

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

Objectives: Pendimethalin and trifluralin are commonly used in many countries to control broadleaf weeds and grassy weed species because of their inhibitor 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 ppia 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. Conclusion: Care should be taken not to exceed the permissible values and residue limits on the food during pendimethalin and trifluralin use in order to reduce the possible carcinogenic effects on humans.

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

Pendimetalin ve Trifluralinin Apoptotik ve Anti-apoptotik Etkilerinin A549 Hücrelerinde *In Vitro* Değerlendirilmesi

Amaç: Pendimetalin ve trifluralin birçok ülkede, büyüme ve hücre bölünmesi üzerindeki inhibitör etkileri nedeniyle, geniş yapraklı yabancı otları ve çimenli ot türlerini kontrol etmek amacıyla yaygın şekilde kullanılmaktadır. 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 ppi'a'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. 100µM 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: Pendimetalin ve trifluralinin insanlar üzerindeki olası kanserojenik etkilerini azaltabilmek için, kullanımları sırasında izin verilen değerlerin ve gıdalar üzerindeki kalıntı limitlerinin aşılmasına dikkat edilmelidir.

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

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 sulfonylaniline 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 cm² cell culture flask and transferred to 75 cm² flask after 24 h under the conditions of 5% CO₂ and 37 °C. 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 IC₅₀ 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 as 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 μ l of the sample was loaded to the base portion fiber terminal. All the samples' OD 260/280 ratio were found in the range of 1,6-1,8.

The cDNA synthesis was performed from the RNA samples with RT² First Strand Kit, QIAGEN as manufacturer's instruction. Shortly, the RNA samples were denaturized at 42 °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°C for 15 minutes and 90°C for 5 minutes. Newly synthesized cDNA samples were stored at -20°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 RT² qPCR primers (bcl-2, bax, casp3, casp9, tp53, birc and ppia), RT² SYBR Green qPCR mastermix and the expression performed with qRT-PCR device under the conditions of hold 95°C 15 min, cycle 95°C 15 sec and 60°C 30 sec, for 40 cycles. The results were recorded at 60°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 RT² Profiler PCR Data Analysis 3.5. $\Delta\Delta$ Ct method was used to interpret the gene expression data (28). All experiments were performed twice.

Results

Average Ct (mean), average $\Delta\Delta$ CT and fold regulation (up-down regulation) values of A549 cell line after exposed to pendimethalin and trifluralin for 24 h were calculated. Δ Ct 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 the $p < 0.05$ significance. The formulas used for the calculations are;

$$\Delta Ct = Ct (\text{Gene of Interest}) - \text{Average } (Ct (\text{Housekeeping} / \text{Reference Gene}))$$

$$\Delta\Delta Ct = \Delta Ct (\text{Test Group } n) - \Delta Ct (\text{Control Group})$$

$$\text{Average } \Delta Ct = (\Delta Ct (\text{Sample1}) + \Delta Ct (\text{Sample2}) + \dots \Delta Ct (\text{Sample } n)) / n$$

Samples

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

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

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 $\mu\text{g/ml}$, bcl-2 gene and on 500 $\mu\text{g/ml}$ 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 proapoptotic 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-3 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 μ M. 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 G₁-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.

Acknowledgement's

"This work was supported by the Hacettepe BAP under Grant number 1603."

References

1. Wallace DR, Trifluralin in Encyclopedia of Toxicology, In: Wexler P, ed. Encyclopedia of Toxicology, 3rd ed. Oxford: Academic Press, pp. 388-389, 2014.
2. Agronomy 317, Intro to Weeds, "Dinitroanilines", Accessed: 03.02.1017, Available via "<http://agron.iastate.edu/-weeds/Ag317-99/manage/herbicide/dnas.html>".
3. T.C. Milli Eğitim Bakanlığı, Çevre Sağlığı, Pestisitler, 850 CK 0054, Ankara, 2012.
4. Kegley S, Hill B, Orme S, Pendimethalin. PAN Pesticide Database, Pesticide Action Network, North America, San Francisco, CA. Accessed 23.12.2015, Available via "<http://www.pesticideinfo.org>".
5. US Environmental Protection Agency (US EPA), R.E.D. Facts: Pendimethalin, US EPA, Washington, DC, 1997.
6. Zimmermann KC, Green DR, How cells die: Apoptosis pathways, J Allergy Clin Immunol 108,99-103, 2001.
7. Martin SJ, Green DR, Protease activation during apoptosis: death by a thousand cuts?, Cell 82, 349-352, 1995.
8. McIlwain DR, Berger T, Mak TW, Caspase functions in cell death and disease, Cold Spring Harb Perspect Biol 5, 2013.
9. Riedl SJ, Shi Y, Molecular mechanisms of caspase regulation during apoptosis, Nat Rev Mol Cell Biol 5, 897-907, 2004.
10. Vermeulen K, Van Bockstaele DR, Berneman ZN, Apoptosis: mechanisms and relevance in cancer, Ann Hematol 84, 627-639, 2005.
11. Kataoka S, Tsuruo T, Physician Education: Apoptosis, Oncologist 1, 399-401, 1996.
12. Wei MC, Wei-Xing Z, Cheng EHY, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB, Korsmeyer SJ, Proapoptotic BAX and BAK: A Requisite Gateway to Mitochondrial Dysfunction and Death, Science (New York, N.Y.) 292, 727-730, 2001.
13. Cory S, Adams JM, The Bcl2 family: regulators of the cellular life-or-death switch, Nat Rev Cancer 2, 647-656, 2002.
14. Hussain SP, Harris CC, Molecular epidemiology of human cancer: contribution of mutation spectra studies of tumor suppressor genes, Cancer Res 58, 4023-4037, 1998.

15. Chi C, Chou CT, Liang WZ, Jan CR, Effect of the pesticide, deltamethrin, on Ca²⁺ signaling and apoptosis in OC2 human oral cancer cells, *Drug Chem Toxicol* 37, 25-31, 2014.
16. Raszewski G, Lemieszek MK, Lukawski K, Juszczak M, Rzeski W, Chlorpyrifos and cypermethrin induce apoptosis in human neuroblastoma cell line SH-SY5Y, *Basic Clin Pharmacol Toxicol* 116, 158-167, 2015.
17. Jang Y, Lee AY, Jeong SH, Park KH, Paik MK, Cho NJ, Kim JE, Cho MH, Chlorpyrifos induces NLRP3 inflammasome and pyroptosis/apoptosis via mitochondrial oxidative stress in human keratinocyte HaCaT cells, *Toxicology* 338, 37-46, 2015.
18. Banerjee BD, Seth V, Ahmed RS, Pesticide-induced oxidative stress: perspectives and trends, *Rev Environ Health* 16, 1-40, 2001.
19. Li Q, Kobayashi M, Kawada T, Carbamate pesticide-induced apoptosis and necrosis in human natural killer cells, *J Biol Regul Homeost Agents* 28, 23-32, 2014.
20. Nick P, Control of plant shape, In *Plant Microtubules: Potential for Biotechnology*, eds. Peter Nick, Berlin- Heidelberg, Springer Berlin-Heidelberg, 25-50, 2000.
21. Tresch S, Plath P, Grossmann K, Herbicidal cyanoacrylates with antimicrotubule mechanism of action, *Pest Management Science* 61, 1052-1059, 2005.
22. EXTOKNET, Extension Toxicology Network Pesticide Information Profiles: Pendimethalin, 1996a. Accessed 23.12.2015, Available via <http://extoxnet.orst.edu/pips/pendimet.htm>
23. EXTOKNET. Extension Toxicology Network Pesticide Information Profiles: Trifluralin, 1996b. Accessed 23.12.2015, Available via <http://extoxnet.orst.edu/pips/triflura.htm>
24. Dimitrov BD, Gadeva PG, Benova DK, Bineva MV, Comparative genotoxicity of the herbicides Roundup, Stomp and Reglone in plant and mammalian test systems, *Mutagenesis* 21, 375-382, 2006.
25. Patel S, Bajpayee M, Pandey AK, Parmar D, Dhawan A, In vitro induction of cytotoxicity and DNA strand breaks in CHO cells exposed to cypermethrin, pendimethalin and dichlorvos, *Toxicol In Vitro* 21, 1409-1418, 2007.
26. Zaidenberg A, Marra C, Luong T, Gomez P, Milani L, Villagra S, Drut R, Trifluralin toxicity in a Chagas disease mouse model, *Basic Clin Pharmacol Toxicol* 101, 90-95, 2007.

27. Weir SM, Yu S, Salice CJ, Acute toxicity of herbicide formulations and chronic toxicity of technical-grade trifluralin to larval green frogs (*Lithobates clamitans*), *Environ Toxicol Chem* 31, 2029-2034, 2012.
28. Livak, K. J., and Schmittgen, T. D. 2001. " Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method." *Methods* 25 (4):402-408.
29. Fridman JS, Lowe SW, Control of apoptosis by p53, *Oncogene* 22, 9030-9040, 2003.
30. Lowe SW, Lin AW, Apoptosis in cancer, *Carcinogenesis* 21, 485-95, 2000.
31. Ricci JE, Munoz-Pinedo C, Fitzgerald P, Bailly-Maitre B, Perkins GA, Yadava N, Scheffler IE, Ellisman MH, Green DR, Disruption of mitochondrial function during apoptosis is mediated by caspase cleavage of the p75 subunit of complex I of the electron transport chain, *Cell* 117, 773-786, 2004.
32. Kirkland RA, Franklin J, Bax and caspases regulate increased production of mitochondria-derived reactive species in neuronal apoptosis: LACK of A role for depletion of cytochrome c from the mitochondrial electron transport chain, *Biochemistry and Biophysics Reports* 4, 158-168, 2015.
33. Chakraborty SM, Mazumdar S, Mukherjee P, Bhattacharjee A, Adhikary A, Manna S, Chakraborty P, Khan A, Sen A, Das T, Restoration of p53/miR-34a regulatory axis decreases survival advantage and ensures Bax-dependent apoptosis of non-small cell lung carcinoma cells, *FEBS Lett* 588, 549-559, 2014.
34. Srinivasula SM, Ahmad M, Fernandes-Alnemri T, Alnemri ES, Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization, *Mol Cell* 1, 949-957, 1998.
35. Cepero EA, King M, Coffey LM, Perez RG, Boise LH, Caspase-9 and effector caspases have sequential and distinct effects on mitochondria, *Oncogene* 24, 6354-6366, 2005.
36. Brentnall M, Rodriguez-Menocal L, De Guevara RL, Cepero E, Boise LH, Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis, *BMC Cell Biology* 14, 1-9, 2013.
37. Wheatley SP, McNeish IA, Survivin: a protein with dual roles in mitosis and apoptosis, *Int Rev Cytol* 247, 35-88, 2005.
38. Monzo M, Rosell R, Felip E, Astudillo J, Sanchez JJ, Maestre J, Martin C, Font A, Barnadas A, Abad A, A novel anti-apoptosis gene: Re-expression of survivin

messenger RNA as a prognosis marker in non-small-cell lung cancers, *J Clin Oncol* 17, 2100-2104, 1999.

39. Tanaka KS, Iwamoto G, Gon T, Nohara M, Iwamoto M, Tanigawa N, Expression of survivin and its relationship to loss of apoptosis in breast carcinomas, *Clin Cancer Res* 6, 127-134, 2000.
40. Krajewska M, Krajewski S, Banares S, Huang X, Turner B, Bubendorf L, Kallioniemi OP, Shabaik A, Vitiello A, Peehl D, Gao GJ, Reed JC, Elevated expression of inhibitor of apoptosis proteins in prostate cancer, *Clin Cancer Res* 9, 4914-4925, 2003.
41. Xing J, Jia CR, Wang Y, Guo J, Cai Y, Effect of shRNA targeting survivin on ovarian cancer, *J Cancer Res Clin Oncol* 138, 1221-1229, 2012.

Uncorrected proof