Evaluation of the Methylation and Acetylation Profiles of Dinitroaniline Herbicides and Resveratrol on the V79 Cell Line

Objective:
Herbicides are among the most widely used pesticide compounds for plant growth control worldwide. Risk assessment of the dinitroaniline-derived herbicides pendimethalin and trifluralin is important for foodborne or other means of exposure. In this study, we aimed to evaluate the methylation and acetylation profiles of pendimethalin and trifluralin, which we have high levels of exposure to in various ways. Furthermore, we also determined the protective effect of resveratrol, an antioxidant compound, against the possible toxic effects of these pesticides.

Materials and Methods:
The effects of pendimethalin and trifluralin alone (25, 50, 100 µM) and in combination with resveratrol (100 µM) on DNA methyltransferase (DNMT) 1, 3a, and 3b; and histone deacetylase (HDAC) 1 and HDAC3 gene expression were evaluated by real-time polymerase chain reaction.

Results:
According to the results, pendimethalin caused a significant decrease in DNMT1, 3a, 3b and HDAC expressions at all concentrations, whereas HDAC1 and 3 expression was increased at the concentration of 25 µM, when applied together with resveratrol. There were no changes in DNMT1 or 3b expression levels. Unlike pendimethalin, trifluralin increased DNMT1 expression in a concentration-dependent manner. While DNMT3a and DNMT3b expression levels increased significantly, HDAC1 and 3 expression levels did not change significantly. The expression levels of HDAC1 and HDAC3 increased at all concentrations of trifluralin combination with resveratrol. Moreover, DNMT levels increased at the concentrations of 50 and 100 µM.

Conclusion:
Epigenetic gene expression results showed that pendimethalin and trifluralin might cause tissue function loss and chromosome damage as a result of direct effects on cell viability by causing expression level changes in all studied genes. It can also be concluded that the changes that occur in gene expression may induce tumor development. Further studies are needed to elucidate the possible toxicity mechanisms of these herbicides, considering the relationship between epigenetic changes and various diseases.

Key words: Pendimethalin, trifluralin, epigenetic, DNA methyltransferase, histone deacetylase

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ÖZ

Amaç: Herbisitler, dünya genelinde bitki büyüme kontrolü için en yaygın kullanılan pestisit bileşiklerindendir. Dinitroanilin türevi herbisitlerin ve resveratrolun metilasyon ve asetilasyon profillerini V79 hücre hattında değerlendirilmesi

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INTRODUCTION

The most important problem for humans since the establishment of residential life has been to produce sufficient nutrients. For this purpose, it is necessary to eliminate insects, fungi, weeds, and other harmful organisms that damage crops in order to increase the quantity and quality of the product. It is also important to combat these pests in terms of health, given the fact that they spread diseases. Although the use of pesticides is necessary, toxic effects can be observed in organisms and in the environment as a result of widespread and uncontrolled use. Due to incorrect or careless use of pesticides, cases of mass poisoning can occur.

In addition, long-term pesticide exposure is linked to cancer, immune system damage, and reproductive toxicity. Herbicides, which are among the most commonly used pesticide compounds in the world, are chemical compounds or cultured biological organisms controlling or suppressing plant growth. Pendimethalin and trifluralin are dinitroaniline herbicides that provide the control of certain broad-leaf and grassy weeds inhibiting mitosis. These herbicides have been used on vegetables, tobacco, oil seed, ornamentals, tomatoes, and cotton for a long time. For this reason, they can affect health via environmental pollution or diet.

Pendimethalin and trifluralin synthesis can cause the formation of reactive compounds known as nitrosamines. Nitrosamines are alkylating agents and can cause DNA damage by formation of adducts. Additionally, epigenetic changes, which are basically related to DNA methylation and histone acetylation mechanisms, are as important as genetic changes because of the 1 genome/n epigenomes relation. The genome-epigenome relationship is thought to play an active role in basic biological functions such as cell viability, cell division, cell differentiation, and phenotypic changes. Although epigenetic research has focused on embryonic development, aging, and cancer, recent research has been advancing in various areas such as the immune system, cardiovascular system, neurodegenerative diseases, obesity, and diabetes.

In the present study, the epigenetic potential of pendimethalin and trifluralin on Chinese hamster lung fibroblast (V79) cells were investigated. We evaluated the DNA methyltransferase (DNMT) 1, 3a, and 3b; and histone deacetylase (HDAC) 1 and 3 levels on V79 cells after 24 h treatment of pendimethalin and trifluralin at the concentrations of 25, 50, and 100 µM, which were determined based on our previous study results from a neutral red uptake assay and comet assay. The effects of resveratrol, a strong antioxidant compound, were also examined at the concentration of 100 µM.

MATERIALS AND METHODS

Pendimethalin, trifluralin, and resveratrol solution preparation

Pendimethalin (98.8% purity, CAS no: 40487-42-1), trifluralin (98.8% purity, CAS no: 1582-09-8), and resveratrol (99% purity, CAS no: R5010) were purchased from Sigma-Aldrich. Pendimethalin stock solution (500 mM) was prepared in dimethyl sulfoxide (DMSO): olive oil (1:3, v/v), and trifluralin (500 mM) and resveratrol stock solution (0.5 mM) were prepared in phosphate buffered saline containing DMSO [final DMSO concentration was 1% (v/v)].

Cell culture

V79 cells obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) were incubated in RPMI 1640 medium supplemented with 1% penicillin-streptomycin solution, 10% heat-inactivated fetal bovine serum (Lot: 094M3288), and 2 mM L-glutamine at 37°C and in 5% CO₂ for 24 h. After 24 h, the cells were harvested and were transferred to 6 well plates as 30,000 cells/2 mL medium of each. Pendimethalin and trifluralin solutions were added to the wells at the concentrations of 25, 50, and 100 µM after 24 h. Moreover, 100 µM resveratrol was used as a single concentration and additionally added to the concentrations of pendimethalin and trifluralin. For the negative control, 1% DMSO and 1% DMSO/3% olive oil were used. The cells were incubated for 24 h and harvested from the wells and centrifuged at 1000 rpm for 5 min.

Evaluation of gene expression profiles by reverse transcription-polymerase chain reaction (RT-PCR) assay

RNA isolation was performed according to the instructions of the RNeasy Mini Kit (QIAGEN). The cell suspensions were filtrated using a gDNA eliminator column after centrifugation. Then they were transferred to the RNeasy spin column and washed with the solutions as given in the kit procedure. The measurement of the amount and quality of the eliminated RNA samples was performed by Maestrogen Nanodrop. Briefly, 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. For the purpose of synthesizing cDNA from the RNA samples, an RT First Strand Kit (QIAGEN) was used according to the instructions. The denaturation of the RNA samples was performed at 42°C for 5 min in the qRT-PCR device. To preserve linearity, the samples were placed on a cold surface. After that, reverse-transcription enzymes were added to the samples and the cDNA synthesis process was performed at 42°C for 15 min and 90°C for 5 min. The synthesized cDNA samples were stored at -20°C. The PCR primers used are listed in Table 1.
For measuring the expression levels of genes, cDNA samples were mixed with RTq SYBR Green qPCR MasterMix and RTq qPCR primers (DNMT1, DNMT3a, DNMT3b, HDAC1, HDAC3, and PPIA) and the expression performed with the qRT-PCR device under the conditions of hold at 95°C 15 min and cycle at 95°C 15 s and 60°C 30 s, for 40 cycles. The results were recorded at 60°C. The threshold limit was set to 0.05 and the cycle threshold (CT) values of the samples were calculated (Table 1).13,14

Statistical analysis

Statistics of the CT values were prepared using the online-based program RTq profiler PCR Data Analysis 3.5. The ∆∆CT method was used to interpret the gene expression data.15 When evaluating the results, the upper limit CT value was taken as 35. Values higher than 35 were evaluated as 35. All experiments were performed twice.

RESULTS

Effects of trifluralin on gene expression

According to the ∆∆CT values, DNMT1 expression in V79 cells increased at a higher level and concentration relative to the control group with 24 h incubation of trifluralin. The levels of DNMT3a and 3b only increased significantly at high concentration. There were no significant changes in HDAC1 or 3 levels.

When resveratrol was administered alone, the levels of DNMT1, 3a, and 3b; and HDAC1 increased significantly compared to the control, but HDAC3 levels remained unchanged.

Furthermore, when trifluralin and resveratrol were co-administered, HDAC1 and 3 expression levels were significantly increased at all concentrations. DNMT levels were increased in 25 and 50 µM trifluralin and resveratrol, whereas 100 µM trifluralin and resveratrol were low in expression.

Generally, when the results of fold regulation and biological significance of trifluralin and resveratrol were examined, a significant increase in expression was observed in all genes except HDAC3 when resveratrol was administered alone. It was observed that trifluralin generally decreased HDAC1 and 3 expression. When trifluralin was combined with resveratrol, it caused an increase in HDAC1 and 3 expression, except 100 µM trifluralin and resveratrol administration. Additionally, DNMT1 showed a significant increase in all studied concentrations, whereas DNMT3a and 3b expression levels increased when 100 µM trifluralin was given. DNMT3b decreased at all concentrations when co-administered with resveratrol, while DNMT3a was significantly reduced only when 25 µM trifluralin and 100 µM resveratrol were co-administered.

The ∆CT, ∆∆CT, fold change, and fold regulation values of the genes are given in Tables 2-5.

Effects of pendimethalin on gene expression

When ∆∆CT values were compared, it was observed that DNMT and HDAC expression levels were significantly decreased in all concentrations of pendimethalin. Moreover, DNMT1 and HDAC1 and 3 expression levels were increased significantly at only 25 µM pendimethalin concentration when given together with resveratrol. However, all gene expression was significantly increased when resveratrol was administered alone.

When the pendimethalin and resveratrol fold-regulation and biological significance results were evaluated, it was seen that

| Gene sequences of primers13,14 |
|------------------------------|
| Gene | Forward | Reverse |
| DNMT1 | 5'-AAC CTT CAC CTA GCC CCA G-3' | 5'-CTC ATC CGA TTT GGC TCT TCA-3' |
| DNMT3a | 5'-CGA CCC ATG CCA AGA CTC ACC TTC CAG-3' | 5'- CCT GGT GGA ATG CAC TGC AGA AGG A-3' |
| DNMT3b | 5'-TAC ACA GAC GTG TCC AAC ATG GGC-3' | 5'-GGA TGC CTT CAG GAA TCA CAC CTC-3' |
| HDAC1 | 5'-CTG TCC GGT ATT TGA TGG CTG-3' | 5'-CAC GAA TCC CAC ACA CGG G-3' |
| HDAC3 | 5'-TCT GAG GAC TAT AGC GAC TCC TCC-3' | 5'-GTC GCC ATC ATA GAA CAC ATG T-3' |
| PPIA | 5'-ATG GTC AAC CCC ACC GTG T-3' | 5'-TCT GCT GTC TTT GCC ACC TTG T-3' |

DNMT: DNA methyltransferase, HDAC: Histone deacetylase, PPIA: Peptidylprolyl isomerase A

| Table 2. ΔCT values of trifluralin and resveratrol the values are expressed in mean ± standard deviation format |
|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Gene          | Control (1% DMSO) | t 25 | t 50 | t 100 | t 25 + r 100 | t 50 + r 100 | t 100 + r 100 |
| PPIA          | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| DNMT1         | 11.29±2.55 | 3.465±19.70 | 6.755±0.346 | 5.085±2.84 | 4.67±0.226 | 3.855±16.94 | 2.07±14.07 | 4.785±14.61 |
| DNMT3a        | 5.68±13.94 | 2.48±21.10 | 6.754±0.347 | 6.375±1.02 | 6.38±0.225 | 9.375±22.21 | 6.3±17.90 | 6.4±13.50 |
| DNMT3b        | 7.3±14.96 | 3.18±20.11 | 6.756±0.344 | 6.374±1.02 | 6.5±0.224 | 9.135±22.54 | 9.6±18.76 | 24.305±3.81 |
| HDAC1         | 5.27±13.88 | 2.83±20.60 | 6.753±0.347 | 6.373±1.01 | 6.48±0.227 | (-) | 3.93±5.99 | (-) | 6.905±5.16 | (-) | 7.115±21.41 |
| HDAC3         | 2.54±8.71 | 2.565±19.93 | 6.755±0.345 | 6.376±1.04 | 6.49±0.223 | (-) | 6.36±1.61 | (-) | 7.875±0.86 | (-) | 6.15±3.58 |

The control gene PPIA value was taken as 0. The concentrations (25, 50, and 100 µM) of trifluralin are shown as t 25, t 50, and t 100. The concentration (100 µM) of resveratrol is shown as r 100. DNMT: DNA methyltransferase, HDAC: Histone deacetylase, PPIA: Peptidylprolyl isomerase A, DMSO: Dimethyl sulfoxide, CT: Cycle threshold
Table 3. ΔΔCT values of trifluralin and resveratrol, the values are expressed as ΔΔCT values

| Gene       | Control (1% DMSO) | r 100 | t 25  | t 50  | t 100 | t 25 + r 100 | t 50 + r 100 | t 100 + r 100 |
|------------|--------------------|-------|-------|-------|-------|--------------|--------------|---------------|
| PPIA       | 1                  | 1     | 1     | 1     | 1     | 1            | 1            | 1             |
| DNMT1      | 0.000399           | 0.090559 | 0.009259 | 0.029462 | 0.039282 | 0.069108 | 0.238159 | 0.036272     |
| DNMT3a     | 0.019505           | 0.179244 | 0.009258 | 0.012049 | 0.039283 | 0.001506 | 0.011842 | 0.011679     |
| DNMT3b     | 0.006346           | 0.110338 | 0.009260 | 0.012048 | 0.039280 | 0.001779 | 0.001236 | 0             |
| HDAC1      | 0.025827           | 0.140632 | 0.009257 | 0.012047 | 0.039283 | 15.242208 | 119.842848 | 70.007239     |
| HDAC3      | 0.171943           | 0.168992 | 0.009259 | 0.012050 | 0.039284 | 82.139257 | 234.753035 | 69.310403     |

The control gene PPIA value was taken as 1. The concentrations (25, 50, and 100 µM) of trifluralin are shown as t 25, t 50, and t 100. The concentration (100 µM) of resveratrol is shown as r 100.

| Gene       | Control (1% DMSO) | r 100 | t 25  | t 50  | t 100 | t 25 + r 100 | t 50 + r 100 | t 100 + r 100 |
|------------|--------------------|-------|-------|-------|-------|--------------|--------------|---------------|
| PPIA       | 1                  | 1     | 1     | 1     | 1     | 1            | 1            | 1             |
| DNMT1      | 226.7565±          | 231.831± | 73.7719± | 98.36±  | 173.0446± | 596.343± | 90.8239±     |
| DNMT3a     | 91896±             | 0.4747* | 0.6177 | 2.0139± | 0.0772* | 0.6071 | 0.5987       |
| DNMT3b     | 17.3878±           | 1.459 | 1.8987 | 6.1903± | 0.2803* | 0.1948* | 0            |
| HDAC1      | 5.4453±            | 0.3585* | 0.4665* | 1.521 | 590.1754± | 4640.2924± | 380.2803±     |
| HDAC3      | 0.9828             | 0.0538* | 0.0701* | 0.2285* | 477.7129± | 1365.2978± | 403.1017±     |

The concentrations (25, 50, and 100 µM) of trifluralin are shown as t 25, t 50, and t 100. The concentration (100 µM) of resveratrol is shown as r 100. A significant increase in gene expression is shown with +, a decrease in gene expression is shown with *. P<0.05 means significantly different from the negative control.

Table 6. ΔCT values of pendimethalin and resveratrol, the values are expressed in mean ± standard deviation format

| Gene       | Control (1% DMSO + %3 olive oil) | p 25  | p 50  | p 100 | p 25 + r 100 | p 50 + r 100 | p 100 + r 100 |
|------------|----------------------------------|-------|-------|-------|--------------|--------------|---------------|
| PPIA       | 0                                | 0     | 0     | 0     | 0            | 0            | 0             |
| DNMT1      | 1.27±11.07                       | 8.88±2.12 | 11.13±0.65 | 7.58±0.52 | 0.98±13.74 | 6.98±0.12 | 7.33±2.03     |
| DNMT3a     | 5.545±16.22                      | 10.28±0.14 | 11.835±0.34 | 13.33±0.41 | 18.09±2.39 | 14.86±0.18 | 12.58±1.86   |
| DNMT3b     | 5.36±18.69                       | 10.215±0.049 | 11.836±0.33 | 13.32±0.40 | 5.43±21.75 | 15.025±0.049 | 15.215±0.64  |
| HDAC1      | 2.405±15.37                      | 10.27±0.13 | 11.834±0.34 | 13.34±0.41 | 18.09±2.39 | 14.86±0.18 | 12.58±1.86   |
| HDAC3      | 8.485±0.34                       | 9.53±0.91 | 11.02±0.80 | 10.76±1.05 | 0.5±11.32 | 9.75±0.17 | 8.39±1.09    | 2.21±20.43   |

The control gene PPIA value was taken as 0. The concentrations (25, 50, and 100 µM) of pendimethalin are shown as p 25, p 50, and p 100. The concentration (100 µM) of resveratrol is shown as r 100. DMSO: Dimethyl sulfoxide, DNMT: DNA methyltransferase, HDAC: Histone deacetylase, PPIA: Peptidylprolyl isomerase A.
of resveratrol at a concentration of 25 µM of pendimethalin, but DNMT3a levels were significantly decreased. There were no changes in DNMT1 or 3b or HDAC3 levels, while expression of other genes was significantly reduced when 50 µM pendimethalin and 100 µM resveratrol were co-administered. The ∆CT, ∆∆CT, fold change, and fold regulation values of the genes are given in Tables 6-9.

**DISCUSSION**

Although genetic material, which is the source of information and life of organisms, is very well protected against degradation by various mechanisms, it may be damaged by exposure to many factors, both internal and external. The DNA repair mechanisms are very active, but they are not sufficient or are repressed in some cases. These types of damage have temporary or permanent effects and may cause minor or major dysfunctions and diseases in the organism and affect future generations besides the organism first affected.

Within the scope of the present study, the possible epigenetic effects of pendimethalin and trifluralin, herbicide compounds that we are frequently exposed to in this country as well as the rest of the world, were investigated in the V79 cell line. It has been evaluated whether resveratrol, an antioxidant substance, has a protective effect on possible methylation and acetylation profile changes of these herbicides.

DNMT and HDAC expression levels were examined to investigate the effects of pendimethalin and trifluralin, dinitroaniline herbicide compounds whose genotoxicity potentials were determined, on epigenetic changes. Based on the genotoxicity...
results, pendimethalin and trifluralin concentrations of 25, 50, and 100 µM were selected for study.

While pendimethalin caused a significant decrease in DNMT levels, trifluralin increased DNMT1 expression and increased all of the DNMT genes at a concentration of 100 µM, causing a decrease in all other genes. Embryo death was observed in mice with increased methylation in DNMT1 gene disorder. Changes in DNMT1 expression lead to X chromosome inactivation and imprinting loss. Disorders in DNMT1 gene expression cause proliferation disorders and mitotic defects leading to cell death. These effects in human colorectal cancer cells have been clearly observed. Similarly, it was reported that mouse fibroblast cells with DNMT1 defect were dragged into apoptosis via the p-53 pathway after several cell divisions, and apoptosis was observed as a result of a decrease in DNMT1 expression in germ cells. Studies have shown that the DNMT1 gene plays a critical role in embryonic development in mice. It was observed that mouse embryos with DNMT3b deficiency died at 9.5 embryonic days and multiple developmental defects occurred; pups without DNMT3a deficiency did not develop and died shortly after birth. Mutations in the DNMT3b gene in humans are the cause of a rare autosomal disease, immunodeficiency, centromere instability, and facial abnormalities syndrome.

Furthermore, mutations in the DNMT3b gene cause a decrease in DNA methylation specific to pericentromeric regions on chromosomes 1, 9, and 16, leading to chromosomal structure and function disorders.

CpG methylation levels were found to be increased in lung cancer patients on two genes, SFTPA1 and SFTPA2, which encode surfactant protein A, associated with lung homeostasis and immunity. In another study, when epigenetic changes were examined in 28 nonsmoking lung adenocarcinoma patients, it was found that methylation levels decreased in tumor tissues compared to neighboring nonmalignant tissues and methylation increased in tumor tissues in CpG islands. Those findings were consistent with the results we obtained, and pendimethalin and trifluralin compounds significantly changed methylation levels. Our evaluation of HDAC gene expression levels showed that both herbicidal compounds cause significant decrease in HDAC1 and 3 levels. HDAC1 and 3 consist of 93% structurally the same proteins and belong to the class I histone deacetylases group. These genes are related to cell cycle control, cell survival, and differentiation. For this reason, the use of HDAC inhibitors for the treatment of cancer as an antineoplastic drug is contemplated. In a study of non-small lung cancer cells, it was observed that HDAC levels were increased in cancer cells and it was possible to fight cancer cells using HDAC inhibitors. However, these results are not consistent with our previous study, which was about the effects of pendimethalin and trifluralin on apoptosis and anti-apoptosis genes (p53, bax, bcl-2, casp3, casp9, and birc). According to our results, trifluralin downregulated the expression of all genes (1-500 µM), but pendimethalin upregulated bcl-2 (100 and 500 µg/mL) and birc5 (500 µg/mL) gene expression and had more effects on anti-apoptosis than trifluralin. These differences in results confirm that in order to reduce the possible carcinogenic effects of pendimethalin and trifluralin in humans, the permissible values and residual limits on foods should not be exceeded.

When the change in the epigenetic expression levels due to resveratrol was examined, the capacity of resveratrol supplementation to reverse the expression changes caused by the herbicides studied was limited. Additionally, normal gene expression levels were not achieved despite resveratrol, especially in HDAC genes. Furthermore, administration of resveratrol alone led to undesirable increases in gene expression possibly as a result of the pro-oxidant effect of resveratrol.

CONCLUSION

Methylation and deacetylation gene expression are among the main pathways of epigenetic changes and they are the main causes of embryonic development disorders and chronic diseases. According to the epigenetic gene expression results, pendimethalin and trifluralin may cause tissue function loss and chromosome damage as a result of direct effects on cell viability by causing expression level changes in all studied genes. Since the groups of cells we studied were healthy lung fibroblast cells, it can be concluded that the changes that occur in gene expression may induce tumor development. Considering the concentrations used, the genotoxic effects appear to be high. However, both herbicidal compounds we investigated are considered group C, as a possible human carcinogen by the Environmental Protection Agency.

In addition to the beneficial effects of antioxidants such as resveratrol against oxidative DNA damage, there is also the risk of causing damage by pro-oxidant effects. Therefore, the use of dinitroaniline herbicides with high genotoxicity and epigenotoxicity potentials should be considered carefully and all the effects of antioxidant compounds should be examined in more detail.

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