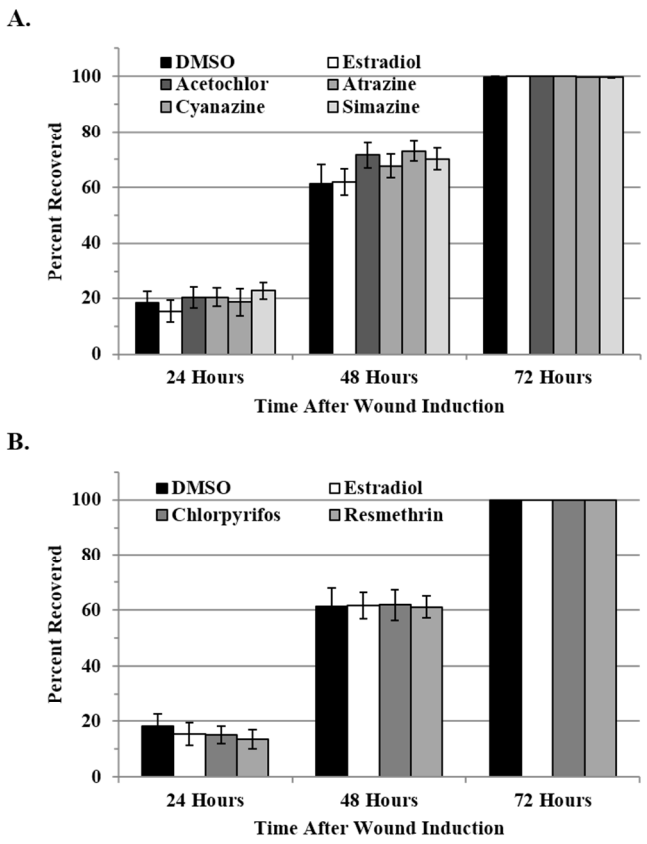

Fig 4. Percent wound recovery in herbicide-treated cells compared to controls. MCF-10A normal breast cells were plated, a wound was induced, and cells were treated with 50 nM herbicide (A) or insecticide (B) or 17β-estradiol, or DMSO vehicle control in DMEM/F-12 media with 5% horse serum. Media was replaced at 48 hours. Wound gaps were photographed twice per well in non-overlapping areas in 24-hour intervals, and open area was measured using ImageJ software (NIH, Bethesda, MD). Results represent four independent experiments performed in triplicate, and are presented as average percent recovery from the initial wound ±SEM.

and eventual metastasis.

Interestingly, the most controversial of the compounds, atrazine, did not demonstrate significant changes in migration, and never varied from the DMSO control. However, the recovery was significantly less than the 17β-estradiol control which may indicate that any actions are through non-estrogenic mechanisms. This is consistent with previous studies which highlight that atrazine does not act as an estrogen agonist [29], yet recent studies have indicated that atrazine and cyanazine exposure are not correlated with reproductive cancers [36, 59].

Discerning the mechanisms of action for these endocrine disrupting chemicals has been quite difficult, as there are several contradictory studies [60]. While breast cancer incidence has often been associated with exposure to estrogenic compounds, over one third of diagnosed breast cancers do not express the estrogen receptor and are often more aggressive [61]. Several studies have found that the presence of ERα in breast cancers is associated with distinctly different risk factors, and therefore, possibly different etiologies [62]. ERα-positive, and not ER-negative, breast cancers were positively associated with toxic air emissions and the proportion of land used for growing crops [62]. Thus, in our study we utilized both an ERα-positive and an ER negative cell line rather than focusing only upon the ERα-positive MCF-7 cell line.

There are reported correlations between some organochlorine pesticides and cell growth in estrogen-dependent cell lines, such as work by Garcia, et.al which showed that hexachlorobenzene (HCB) induced cell growth only in ERα-containing MCF-7 cells, and not in the MDA-MB-231 cell line [63]. However, it is now believed that HCB may act as a ligand of the aryl hydrocarbon receptor, and not through ER [64]. Other work has indicated that endocrine disrupting pesticides may reduce fertility in humans [65, 66].While the specific mechanisms of action have not been identified, it was hypothesized that these compounds may potentially activate the aryl hydrocarbon receptor or insulin growth factor-1 [63], and there has been clear evidence that the cellular changes attributed to activation of estrogenic pathways may be due to changes in aromatase activity rather than a direct interaction between these chemicals and either ERα or ERβ [29, 67].

Although minor differences in recovery over 72 hours were observed, there was no statistically significant difference in the migration of the pesticide-exposed cells back into the wound after more than 24 hours. Thus, we feel that our results show a failure of physiologically relevant concentrations of these compounds to alter

cell migration rates, both in cancerous and non-cancerous cells, and in both the presence and absence of ERα.

Conclusion

Although pesticides have often been shown to cause alterations in cell behavior, our research fails to show a significant change in cell migration after wound induction post-exposure to physiologically relevant concentrations of the organochlorine herbicides acetochlor, atrazine, cyanazine, simazine, as well as the insecticides chlorpyrifos and resmethrin.

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