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The Apoptotic and Anti-Apoptotic Effects of Pendimethalin and Trifluralin on A549 Cells *In Vitro*

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Objectives: Pendimethalin and trifluralin are commonly used in many countries to control broadleaf weeds and grassy weed species because of their inhibitory effects on growth and cell division. In this study, we examined the apoptotic and the anti-apoptotic potentials of pendimethalin and trifluralin on A549 human non-small lung cancer cells with several concentrations *in vitro*. Materials and Methods: The expression levels of apoptosis-related genes bcl-2, bax, casp3, casp9, tp53, birc and hsppia were examined by quantitative RT PCR after 24h treatment of 1, 10, 50, 100 and 500 µM pendimethalin and trifluralin. Results: The effects of pendimethalin were found more repressive than trifluralin on all the studied concentrations. 24 hours of exposure with 100 µM pendimethalin and trifluralin altered the gene expressions in the way to suppress the apoptosis and lead cancer cells to grow and proliferate. Conlusion: Care should be taken not to exceed the permissible values and residue limits on the food during pendimethalin and trifluralin using in order to reduce the possible carcinogenic effects on humans.

Keywords: Pendimethalin, trifluralin, apoptosis, A549, gene expressions

Pendimetalin ve Trifluralinin Apopitotik ve Anti-apopitotik Etkilerinin A549 Hücrelerinde *İn Vitro* Değerlendirilmesi

Amaç: Pendimetalin ve trifluralin birçok ülkede, büyüme ve hücre bölünmesi üzerindeki inhibitör etkileri nedeniyle, geniş yapraklı yabani otları ve çimenli ot türlerini kontrol etmek amacıyla yaygın şekilde kullanmaktadır. Bu çalışmada, pendimetalin ve trifluralinin apoptotik ve anti-apoptotik potansiyelleri A549 insan küçük olmayan akciğer kanseri hücreleri üzerinde çeşitli konsantrasyonlarda in vitro incelendi. Gereç ve Yöntem: Apoptoz ile ilişkili genler bcl-2, bax, casp3, casp9, tp53, birc ve ppia'nın ekspresyon seviyeleri, 24 saat 1, 10, 50, 100 ve 500 µM pendimetalin ve trifluralin uygulamasından sonra kantitatif gerçek zamanlı RT PCR ile incelendi. Bulgular: Çalışılan tüm konsantrasyonlarda pendimetalinin etkileri trifluralinin etkilerine kıyasla daha fazla baskılayıcı bulundu. 100 µM pendimetalin ve trifluraline 24 saat boyunca maruz bırakılan hücrelerde gen ifadesi, apoptozu baskılayacak ve kanser hücrelerinin büyüme ve çoğalmasına yol açacak şekilde değişikliğe uğradı. Sonuç: Pendimethalin ve trifluralinin insanlar üzerindeki olası kanserojenik etkilerini azaltabilmek için, kullanımları sırasında izin verilen değerlerin ve gıdalar üzerindeki kalıntı limitlerinin aşılmamasına dikkat edilmelidir.

Anahtar kelimeler: Pendimetalin, trifluralin, apopitoz, A549, gen ifadesi

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Introduction

Dinitroaniline herbicides were first discovered when dye and dye chemical intermediates were being evaluated. Chemicals in dinitroaniline herbicide family have essentially bright yellow color depending on the two nitro groups of the phenyl ring. They are generally referred to as "yellow compounds". The most important, and the first, herbicide in dinitroaniline family is trifluralin which was made known in 1963 (1). Dinitroaniline herbicides are separated into two groups as methylaniline herbicides and sulfonyllanine herbicides. Pendimethalin and trifluralin are herbicidal compounds in the

group of methylaniline (2). Herbicides such as pendimethalin and trifluralin are used to control broadleaf weeds and grassy weed species in cabbage, celery, corn, cotton, garlic, lettuce, radish, rice, sorghum, tobacco, brassicas, carrots, cereals, citrus, onions, peas, peanuts, pome fruits, potatoes, soybeans, stone fruits, and tomatoes. Both herbicide compounds are also used in Turkey. Pendimethalin is used for growing apple, walnut, hazelnut, peanut, potato, soy, citrus, grape and asiatic seed; trifluralin is used for growing cotton, soybean, sunflower seed, sugar cane, citrus, tomato, pepper, onion, aubergine bean, carrot, cumin and sesame in Turkey (3)

Dinitroaniline group herbicide compounds pendimethalin and trifluralin can cause nitrosamine synthesis in animals and humans. Nitrozamines are highly reactive, harmful chemical species. They can act as carcinogenic substances by removing amino groups from the nucleotide bases of DNA. At the same time they can act as toxic alkylating agents (2). For these reasons, cancer is the suspected health effect and the risk of dietary exposure to pendimethalin and trifluralin. Because of the widespread use of various formulations, pendimethalin and trifluralin are also present as contaminant in soil, ground water, surface water, and air (4). Pendimethalin is classified as slightly toxic compound (class III) to mammals. Trifluralin has no acute toxicity on oral, dermal and ocular exposures to mammals; although it is highly toxic to cold and warm water aquatic organisms as reported by the US EPA. Pendimethalin and trifluralin have also been classified in group C - possible human carcinogen (5).

Apoptosis is programmed cell death defined by important morphologic changes; blebbing, chromatin condensation, nuclear fragmentation and cell shrinkage. It is an essential process for normal development and also related to chronic diseases with various pathologic situations such as cardiovascular, immunological, neurodegenerative diseases and cancer (6).

Apoptotic mechanism is activated with many biochemicals, the best defined pathway factors are caspases (7). As caspase-3 is the main protease on the cell death process, where caspase-6 and caspase-7 are also contributory for the coordination of apoptosis. Thus, these three caspases are known the ‘executioner caspases’. Besides, caspase-8 and caspase-9 play a role on initiation step (8). The time that the initiative caspases activate the executioner caspases, the apoptotic process gets started with other enzyme activations (9). The apoptosis process continues on the extrinsic or intrinsic (mitochondrial) pathway and results in cell death (10). As well as it is a programmed process, apoptosis can occur after different kinds of irritations, such as radiation,

anticancer drugs that causes DNA damage and deprivation of cytokines that provide survival signals (11).

In addition to enzymatic changes, apoptotic pathway is directly related to some gene expressions. As an example, BCL-2 family proteins control the intrinsic pathway on the antiapoptotic side; however, BAX and BAK proteins are the promoter of the cell death (12,13). One of the most important protein at the cell cycle checkpoint is p53 tumor suppressor protein, which can be activated by DNA damage, hypoxia and apoptosis (14).

Pesticides are known to lead the cells to apoptosis both intrinsic and extrinsic pathways (15- 17). Mainly, the compounds enhance the mitochondrial oxidative stress mediators and activate cytochrome-C pathway resulting in intrinsic apoptosis (18, 19). Dinitroaniline herbicides are used as weed controller. Their mechanisms of action is based on cell division and decreasing cell elongation and growth with mitotic disruption during mitosis (20,21).

In this study, we measured the expression levels of p53, bax, bcl-2, casp3, casp9, birc and ppia (housekeeping) genes related to apoptosis on A549 Human Lung Carcinoma Cells after exposed to pendimethalin and trifluralin which are two commonly used dinitroaniline herbicides.

Material-method

Solution preparation

Pendimethalin is highly soluble in oil and organic solvents (22). The solution was prepared in DMSO : olive oil (1:3, v/v) mix. Trifluralin is soluble in organic solvents and less soluble in water (23). The trifluralin solution was prepared in PBS (1% DMSO).

Dulbecco's Modified Eagle's medium (DMEM) with 10% Foetal calf serum and 1% Penicillin-streptomycin mixture were used as cell culture medium.

Cell culture and treatment

A549 cells were cultured in a 25 cm^2 cell culture flask and transferred to 75 cm^2 flask after 24 h under the conditions of 5% CO_2 and 37 ^0C. After 24 hours, the cells were harvested and were transferred to 6-well plates as 10.000 cell/2ml medium of each. Cell count was practiced with tripan blue (0.4% w/v in distilled water) on Neubauer Chamber. One day later when the cell count multiplied 2 folds and reached to

20.000/well, pendimethalin and trifluralin solutions were added the wells as the last concentrations were 1, 10, 50, 100 and 500 µM. These concentrations were chosen according to their IC_{50} and toxicity levels (24-27). The cells were incubated for 24 hours and harvested from the wells and centrifugated at 1200 rpm for 5 min.

RNA isolation, cDNA synthesis and gene expression

RNA isolation were performed with RNeasy Mini Kit, QIAGEN a manufacturer's instruction. Briefly, after the centrifugation, cell suspension was filtrated from gDNA eliminator column, then transferred and attached to RNeasy spin column and washed with the solutions as instructed.

The amount and quality of the eliminated RNA samples were measured by Maestrogen Nanodrop. For this measurement, 1 µl of the sample was loaded to the base portion fiber terminal. All the samples' OD 260/280 ratios were found in the range of 1,6-1,8.

The cDNA synthesis was performed from the RNA samples with RT^2 First Strand Kit, QIAGEN as manufacturer's instruction. Shortly, the RNA samples were denaturized at 42 ^{0}C for 5 min in qRT-PCR device. The samples were placed to cold surface to protect the linearity. Then reverse-transcription enzymes were added and the cDNA synthesis process was performed at the degrees of 42^{0}C for 15 minutes and 90^{0}C for 5 minutes. Newly synthesized cDNA samples were stored at -20^{0}C. The PCR primers used in this study were listed in Table 1.

To measure the expression levels of apoptosis-related genes, cDNA samples were mixed with RT qPCR primers (bcl-2, bax, casp3, casp9, tp53, birc and ppia), RT^2 SYBR Green qPCR mastermix and the expression performed with qRT-PCR device under the conditions of hold 95^{0}C 15 min, cycle 95^{0}C 15 sec and 60^{0}C 30 sec, for 40 cycles. The results were recorded at 60^{0}C. The threshold limit was set to 0,05 and the Ct values of the samples were calculated.

Statistical Analysis

Statistics of the Ct values were prepared with the on-line based program RT^2 Profiler PCR Data Analysis 3.5. ΔΔCt method was used to interpret the gene expression data (28). All experiments were performed twice.

Results

Average Ct (mean), average ΔΔCT and fold regulation (up-down regulation) values of A549 cell line after exposed to pendimethalin and trifluralin for 24 h were calculated. ΔCt calculation was preferred to normalize the raw data and Ct value of the housekeeping gene, ppia, was used as the normalization factor. Fold regulation is the ratio of the relative gene expression between the housekeeping gene and the treatment group. This calculation illustrated the up-and down-regulated expressions within th $p<0.05$ significance. The formulas used for the calculations are;

ΔCt = Ct (Gene of Interest) – Average (Ct (Housekeeping / Reference Gene))

ΔΔCt = ΔCt (Test Group n) – ΔCt (Control Group)

Average ΔCt = (ΔCt (Sample1) + ΔCt (Sample2) + … ΔCt (Sample n)) / n Samples

Average $\Delta\Delta Ct = 2^{(-\text{Average } \Delta Ct)}$

Fold regulation = $2^{(-\Delta\Delta Ct)}$

The results were given on Table 2-7.

Due to the fold regulation results of trifluralin, all gene expressions down-regulated at the examined concentrations (1-500 µM). However, pendimethalin showed different regulation profiles on the same genes. The concentrations of 100 and 500 µg/ml, bcl-2 gene and on 500 µg/ml birc5 gene expressions found up-regulated compared to control gene ppia, where other concentrations of pendimethalin the examined genes are down-regulated.

Discussion

Pesticide usage came out with both positive and negative results on human health and environment. They led the increase of the amount and quality of the agricultural products with various health problems and disruption of the soil and water.

In this study, we determined the changes of apoptosis related gene expressions with dinitroaniline herbicides. After 24 h of incubation, at the concentration of 100 µM pendimethalin down-regulated bax, p53, cas3 significantly. Although the cas9 levels did not show any significant change, bcl-2 and birc5 levels are up-regulated with pendimethalin exposure. On the other hand, trifluralin exposure down-regulated all the examined gene levels at all concentrations. It has been showed that p53 is essential for the normal cell apoptosis regulation with the ability to control bax regulation-the

proapoptoic member of bcl-2 family (29). Decreased p53 levels gave rise to cellular viability, lifespan and chromosomal instability (30). This can be stated that increased Bcl-2 expressions come with the decrease of p53 and bax levels and prevent the A549 cells from apoptosis. Also, bax can induce the caspase activation and increase the cellular reactive oxygen species by caspase cleavage (31). The studies demonstrated that cas-3 activation mediates the bax mediated pro-oxidant effects (32) and have an important role on inducing apoptosis via mitochondrial cascade (33). In our study, cas-expressions were found down-regulated with bax which in turn lower the probability of apoptosis on A549 non-small lung cancer cells.

Caspases can initiate the degradation phase of apoptosis with DNA fragmentation and blebbing (34). Cas-9 inhibition was shown to decrease the ROS production in mitochondria (35), and the up-regulation was resulted with induced ROS production and activation of cas-3 and cas-7 (36).

Birc-5 (survivin) showed different effects with the herbicide exposure at the concentration of 100 µM. Although pendimethalin caused up-regulation, trifluralin exposure down-regulated the birc-5 levels significantly. This has been known that Birc-5 is responsible for cell division regulation during the G_1-S phase and it is also considered for anticancer therapies (37). Expressed levels of birc-5 were found higher than normal healthy cells in various tumors such as lung, breast, ovarian and prostate cancers (38-41). One of the study stated that birc-5 silencing suppressed the cell proliferation in A549 non-small lung cancer cells (38). Comparing to our results, we can state that even though pendimethalin reduced the apoptotic cycles with birc-5 up-regulation, trifluralin exposure could not deactivate the programmed cell death in A549 cells at birc-5 level.

According to our findings and the previous studies, pendimethalin and trifluralin exposure was resulted with reduced-apoptosis which in turn lead to tumor growth in A549 cells *in vitro.* As stated before, both of the herbicides significantly changed the expression levels, but pendimethalin has more effects on anti-apoptosis than trifluralin. This study stated that tumor suppression genes can be altered by environmental exposure and further studies will enlighten us about the connection between dinitroaniline herbicides and lung cancer.

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Table 1. The PCR primers we used in this study.

p53	5′-CCCAGCCAAAGAAGAAACCA-3′- (fwd)
	5′-TTCCAAGGCCTCATTCAGCT-3′ (rv);
birc5	5′-AGAACTGGCCCTTCTTGGAGG-3′-(fwd)
	5′-CTTTTTATGTTCCTCTATGGGGTC-3′ (rv)
bax	5′-TGCTTCAGGGTTTCATCCAG-3′ (fwd)
	5′-GGCGGCAATCATCCTCTG-3′ (rv)
bcl-2	5′-AGGAAGTGAACATTTCGGTGAC-3′ (fwd)
	5′-GCTCAGTTCCAGGACCAGGC-3′ (rv)
caspase-3	5′-ACATGGCGTGTCATAAAATACC-3′ (fwd)
	5′-CACAAAGCGACTGGATGAAC-3′ (rv)
caspase-9	5′-CCAGAGATTCGCAAACCAGAGG-3′ (fwd)
	5′-GAGCACCGACATCACCAAATC[illegible]3′ (rv)
housekeeping ppia	5′-AAGGGTTCCTGCTTTCAC-3′ (fwd)
	5′-GGACCCGTATGCTTTA[illegible]G[illegible]′ (rv)

Table 2. Average Ct values of Pendimethalin.

AVG Ct Pendimethalin						
Symbol	**Control**	**PM 50**	**PM 100**	**PM 50**	**PM 10**	**PM 1**
bax	19.48	34.74	33.89	25.66	23.81	19.97
bcl-2	26.87	30.87	30.88	29.97	28.94	24.98
birc5	25.79	33.05	*N/A	31.78	29.99	22.99
p53	20.35	34.43	34.85	26.59	26.04	19.93
cas3	22.79	*N/A	*N/A	28.57	25.94	22.05
cas9	24.27	*N/A	33.31	30.22	25.7	22.9
ppia	23.[illegible]	*N/A	*N/A	27.48	26.36	22.27

*N/A greater than 35

Table 3. Average ΔΔCt values of Pendimethalin.

2^(-Avg.(Delta(Ct)) Pendimethalin						
Symbol	**Control**	**PM 500**	**PM 100**	**PM 50**	**PM 10**	**PM 1**
bax	47.01	1.20	2.17	3.53	5.86	4.92
bcl-2	0.28	17.57	17.39	0.18	0.17	0.15
birc5	0.59	2.07	1.00	0.05	0.08	0.61
p53	25.72	1.49	1.11	1.86	1.24	5.06
cas3	4.74	1.00	1.00	0.47	1.33	1.16
cas9	1.69	1.00	3.24	0.15	1.58	0.65
ppia	1.00	1.00	1.00	1.00	1.00	1.0

Table 4. Fold regulation (up-down) values of Pendimethalin.

Up-Down Regulation (comparing to control group) Pen imeth in					
	PM 500	**PM 100**	**PM 50**	**PM 10**	**PM 1**
Symbol	**Fold Regulation**	**Fold Regulation**	**Fold Regulatio**	**Fold egulation**	**Fold Regulation**
bax	-39.1245*	-21.7057*	-13.3152*	-8.0 8*	-9.5467*
bcl	62.6829**	62.0345**	-1.569	818***	-1.834***
birc5	3.4943**	1.6876***	-11.6 8	-7.336	1.0281***
p53	-17.2677*	-23.1831*	5.83	-20.6776*	-5.0806*
cas3	-4.7404*	-4.7404*	-1 0561	-3.5554*	-4.0699*
cas9	-1.6935***	1.9119***	-11.2 46*	-1.0718***	-2.6208*
ppia	1	1	1	1	1

* Downregulated expression compared to the control g p (ppia) p 0.05

** Upregulated expression compared to the c tro g p (p 0.05

*** No significant changes observed compa d to e co rol group

Table 5. Average C values f Trifluralin.

AVG Ct Trifluralin						
Symbol	**C ntrol G oup**	**TF 500**	**TF 100**	**TF 50**	**TF 10**	**TF 1**
bax	19.48	22.83	18.29	18.12	18.79	18.35
bcl	26. 7	28.78	25.21	25.78	26.36	25.97
5	25 9	29.42	24.85	24.52	24.49	23.77
p53	20.35	24.86	19.87	19	19.7	19.33
as3	22.79	26.05	21.41	21.9	22.77	22.33
ca 9	24.27	26.12	22.55	22.65	23.21	23.16
ppia	25.03	24.96	20.74	20.26	20.94	21.07

Table 6. Average ΔΔCt values of Trifluralin.

2^(-Avg.(Delta(Ct)) Trifluralin						
Symbol	**Control Group**	**TF 500**	**TF 100**	**TF 50**	**TF 10**	**TF 1**
bax	47.01	4.38	5.45	4.42	4.44	6.61
bcl-2	0.28	0.07	0.05	0.02	0.02	0.03
birc5	0.59	0.05	0.06	0.05	0.09	0.15
p53	25.72	1.07	1.83	2.39	2.35	3.35
cas3	4.74	0.47	0.63	0.32	0.28	0.42
cas9	1.69	0.45	0.28	0.19	0.21	0.24
ppia	1.00	1.00	1.00	1.00	1.00	1.00

Table 7. Fold regulation (up-down) values of Trifluralin.

Up-Down Regulation (comparing to control group) Trifluralin					
	TF 500	**TF 100**	**TF 50**	**TF 10**	**TF 1**
Symbol	**Fold Regulation**	**Fold Regulation**	**Fold Regulation**	**Fold Regulation**	**Fold Regulation**
bax	-10.7406*	-8.6338*	-10.6295*	-10.5927*	-7.1107*
bcl-2	-3.9449*	-6.2118*	-12.8616	-[illegible]0003*	-8.3397*
birc5	-13.0412*	-10.2319*	-[illegible]*	-6.9644*	-3.8504*
p53	-24.0006*	-14.0744*	-[illegible]0.740[illegible]	-10.9283*	-7.6741*
cas3	-10.091*	-7.5685*	-[illegible]4.[illegible]741*	-16.8538*	-11.3137*
cas9	-3.7842*	-5.9587*	-[illegible]766*	-8.1965*	-7.1851*
ppia	1	1	1	1	1

* Downregulated expression compared to the control group (ppia) p<0.[illegible]

** Upregulated expression compared to the control group (ppia) p<[illegible]05

*** No significant changes observed compared to the control group