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In vitro genotoxicity assessment of dinitroaniline herbicides pendimethalin and trifluralin

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ABSTRACT

Pendimethalin and trifluralin are widely used dinitroaniline herbicides. Both compounds can be found as residue levels in agricultural products. This study was conducted in order to provide necessary information for the risk assessment of pendimethalin and trifluralin. In this study, reactive oxygen species (ROS) levels were measured to examine the potential of both compounds to induce oxidative damage in Chinese hamster lung fibroblast (V79) cells. Also, the genotoxic effects of pendimethalin and trifluralin at the concentration range of 1-500 μM was determined. Single cell gel electrophoresis (comet) and micronucleus assays were used on human peripheral lymphocytes and V79 cells for the genotoxicity assessment. The cell viability of two dinitroaniline herbicides were determined by the use of neutral red uptake assay on V79 cells. IC_{50} values were determined as 66 μM and 128 μM for pendimethalin and trifluralin, respectively. They significantly increased ROS levels on V79 cells for 1-24 h. Both herbicides significantly induced the DNA damage and showed genotoxicity on lymphocytes and V79 cells. Micronucleus frequency increased significantly after pendimethalin and trifluralin treatment of the lymphocytes and V79 cells. Therefore, we concluded that both of the herbicides induced the genotoxicity through the activation of oxidative stress pathway and chromosomal damage.

1. Introduction

In the early twentieth century, herbicides were developed to control weeds selectively and became the most beneficial chemical as plant growth controller (Burke and Bell, 2014). According to the report of the United States Department of Agriculture (USDA) in May 2014, The total quantity of pesticides for 21 crops analyzed increased from 196 million pounds of pesticide active ingredients in 1960 to 632 million pounds in 1981. The herbicide usage in the U.S. received 18 percent of the pounds in 1960, while it was 76 percent in 2008 (Jorge Fernandez et al., 2014).

Herbicides are one of the common-used pesticide groups all over the world. They are used for prevention from pests and getting more and healthy agricultural products. Pendimethalin (N-(1-ethylpropyl)-3,4-dimethyl-2,6-dinitrobenzenamine) is a synthetic, selective dinitroaniline herbicide primarily used to control grasses and certain broadleaf weeds (Ramasahayam, 2014). The oral LD_{50} value is 1250 mg kg^{-1} in male rats, while dermal LD_{50} value is greater than 5000 mg kg^{-1} in rabbits, which means pendimethalin is slightly toxic by oral exposure and nontoxic by skin exposure (US-EPA, 1997). The oral Reference Dose (RfD) for pendimethalin was last revised on 06.03.1988 and it is reported as 4E-2 mg/kg/day. Critical health effects of pendimethalin are increasing in serum alkaline phosphatase and liver weight, and hepatic lesions (IRIS, Pendimethalin, 2017). Tolerable daily intake (TDI) of this widely used herbicide is 0.02 mg/L (WHO, 2003a,b). Pendimethalin has low acute toxicity, but causes thyroid follicular cell adenomas in male and female rats and it has classified as “suggestive evidence of carcinogenic potential” a possible human carcinogen (group C) (US-EPA, 1997, 2014). However, overall cancer incidence did not increase with increasing lifetime pendimethalin use and there was no clear evidence of an association between pendimethalin and cancer (Hou et al., 2006). The support for human carcinogenicity of pendimethalin is missing. It is classified as ‘probably not carcinogenic’, although it has not been assessed under IRIS. It is still widely used herbicide worldwide, although there are reports of the pendimethalin genotoxicity (USGS, 2015). There are studies reporting that it increases the micronucleus frequency and causes chromosomal damage. When considering the relationship between genotoxicity and cancer, the risk assessment of pendimethalin should be considered. A genotoxicity study has shown that 489 mg kg^{-1} pendimethalin significantly increased the chromosomal aberrations in mouse bone marrow and induced the micronucleus (MN) frequency in plant cell and mouse bone marrow polychromatic erythrocytes (Dimitrov et al., 2006). Another genotoxicity study has shown that pendimethalin decreased the root bundle length, increased the mitotic index and the percentage of chromosome aberrations dose-dependent

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manner in maize and onion (Promkaev et al., 2010). Pendimethalin has been reported to be a contaminant for the environment, and it has been found highly toxic for fish and aquatic invertebrates (Singh and Singh, 2014).

Trifluralin is another dinitroaniline herbicide used for annual grasses and broadleaf weeds to protect crops since it has been approved in 1963 in the United States (US) (Wallace, 2014). Trifluralin is registered for use and has been used in several countries including the US, Belgium, Switzerland, Denmark, Canada and Turkey. The use of trifluralin was discontinued in Sweden in 1990; Germany, Finland, France, Ireland and Spain in 2008; Croatia, Cyprus, Czech Republic and Estonia in 2009. Slovenia reported that trifluralin has never been used but it was registered for use in several crops. (UNECE, 2007, 2010; EMO Trifluralin, 2010). According to US-EPA reports, there are no acute toxicity in mammals by the oral, dermal and ocular exposure routes, while it has been classified as highly to very highly toxic to both of coldwater and warmwater fishes. LC_{50} values are 41, 58 and 145 ppb for rainbow trout, bluegill sunfish, and goldfish, respectively. The oral RfD for trifluralin was reported as 7.5E-3 mg/kg/day. Critical health effects of trifluralin are increase in liver weight and methemoglobin levels (IRIS, trifluralin, 2017). TDI of trifluralin is 0.02 mg/L (WHO, 2003a,b). Trifluralin has also been classified as group C 'a possible human carcinogen', since there are limited evidence of carcinogenicity (US-EPA, 1996). Könen and Çavaş evaluated the genotoxic effects of trifluralin and its commercial formulation treflan by MN test in fish species, *Oreochromis niloticus* (Nile Tilapia). They found that both treflan and trifluralin treatments at the doses of 1, 5 and 10 μg/L for 3, 6 and 9 days significantly increased the MN frequencies in peripheral erythrocytes of *O.niloticus*. Furthermore, they informed that the genotoxicity of the active ingredient, trifluralin, was observed to be higher than the commercial formulation Treflan (Könen and Çavaş, 2008). Ribas et al., reported that 0–200 μg/mL trifluralin with or without metabolic activation of S9 mix was able to show weak cytotoxicity and genotoxicity in cultured human lymphocytes (Ribas et al., 1996).

In this study, we tried to analyze the genotoxic effects of commonly used dinitroaniline herbicides belonging to the same chemical group and carcinogenicity class with different validated methods, *in vitro*. Among these compounds the use of trifluralin has been banned in many countries, but still in use in Turkey. However, pendimethalin continues to be widely used throughout the world. We performed this study to see if there was a difference between the genotoxicity capacities of these two compounds. The authors believe that, this study will provide additional information to the literature about the genetic toxicity of pendimethalin and trifluralin and the data of this study may contribute to the risk assessment process.

2. Materials and methods

2.1. Chemicals

The chemicals used in the experiments were purchased from the following suppliers: pendimethalin (98.8% purity, CAS NO: 40487-42-1) and trifluralin (98.8% purity, CAS NO: 1582-09-8), cytochalasin B (Cyt-B), 2′,7′-dichlorodihydrofluorescein (DCHF), dimethyl sulfoxide (DMSO), ethidium bromide (EtBr), ethanol, ethylenediamine tetra acetic acid disodium salt dihydrate (EDTA), fetal calf serum (FCS) (Lot:094M3288), formaldehyde, giemsa, glacial acetic acid, low melting point agarose (LMA), methanol, neutral red (3-amino-7-dimethyl-amino-2-methylphenazine hydrochloride), N-lauryl sarcosinate, normal melting point agarose (NMA), phosphate buffered saline (PBS) tablets, potassium chloride (KCl), sodium chloride (NaCl), sodium hydroxide (NaOH), Tris, Triton X-100, VA (97% purity, CAS Number: 121-34-6) from Sigma-Aldrich (St. Louis, MO); Dulbecco's modified Eagle'smedium (DMEM), trypsin-EDTA, RPMI 1640, penicillin–-streptomycin, L-glutamine, phytohaemagglutinin M (PHA-M) from Biological Industries (Kibbutz Beit-Haemek, Israel).

2.2. Determination of cytotoxicity

The cytotoxicity of pendimethalin and trifluralin was assessed by neutral red uptake (NRU) assay. NRU assay was first described by Borenfreund and Puerner and it is one of the most common used cytotoxicity tests in biomedical areas (Borenfreund and Puerner, 1985; Repetto et al., 2008).

The V79 cells obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) were incubated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Lot:094M3288), 1% penicillin-streptomycin solution, and 2 mM L-glutamin at 37 °C and 5% CO_2 for 24 h. The culture medium was changed every 3–4 days. The passage numbers of V79 cell lines was between 6 and 10.

The cells were treated with pendimethalin or trifluralin at the concentrations of 1, 2.5, 5, 7.5, 10, 25, 50, 100, 250, 500 and 1000 μM in the culture medium with 1% DMSO. 1% DMSO and 50 μM H_2O_2 was used as negative control and positive control, respectively. After 18 h incubation, the cells were washed with phosphate buffer saline (PBS) and incubated with neutral red (NR) solution for 3 h, then the solution was removed and the plate was washed with PBS for 3 times. For fixation, NR fixative solution (glacial acetic acid:ethanol:water, 1:49:50, v/v/v) was added followed by gently rocking for 20 min.

The absorbance was measured at 540 nm by spectrofluorometer (SpectraMax M2, Molecular Devices Limited, Berkshire, UK). Cell viability was calculated as percent survival, determined by the number of treated over negative control X 100 (% cell viability). IC_{50} values, the concentration reducing the absorbance of treated cells by 50% with reference to the control (untreated cells), were determined according to the absorbance-concentrations relationship (Rao, 2003).

Percentage of cell viability (% cell viability) = $(A_{samples}–A_{blank})/(A_{control}–A_{blank})$ x 100 (Rao, 2003)

2.3. Determination of ROS generation

Reactive oxygen species (ROS) generation was assessed in V79 cell line by fluorescent assay according to the procedure of Thannickal and Fanburg based on the oxidation of nonfluorescent 2′,7′-dichlorodihydrofluorescein (DCHF) to the fluorescent 2′,7′-dichlorofluorescein (DCF) for 24 h at 37 °C and 5% CO_2 (Thannickal and Fanburg, 2000). Briefly, V79 cells were cultured in RPMI 1640 medium with 2 mM L-glutamine, 10% FBS and 10 μM final concentration of 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA) dye at 37 °C for 2 h. After the incubation with the dye, the cells were washed with PBS and treated with 1% DMSO as negative control, 100 μM H_2O_2 as positive control, and pendimethalin or trifluralin at the concentrations between 1 μM and 500 μM at 37 °C up to 24 h. Fluorescence intensity was measured with an excitation/emission wavelengths: 485nm/535 nm using spectrofluorometer at 0, 2, 4, 18 and 24 h. The caveats is ROS measurement by fluorescent probes were accounted for, as mentioned in the study of Kalyanaraman et al. (2012).

2.4. Determination of DNA damage by the single cell gel electrophoresis technique

We used Single Cell Gel Electrophoresis technique (comet assay) and formamido pyrimidine DNA glycosylase (FPG) modified comet assay in both V79 cells and human lymphocytes as described by Singh et al. (1988). Lymphocytes were isolated from healthy donor's heparinized whole blood sample with Ficoll-Hypaque density gradient and then washed and diluted with PBS buffer solution (Boyum, 1976). Oxidative damage was introduced by replacing the medium with PBS containing H_2O_2 at the concentrations of 100 μM for 5 min on ice, then the H_2O_2 solution was removed. The cell suspension (2×10^6 cells/mL)

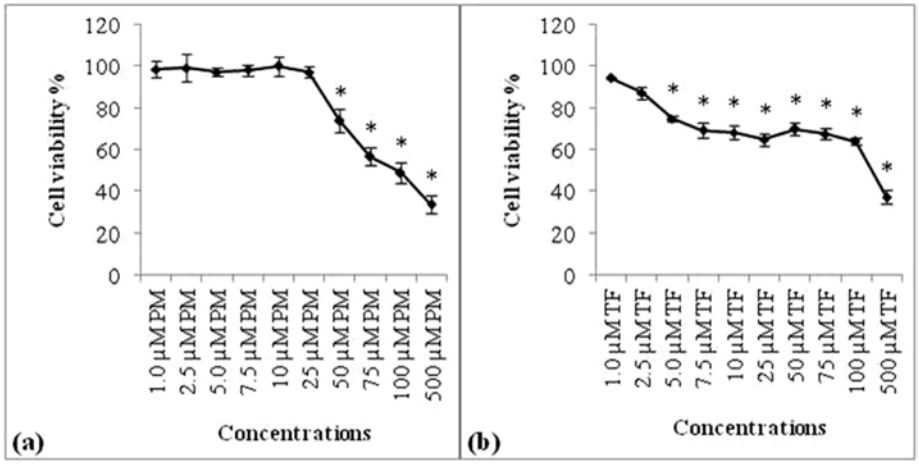

Fig. 1. Effects of pendimethalin (a) and trifluralin (b) on V79 cells viability using NRU assay. The results were expressed as percentage of cell growth inhibition from three independent experiments. Cell viability was plotted as percent of negative control (assuming data obtained from untreated cells as 100%). Results were given as the mean ± standard deviation. Differences between the means of data were compared by the one way variance analysis (ANOVA) test and post hoc analysis of group differences by least significant difference (LSD) test. *$p < 0.05$, compared to negative control. NRU: neutral red uptake, PM: pendimethalin, TF: trifluralin. Negative control (1% DMSO), positive control (50 µM H_2O_2). The cell viabilty of positive control was 37.7%.

with 0.5% low melting agarose (LMA) was embedded on 1% normal melting agarose (NMA) coated slides and then kept on an cold tray to solidify. The slides were immersed in cold lysing solution (2,5 M NaCl, 100 mM ethylenediaminetetraacetic acid (EDTA), 100 mM Trizma® base, 1% sodium sarcosinate, pH 10.0) at 4 °C for 1 h with 1% Triton X-100 and 10% dimethyl sulfoxide (DMSO) added before the process started. The slides were left in the alkaline electrophoresis solution (1 mM sodium EDTA, 300 mM NaOH, pH13.0) at 4 °C for 20 min and electrophoresis procedure was started in the same solution for 20 min under 24 V/300 mA. Neutralization was practiced in the solution of 0.4 M Trizma® base-HCL (pH 7.5) for 5 min at room temperature. For fixation of the cells, the slides were left ethanol solution at the concentrations of 50, 75, and 98% for 5 min each, successively. After the slides got dried, they were stained with ethidium bromide (EtBr) (20 µg/mL in distilled water) per slide and covered with cover glass to determine the DNA fragments occurred with DNA damage after the electrophoresis. We assayed 100 cells (50 cells from each of two replicate slides from each sample) using Leica® fluorescence microscope under green light at 60x magnification and measured the DNA damage with Comet Analysis Software (version 3.0, Kinetic Imaging Ltd, Liverpool, UK). DNA damage was expressed as DNA % Tail (DNA tail intensity).

To determine FPG-sensitive sites, FPG-modified comet assay was carried out after lysation step (Collins et al., 1993). The slides were washed 3 times for 5 min at room temperature with enzyme buffer 40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 mM KCl, 0.5 mM EDTA and 0.2 mg/mL bovine serum albumin and incubated at 37 °C for 30 min with FPG (1 µg/mL) in enzyme buffer and without FPG in enzyme buffer as control. Then the electrophoresis procedure steps were followed as described before.

To measure the effects on V79 cell lines, the cells were seeded in 6-well plates, each well contained 10.000 cells, exposed to one concentration of pendimethalin or trifluralin for 30 min and 24 h. After the incubation period, the cells were harvested with trypsin and centrifuged at 1200 rpm for 5 min. The supernatant was discarded and cells were suspended with PBS one volume. Then the same procedure was followed with the lymphocyte cultures.

2.5. Determination of clastogenicity by cytokinesis-block micronucleus assay

The Cytokinesis-block micronucleus assay (CBMN) was carried out according to Fenech method in the human whole blood cells (Fenech, 2007). Blood samples were incubated at 37 °C, 5% CO_2 for 72 h. Cytochalasin B was added to the cultures at 44th h at the final concentration of 5.2 µg/mL and cytokinesis was arrested. The incubation process was finished with 0.075 M KCl hypotonic solution at 72th h and the fixation treatment was processed with methanol: glacial acetic acid (3:1, v/v). To score the cells, slides were stained with 5% Giemsa and 1000 binucleated cells were counted per concentration via Organisation for Economic Co-operation and Development (OECD) guideline (OECD TG 487, 2014).

MN assay were practiced for V79 cell lines using *in vitro* MN method described by Fenech (2000). Briefly, 10.000 cell/well in 6 well-plate were used for each concentration. After the treatment with compounds for 18 h, the cells were trypsinized and harvested. The cells were centrifuged at 1000 rpm for 5 min. Then the supernatant was thrown away and 0,4 KCl hypotonic solution was added very gently using vortex. For fixation, similar with CBMN assay we used methanol: glacial acetic acid (3:1, v/v). For counting the micronuclueated cells, slides were dyed with acridine orange and 1000 cells for each concentration was counted to obtain the micronucleuses.

2.6. Statistical analysis

The SPSS for Windows 20.0 computer program was used for statistical analysis. The experiments were repeated three times. Differences between the means of data were compared by the one way variance analysis (ANOVA) test and post hoc analysis of group differences by least significant difference (LSD) test. Results of the MN assay as the percentage of MN and NDI were analyzed with z-test. The results were given as the mean ± standard deviation. P value of less than 0.05 was considered as statistically significant.

3. Results

3.1. Cytotoxicity of pendimethalin and trifluralin in V79 cells

The cytotoxic effects of different concentrations of pendimethalin and trifluralin on V79 cell lines measured by NRU assay are given in Fig. 1. A concentration-dependent toxicity was observed in V79 cells after 18 h exposure to pendimethalin and trifluralin. The concentrations up to 25 µM for pendimethalin and 2.5 µM for trifluralin had no effect on V79 cell viability during 18 h exposure. The IC_{50} values of pendimethalin and trifluralin in V79 cell lines were found to be 66 µM and 128 µM, respectively.

3.2. Cellular ROS generation in V79 cells treated with pendimethalin and trifluralin

We determined the alterations in ROS levels up to 24 h (0 h, 2 h, 4 h, 18 h, and 24 h) for pendimethalin and trifluralin in V79 cell lines (Fig. 2.). Fluorescence intensity for pendimethalin and trifluralin were found to be increased in dose-dependent manner. The increase in

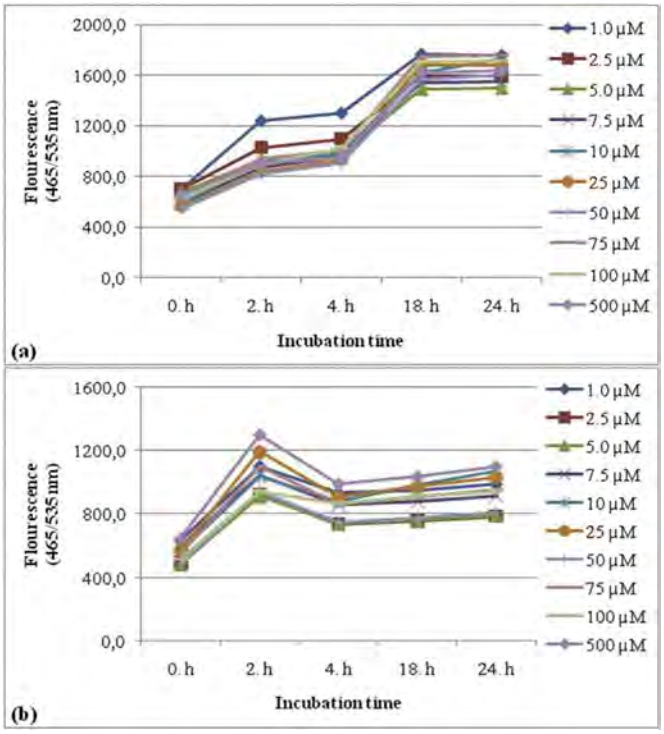

Fig. 2. Fluorescence levels in ROS assay in V79 cells treated with pendimethalin (a) and trifluralin (b) up to 24 h by a fluorescence microplate reader at Ex/Em = 465/535 nm.

fluorescence intensity for trifluralin treatment was lower than that of pendimethalin treatment. The fluorescence measurements at 2nd h and 24th h were evaluated due to significant differences in the changes of ROS generation capacity for statistical evaluation (Fig. 3), Treatment with 100 µM H_2O_2 (as positive control) significantly increased the intracellular ROS level compared to negative control (1% DMSO) at both 2nd h and 24th h ($p < 0.05$). At 2nd h and 24th h, at the concentrations between 1 and 500 µM pendimethalin increased ROS levels significantly when compared to negative control ($p < 0.05$). At the concentrations between 2.5 and 500 µM trifluralin increased ROS levels only at 24th h significantly when compared to negative control ($p < 0.05$).

3.3. Assessment of DNA damage in V79 cell lines and lymphocytes treated with pendimethalin and trifluralin by the single cell gel electrophoresis technique

The comet assay was performed to assess DNA damage in V79 cell lines and the lymphocytes against H_2O_2 and the FPG-modified treatment was used to investigate oxidative DNA damage after incubation period for 30 min and 24 h. The results are shown in Figs. 4–7.

In the lymphocytes, pendimethalin treatment was found to cause significant increases in DNA damage for 30 min and 24 h incubation periods (Fig. 4). After 30 min exposure, the DNA damage were found to be significantly higher than negative control at the concentrations of 7.5–500 µM (7.5–50 µM, $p < 0.05$; 75–500 µM, $p < 0.01$) for standard comet assay and at 10–500 µM (10, 50, 75 µM, $p < 0.05$; 25, 100, 500 µM, $p < 0.01$) for FPG-modified comet assay (Fig. 4a). After 24 h exposure, the DNA tail intensity were higher than the negative control at the concentrations of 7.5, 10, 25, 50, 500 µM ($p < 0.05$) pendimethalin for standard comet assay and 7.5–500 µM (7.5 µM, $p < 0.05$; 10–500 µM, $p < 0.01$) for FPG-modified comet assay (Fig. 4b). FPG sensitive sites were found to be statistically higher at higher concentrations (25 µM for 30 min and 10–500 µM for 24 h) on both incubation periods in the lymphocytes (Fig. 4a and b).

In V79 cells, the effect of pendimethalin on DNA damage after 30 min and 24 h were found to be similar to the result of lymphocytes treated with pendimethalin (Fig. 5). After 30 min, the DNA damage were found to be significantly higher than the negative control at the concentrations of 1–500 µM pendimethalin ($p < 0.05$) for standard and FPG-modified comet assays ($p < 0.05$) (Fig. 5a). After 24 h exposure, the DNA damage was increased with pendimethalin treatment at the concentrations of 5.0, 7.5, 10, 25, 100, 500 µM (5.0, 7.5, 25, 100, 500 µM, $p < 0.05$; 10 µM, $p < 0.01$) for standard comet assay and at the concentrations of 1–50, 500 µM (2.5, 5.0, 7.5, 50 µM $p < 0.05$; 10,

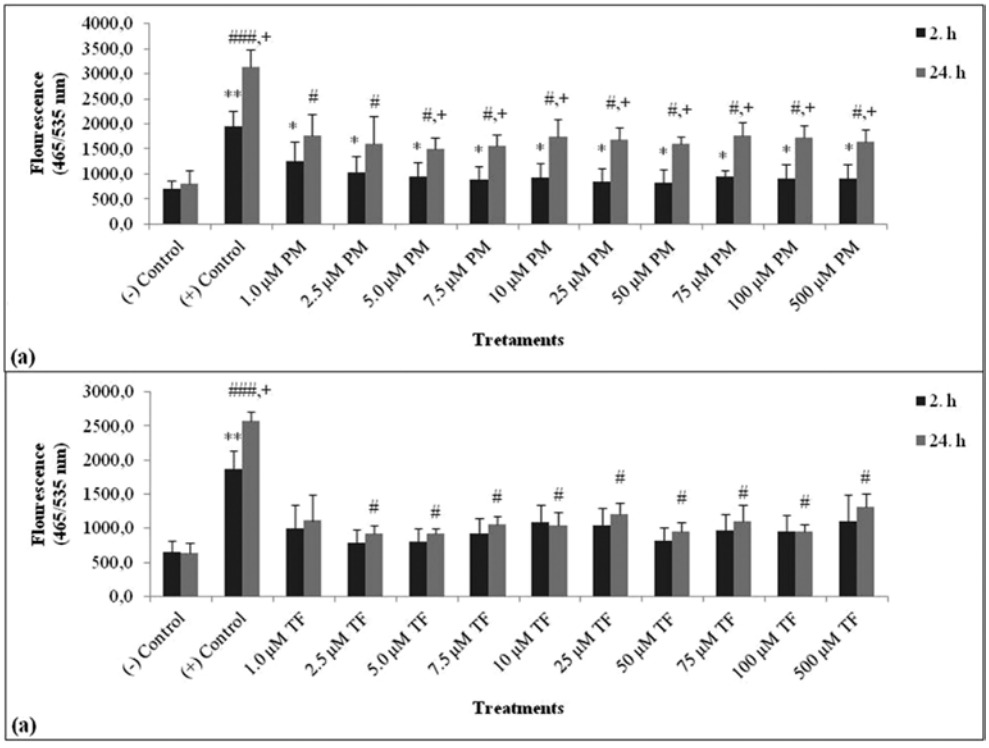

Fig. 3. Changes of ROS in V79 cells treated with pendimethalin (a) and trifluralin (b) for 2 h and 24 h by a fluorescence microplate reader at Ex/Em = 465/535 nm. Results were given as the mean ± standard deviation. Differences between the means of data were compared by the one way variance analysis (ANOVA) test and post hoc analysis of group differences by least significant difference (LSD) test. *$p < 0.05$; **$p < 0.05$, compared to negative control for 2 h and #$p < 0.05$; ###$p < 0.001$, compared to negative control group for 24 h +$p < 0.05$, significant differences between 2 h and 24 h. At 2nd h and 24th h, at the concentrations between 1 and 500 µM pendimethalin (PM) increased ROS level significantly when compared to negative control. At 24th h, at the concentrations between 2.5 and 500 µM trifluralin (TF) increased ROS level significantly when compared to negative control. The treatment with H_2O_2 significantly increased ROS level compared to negative control. Negative control (1% DMSO), positive control (100 µM H_2O_2).

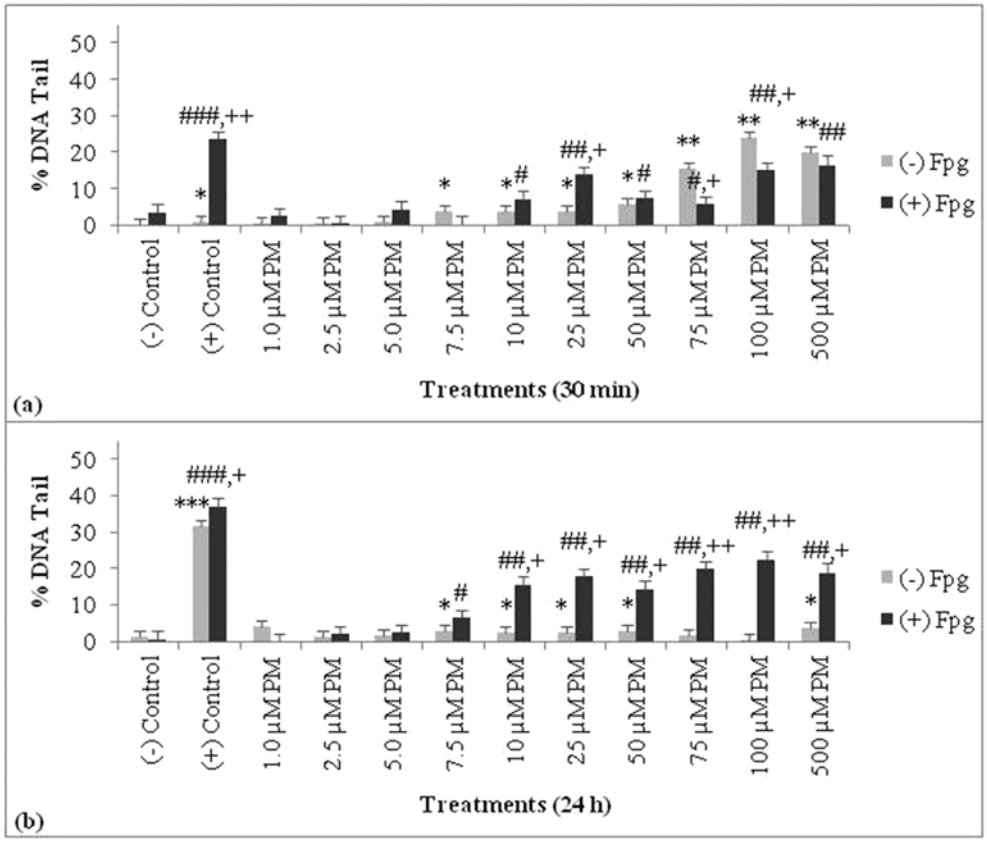

Fig. 4. Genotoxic effects of pendimethalin for 30 min (a) and 24 h (b), and with or without Fpg in human peripheral lymphocytes. DNA damage was expressed as % DNA Tail (Tail intensity). Results were given as the mean ± standard deviation. Differences between the means of data were compared by the one way variance analysis (ANOVA) test and post hoc analysis of group differences by least significant difference (LSD) test. *p < 0.05; **p < 0.01, compared to negative control for comet assay without Fpg. #p < 0.05; ##p < 0.01, compared to negative control for comet assay with Fpg. +p < 0.05; ++p < 0.01, significant differences between with and without Fpg treatments. PM: pendimethalin. Negative control (1% DMSO), positive control (50 µM H_2O_2).

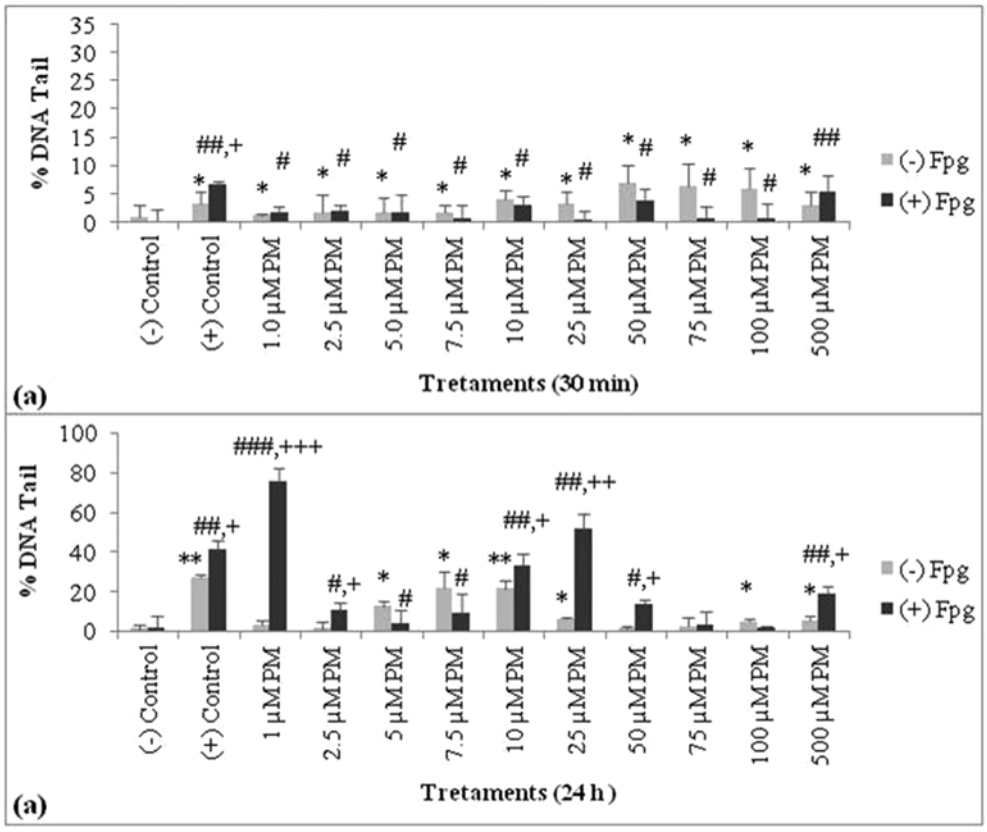

Fig. 5. Genotoxic effects of pendimethalin for 30 min (a) and 24 h (b), and with or without Fpg in V79 cells. DNA damage was expressed as % DNA Tail (Tail intensity). Results were given as the mean ± standard deviation. Differences between the means of data were compared by the one way variance analysis (ANOVA) test and post hoc analysis of group differences by least significant difference (LSD) test. *p < 0.05; **p < 0.01, compared to negative control group for comet assay without Fpg. #p < 0.05; ##p < 0.01; ###p < 0.001, compared to negative control group for comet assay with Fpg. +p < 0.05; ++p < 0.01; +++p < 0.001, significant differences between with and without Fpg treatments. PM: pendimethalin. Negative control (1% DMSO), positive control (50 µM H_2O_2).

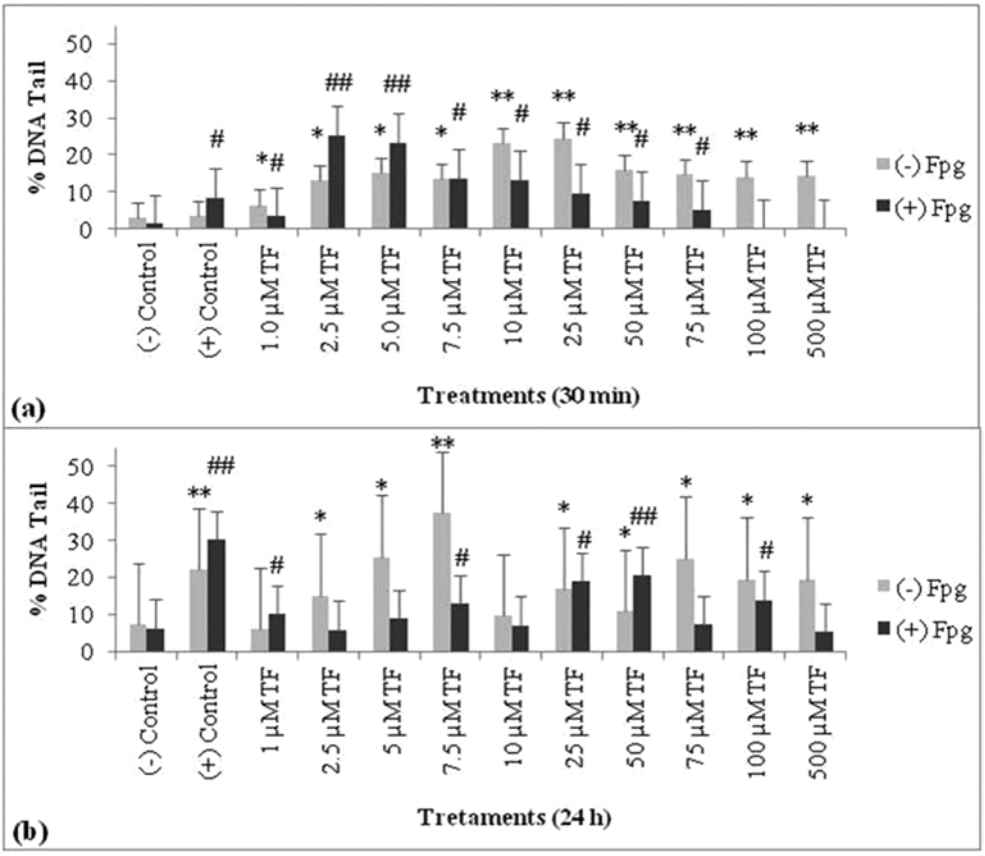

Fig. 6. Genotoxic effects of trifluralin for 30 min (a) and 24 h (b), and with or without Fpg in human peripheral lymphocytes. DNA damage was expressed as % DNA Tail (Tail intensity). Results were given as the mean ± standard deviation. Differences between the means of data were compared by the one way variance analysis (ANOVA) test and post hoc analysis of group differences by least significant difference (LSD) test. *p < 0.05; **p < 0.01, compared to negative control group for comet assay without Fpg. #p < 0.05; ##p < 0.01, compared to negative control group for comet assay with Fpg. +p < 0.05, significant differences between with and without Fpg treatments.TF: trifluralin. Negative control (1% DMSO), positive control (50 µM H_2O_2).

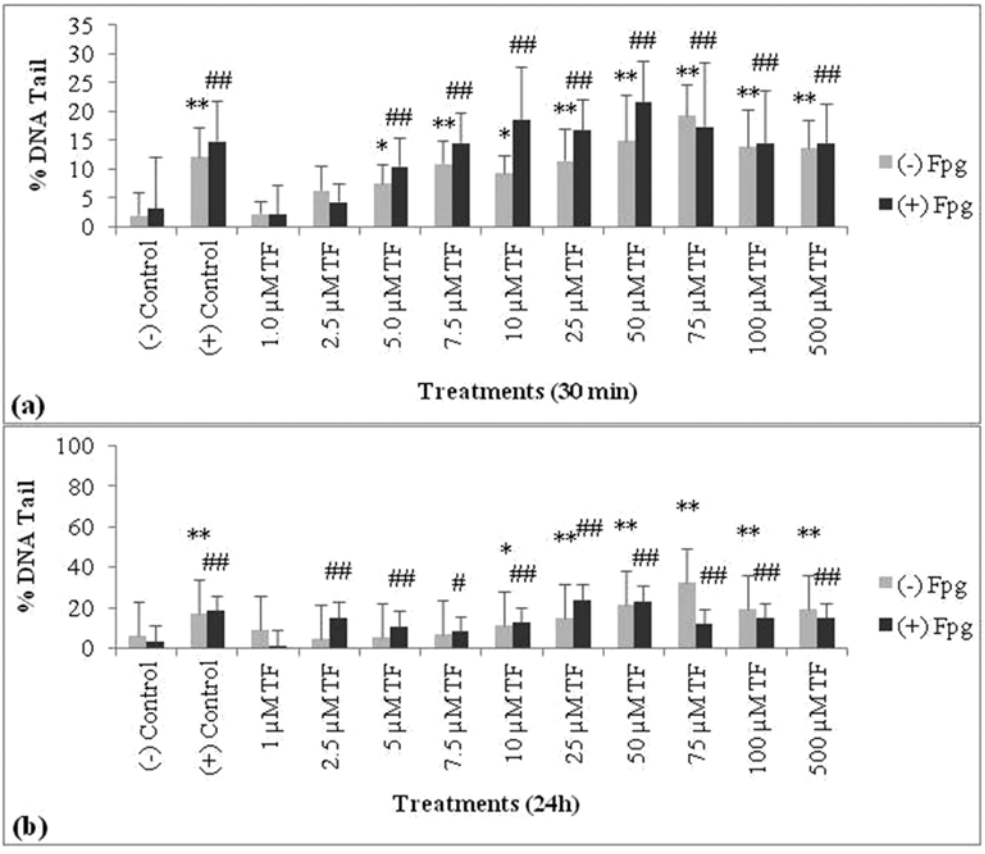

Fig. 7. Genotoxic effects of trifluralin for 30 min (a) and 24 h (b), and with or without Fpg in V79 cells. DNA damage was expressed as % DNA Tail (Tail intensity). Results were given as the mean ± standard deviation. Differences between the means of data were compared by the one way variance analysis (ANOVA) test and post hoc analysis of group differences by least significant difference (LSD) test. *p < 0.05; **p < 0.01, compared to negative control group for comet assay without Fpg. #p < 0.05; ##p < 0.01, compared to negative control group for comet assay with Fpg. +p < 0.05, significant differences between with and without Fpg treatments.TF: trifluralin. Negative control (1% DMSO), positive control (50 µM H_2O_2).

25, 500 μM $p < 0.01$; 1 μM $p < 0.001$) in V79 cells compared to negative control for FPG-modified comet assay (Fig. 5b). In V79 cells, there were no differences between with and without FPG treatments for 30 min, however FPG sensitive sites were found to be higher at 1.0, 2.5, 10, 25, 50, 500 μM doses for 24 h (Fig. 5a and b).

Pendimethalin has been caused to oxidative DNA damage even low exposure conditions such as 1 μM. However, the effects of FPG treatment on pendimethalin treated V79 cells varied from dose to dose, and there was not a constant tendency. When the treatment concentrations of pendimethalin increased too much, DNA tail intensity was found to decrease which caused cell death. Which means that, pendimethalin had an apoptotic effect.

In the lymphocytes, trifluralin treatment was found to cause significant increases in DNA damage for 30 min and 24 h incubation periods (Fig. 6). After 30 min exposure, DNA damage were found to be significantly higher than the negative control at the concentrations of 1–500 μM (1.0–7.5, $p < 0.05$; 10–500 μM, $p < 0.01$) trifluralin for standard comet assay and at the concentrations of 1.0–75 μM (1.0, 7.5–75 μM, $p < 0.05$; 2.5, 5.0 μM, $p < 0.01$) trifluralin for FPG-modified comet assay (Fig. 6a). After 24 h exposure DNA damage were found to be significantly higher than the negative control at the concentrations of 2.5–500 μM (2.5, 5.0, 25–500 μM, $p < 0.05$; 7.5 μM, $p < 0.01$) trifluralin for standard comet assay and at the concentrations of 1.0, 7.5, 25, 50, 100 μM (1.0, 7.5, 25, 100 μM, $p < 0.05$; 50 μM, $p < 0.01$) trifluralin for FPG-modified comet assay (Fig. 6b).

Likewise, the results from V79 cell incubations were similar to the results of lymphocytes treated with trifluralin (Fig. 7). After 30 min exposure, DNA damage were found to be significantly higher than the negative control at the concentrations of 5.0–500 μM (5.0, 10, $p < 0.05$; 7.5, 25–500 μM, $p < 0.01$) trifluralin for standard comet assay and at the concentrations of 5–500 μM ($p < 0.01$) trifluralin for FPG-modified comet assay (Fig. 7a). After 24 h exposure DNA damage were found to be significantly higher than the negative control at the concentrations of 10–500 μM (10 μM, $p < 0.05$; 25–500 μM, $p < 0.01$) trifluralin for standard comet assay and at the concentrations of 2.5–500 μM (7.5 μM, $p < 0.05$; 2.5, 5.0, 10–500 μM, $p < 0.01$) trifluralin for FPG-modified comet assay (Fig. 7b).

In the lymphocytes and V79 cells treated with trifluralin, there were no differences between with and without FPG treatments at all studied doses for 30 min and 24 h incubation periods (Figs. 6 and 7). It seems that the causation of the comet may be independent of the oxidative DNA damage in human lymphocytes and V79 cells.

3.4. Determination of clastogenicity by cytokinesis-block micronucleus assay in V79 cell lines and lymphocytes treated with pendimethalin and trifluralin

We determined the MN producing capacity of pendimethalin and trifluralin in the lymphocytes after 48 h incubation (Fig. 8) and V79 cell lines after 18 h (Fig. 9) *in vitro*. In our study, in the lymphocytes, MN frequency was found to be increased significantly at 7.5 μM ($p < 0.05$) and 10, 25, 50 μM ($p < 0.01$) pendimethalin (Fig. 8a) and at 25, 50, 75 μM ($p < 0.05$) and 100, 500 μM ($p < 0.01$) trifluralin (Fig. 8b).

In V79 cells, MN frequency was found to be increased significantly at 25, 7.5 μM ($p < 0.05$) and 50 μM ($p < 0.01$) pendimethalin (Fig. 9a) and at 75, 100 μM ($p < 0.05$) and 500 μM ($p < 0.01$) trifluralin (Fig. 9b).

All the data from MN assay showed similar results with comet assay. Pendimethalin decreased the cell amounts after 100 μM concentrations as MN frequency did not show a significant change. But trifluralin had notable changes at the concentrations of 25–500 μM which were well-matched with the comet assay results.

4. Discussion

In this study, we determined the cytotoxicity and genotoxicity of dinitroaniline herbicides pendimethalin and trifluralin. When cytotoxic effect was evaluated in V79 cells by NRU method, trifluralin was found to have lower cytotoxic effect than pendimethaline, The IC_{50} values of pendimethalin and trifluralin in V79 cell lines were 66 μM and 128 μM, respectively.

According to our results, these two herbicides have a potential to damage DNA directly. We found these chemicals induced tail intensity in both the lymphocytes and V79 cell lines. DNA damage were increased dose-dependent manner up to 100 μM concentrations of pendimethalin. The percentage of the damaged cells were found to be low above the concentrations of 100 μM, since cell viability significantly decreased above that concentration.

When we used the FPG enzyme to detect the oxidative damage, the quantity of damage increased compared to the FPG negative samples (Speit et al., 2004). Also, the positive control chemical, H_2O_2 acted as a strong oxidant after incubation with FPG enzyme as known (Sies, 2014). In our results, it seems that the causation of the comet in human lymphocytes and V79 cells may be dependent on the oxidative DNA damage induced by pendimethalin, while it may be independent of the oxidative DNA damage induced by trifluralin according to FPG-modified comet assay used to investigate oxidative DNA damage. The ROS assay showed similar results with the comet assay; an increase on ROS production at a concentration of up to 100 μM and decrease above

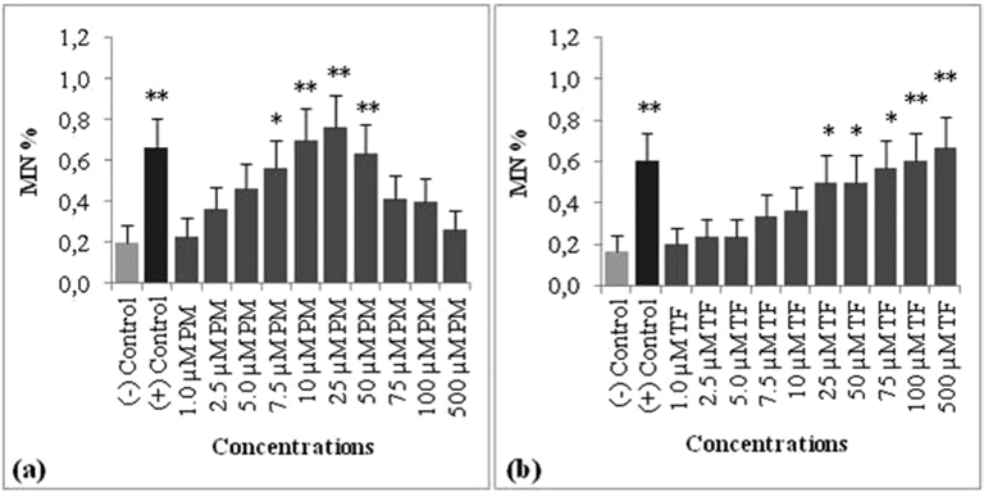

Fig. 8. Clastogenic effects of pendimethalin (a) and trifluralin (b) in human peripheral lymphocytes using CBMN. *$p < 0.05$; **$p < 0.01$, compared to negative control group. The results were given as the mean ± standard deviation. For statistical analysis of the CBMN assay results, the z-test were applied for the percentage of MN. CBMN: cytokinesis-block micronucleus assay, PM: pendimethalin, TF: trifluralin. Negative control (1% DMSO), positive control (50 μM H_2O_2).

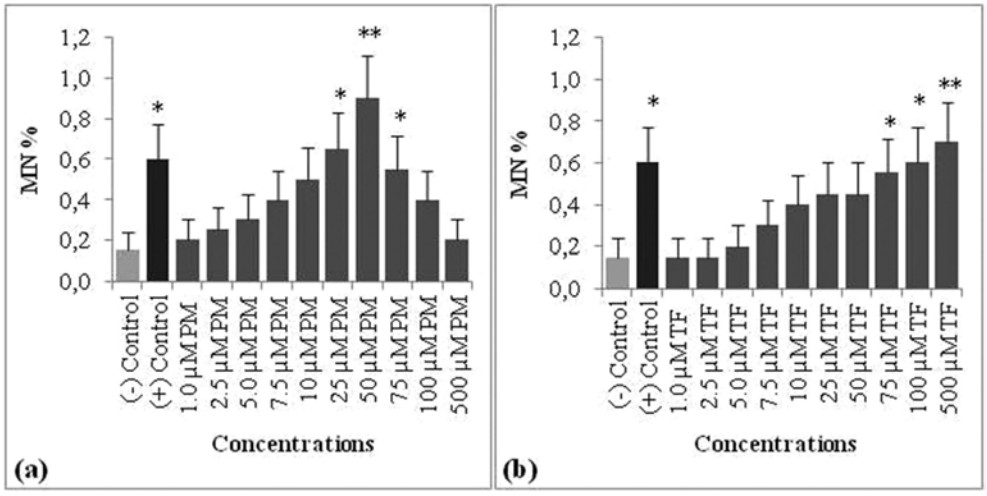

Fig. 9. Clastogenic effects of pendimethalin (a) and trifluralin (b) in V79 cells using CBMN. The results were given as the mean ± standard deviation. For statistical analysis of the CBMN assay results, the z-test was applied for the percentage of MN. *p < 0.05; **p < 0.01, compared to negative control group. CBMN: cytokinesis-block micronucleus assay, PM: pendimethalin, TF: trifluralin. Negative control (1% DMSO), positive control (50 µM H_2O_2).

100 µM were determined by pendimethalin, because the treatment of pendimethalin above 100 µM concentrations caused cell death. Increased ROS production can lead to cell death and organ injuries (Lefort et al., 2010; Ferrari and Andrade, 2015).

On the other hand, it was observed that these compounds increased the MN frequency when compared to control group. This means, the examined compounds also had a chromosomal damage capacity on cell line (Fenech, 2008). This type of damage in cells classified as cancer markers, which are known as double strand breaks from the ionizing radiation type damage (Bryant, 2004). Also, comet assay helped us to determine the amounts of single and double strand breaks (Pu et al., 2015). There are such evidences that the DNA strand breaks lead the way to cell death unless the repair mechanisms come into play (Chapman et al., 2012).

We found that dinitroaniline herbicides pendimethalin and trifluralin have a potential to induce DNA damage and chromosomal damage on V79 cell line and human lymphocytes. Both investigated herbicidal compounds pendimethalin and trifluralin are in the group of dinitroaniline herbicides. There are two nitrogen dioxide (-NO_2) and one aniline group in their chemical structures.According to other researchers, -NO_2 causes strand breaks, and EPA gave the information that -NO_2 effects lungs on irritation level and reduces the resistance of respiratory system (Görsdorf et al., 1990; US-EPA, 2015). The other probable group which can cause damage is the aniline group. Researchers showed aniline-caused DNA damage on various studies (Przybojewska, 1997, 1999). Wu et al. demonstrated that the capacity of oxidative DNA damage of aniline on rats, which is similar to our results (Wu et al., 2005).

The minimum doses at which positive responses seen in each experiment are as follows; 50 µM pendimethalin and 5.0 µM trifluralin for cytotoxicity (NRU assay) in V79 cells; 1.0 µM/1.0 µM ± FPG for pendimethalin and 2.5 µM/10 µM ± FPG for trifluralin in comet assay (24 h); 25 µM for pendimethalin and 75 µM for trifluralin in micronucleus test and 1.0 µM for pendimethalin and 2.5 µM for trifluralin for ROS generation. According to this data, it may be seen that for both compounds ROS induction is seen at the same or lower doses than those of genotoxicity endpoints (Comet and micronucleus); hence it can be claimed that oxidative damage may lead to genotoxicity as mentioned in the Bradford-Hill criterion (Becker et al., 2015). However, the comparison between genotoxicity and cytotoxicity endpoints for trifluralin suggests that cytotoxicity may lead to genotoxicity in V79, because cytotoxicity is already evident at 5 µM, but no signs of genotoxicity (micronucleus) is observed until 75 µM. Oxidative stress may lead to genotoxicty of both compounds.

Our study has been performed in order to contribute to literature related to the genotoxic effects of pendimethalin and trifluralin. Our results could be classified as the first genetic toxicity capacity information about pendimethalin and trifluralin on human peripheral lymphocytes and V79 cells using the comet and micronucleus assays. Findings from our *in vitro* studies are not sufficient to assess the risk of pendimethalin and trifluralin. Long-term exposure studies in experimental animals are needed to perform risk assessment and following in vivo studies, carcinogenic potency of these herbicides can be estimated (Vardavaş et al., 2016; Soeterman-Hernandez et al., 2016).

5. Conclusion

Dinitroaniline herbicides pendimethalin and trifluralin induced DNA damage and chromosomal damage, showed genotoxicity on human peripheral lymphocytes and V79 Chinese hamster lung fibroblast cell lines at the concentration range of 1–500 µM. These compounds belonging to the same chemical group (dinitroanilin herbicides) and carcinogenicity class ‘a possible human carcinogen’ (group C) by US-EPA. While the use of trifluralin has been stopped in many countries, it continues to be used in many countries, including Turkey. On the other hand, pendimethalin is widely used in many countries. In our study, it was found that both compounds showed DNA damage and chromosome damage at low doses in human lymphocytes and V79 cells. According to the results of this study, oxidative stress may lead to genotoxicty of both compounds.

This study provides important results for the low concentration genotoxicity assessment of pendimethalin and trifluralin and may contribute to the risk assessment process of these compounds.

Declaration of interest

The authors declare that there are no conflicts of interest.

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