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ORIGINAL ARTICLE

Assessment of Genotoxic Effects of Pendimethalin in Chinese Hamster Over Cells by the Single Cell Gel Electrophoresis (Comet) Assay

Pendimetalinin Genotoksik Etkilerinin Çin Hamster Over Hücrelerinde Tek Hücre Jel Elektroforez (Comet) Yöntemiyle Değerlendirilmesi

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ABSTRACT

Objectives: Pendimethalin (*N*-(1-ethylpropyl)-3,4-dimethyl-2,6-dinitrobenzeneamine) is a dinitroaniline herbicide compound which selectively controls weeds. It is a cell division and growth inhibitor. It descends plants in a short time after seedling. It is a soil and water pollutant due to the widespread use of formulations in Turkey and around the world. Pendimethalin is manufactured in and imported by Turkey. Pendimethalin is a slightly toxic compound that is classified in toxicity class 3 by the United States Environmental Protection Agency (USEPA). Even though it is classified as group C (human possible carcinogen) compound by the USEPA, there are limited number of studies about its genotoxic effects. The aim of this study was to evaluate *in vitro* genotoxic effects of different concentrations of pendimethalin in Chinese hamster over (CHO) cells by the single cell gel electrophoresis (comet) assay.

Materials and Methods: The cells are incubated with 1, 10, 100, 1000 and 10000 µM concentrations of pendimethalin for 30 min at 37°C and DNA damage was compared with CHO cells untreated with pendimethalin. 50 µM hydrogen peroxide was used as positive control.

Results: No significant cytotoxic effects were observed within the concentration ranges studied. The DNA damage in CHO cells was significantly increased in the pendimethalin concentrations of 1, 100, 1000 and 10000 µM, however, a significant decrease was observed in 10 µM pendimethalin concentration.

Conclusion: Our results show that 1-10000 µM concentrations of pendimethalin induce DNA damage in CHO cells, which was assessed by comet assay.

Key words: Pendimethalin, herbicide, DNA damage, comet assay, CHO cells

ÖZ

Amaç: Pendimetalin (*N*-(1-etilpropil)-3,4-dimetil-2,6-dinitrobenzenamin) seçici bitki kontrolü yapan dinitroanilin türevi bir herbisit bileşiktir. Hücre bölünmesi ve büyümesi engelleyicisidir. Bitkilerin filizlenme aşamasından kısa süre sonra ölmelerine neden olur. Türkiye'de ve dünyada formülasyonlarının yaygın kullanımı nedeniyle toprak ve su kirleticisidir. Pendimetalin Türkiye'de üretilmekte ve ithal edilmektedir. Pendimetalin az toksik bir bileşiktir ve Amerika Birleşik Devletleri Çevre Koruma Ajansı'na (USEPA) göre toksisite sınıfı 3'tür. USEPA tarafından grup C (insanda olası kanserojen) olarak sınıflandırılmasına rağmen genotoksik etkileri sınırlı sayıda çalışılmıştır. Bu çalışmanın amacı, farklı konsantrasyonlardaki pendimetalinin Çin hamster over (CHO) hücrelerindeki *in vitro* genotoksik etkilerini tek hücre jel eletroforez (comet) yöntemiyle değerlendirmektir.

Gereç ve Yöntemler: Hücreler 1, 100, 1000 ve 10000 µM konsantrasyonlardaki pendimetalin ile 37°C'de 0.5 saat inkübe edilmiş ve DNA hasarı pendimetalin uygulanmayan CHO hücreleriyle karşılaştırılmıştır. 50 µM hidrojen peroksit pozitif kontrol olarak kullanılmıştır.

Bulgular: Çalışılan konsantrasyon aralığında önemli sitotoksik etki gözlenmemiştir. CHO hücrelerindeki DNA hasarı 1, 100, 1000 ve 10000 µM konsantrasyonlarda önemli derecede artarken, 10 µM pendimetalin DNA hasarını önemli derecede azaltmıştır.

Sonuç: Sonuçlarımız, 1-10000 µM konsantrasyon aralığındaki pendimetalinin CHO hücrelerinde comet yöntemiyle değerlendirilebilen DNA hasarını indüklediğini ortaya koymaktadır.

Anahtar kelimeler: Pendimetalin, herbisit, DNA hasarı, comet yöntemi, CHO hücreleri

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INTRODUCTION

Pendimethalin (PM), whose chemical name is (*N*-(1-ethylpropyl)-3,4-dimethyl-2,6-dinitrobenzeneamine), is a dinitroaniline herbicide. It is an inhibitor of cell division and cell elongation which selectively terminates weeds. This pre- and early post-emergence herbicide is used to control broadleaf weeds and grassy weed species in cabbage, carrots, celery, cereals, citrus, corn, cotton, garlic, lettuce, onions, peanuts, peas, pome fruits, potatoes, radish, rice, sorghum, soybeans, tomatoes, tobacco and stone fruits. Moreover, it is also used in nonagricultural areas, residential lawns and ornamentals. PM is a widely used herbicide in different formulations; so that, it can be detected as a contaminant in surface and ground water, in soil and air with increasing amount.[1,2] It may disperse through leaching, drift, evaporation and runoff after application to soil. It is also degraded by photo- and bio-degradation or volatilization.[3,4] PM is classified as a slightly toxic compound and also classified in group C carcinogen (human possible carcinogen) by Environmental Protection Agency.[5] In spite of this classification only a few works have been published on the genotoxic effects of PM. PM induces cytotoxicity in rat hepatocytes evaluated by altered mitochondrial respiration.[6] It also shows cytotoxic effect in FRTL 5 (rat thyroid)[7] and Chinese hamster over (CHO) cell line.[8] Dimitrov et al.[9] (2006) has shown that PM significantly increased the chromosomal aberrations at 489 mg/kg dose in mouse bone marrow. In addition, it induced the micronucleus frequency in plant cell and mouse bone marrow polychromatic erythrocytes. PM achieved, concentration dependent (0.1-100 mM) induction in DNA damage evaluated by Comet assay in CHO cells.[8] PM decreased the root bundle length and increased the mitotic index and the percentage of chromosome aberrations dose dependently in maize and onion.[10] It has been reported to be a contaminant for the environment and found highly toxic for fish and aquatic invertebrates.[11] PM exposure has been correlated with an increased incidence of cancer.[12,13] On the other hand, Hou et al.[14] detected no association of exposure of PM during lifetime either with specific cancer sites or with overall cancer incidence among pesticide applicators in North Carolina and Iowa.

The Alkaline Single-cell Gel Electrophoresis technique or comet assay is a versatile tool for assessing DNA damage. Comet assay measures strand breaks, incomplete excision repair events, alkaline labile sites and cross-linking in individual cells. It has been shown to be a method commonly used for measuring the genetic damage induced *in vitro* by different genotoxic agents and also for determining DNA repair under a variety of experimental conditions.[15,16] This assay is also widely used for the evaluation of DNA damage in studies to characterize DNA lesions with and without the addition of the repair enzymes such as formamidopyrimidine N-glycosylase (FPG). FPG is a base excision repair enzyme, it initiates the repair of oxidized bases by recognizing, excising them and cutting the sugar-phosphate backbone of the DNA molecule. At the location of oxidized DNA bases, additional DNA strand breaks occurs and these leads to DNA migration. The determination of FPG-sensitive DNA lesions indicates the appearence of oxidized purine bases.[17-19]

In this study, the genotoxic potentials of dinitroaniline herbicide PM is investigated at different concentrations in CHO cells by using the comet assay with and without the addition of the repair enzyme FPG.

EXPERIMENTAL

Chemicals

The cells were purchased from Republic of Turkey, Ministry of Food, Agriculture and Livestock. The chemicals used in the experiments were purchased from the following suppliers: Low melting agarose (LMA) and normal melting agarose (NMA) (respectively) from Boehringer Manheim (Manheim, Germany); sodium hydroxide (NaOH) and sodium chloride (NaCl) from Merck Chemicals (Darmstadt, Germany); RPMI 1640 medium, fetal calf serum (FCS), trypsin-EDTA, penicillin-streptomycin, dimethylsulfoxide (DMSO), hydrogen peroxide (H_2O_2), ethidium bromide, Triton X-100, phosphate-buffered saline tablets, ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA), *N*-lauroyl sarcosinate, Tris HCl, bovine serum albumin (BSA) and FPG enzyme from Sigma-Aldrich (St. Louis, MO, USA); olive oil from Egaş (Ankara, Turkey).

Cell culture

CHO cells were seeded in 75 cm^2 flasks in 20 mL RPMI 1640 medium (phenol red-free, with L-glutamine and 10% FCS) for 24 h in a 5% CO_2 atmosphere at 37°C.

Treatment of cells

CHO cells were treated for 30 min with 1, 10, 100, 1000 and 10000 mM concentrations of PM which dissolved in 0.5% DMSO and 0.02% olive oil mixture. 0.5% DMSO and 0.02% olive oil mixture were used as (-) controls. As positive control, to create oxidative DNA damage, 50 mM H_2O_2 solution was applied to cells for 5 min on ice, then H_2O_2 solution was removed. CHO cells were disaggregated with trypsin EDTA and resuspendated in 10% FCS containing medium. Cells were centrifugated for 3 min at 3000 rpm, the supernatant was removed.

Evaluation of cell viability

Trypan blue dye exclusion technique was performed for evaluation of cell viability.[19]

Comet assay

The basic alkaline comet assay of Singh et al.[20], as later mentioned by Collins et al.[21], was performed in the standard version and post-treatment with FPG protein to evaluate oxidative DNA damage was used as described in.[16,22,23] The precipitated cells were resuspended and mixed with 80 mL of LMA for embedding on slides which were covered with 1% NMA. Approximately 25.000 cells mixed with 80 mL of 1% LMA were rapidly pipetted onto this slide as the second layer. Slides were covered with cover slips, then kept on an ice-cold flat tray for 5 min to solidify. Cover slips were removed and slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% sodium laurylsarcosinate, pH 10) with 1.5% Triton X-100 and 10% DMSO was added just before use, for a minimum period of 1 h at 4°C. H_2O_2 treated positive

control cells were immersed in a seperate cold lysing solution. Following lysis, slides were washed in enzyme buffer (20 mM Tris HCl, 1 mM Na_2EDTA, 100 mM NaCl, 0.5 mg BSA/mL, pH 7.5) at 4˚C, four times for 5 min, 20 min in total. Slides were covered with 200 mL of FPG protein (1 mg/mL) in enzyme buffer for detection of FPG-sensitive DNA lesions, and incubated for 30 min at 37˚C, then cover slips were removed. Slides were placed in a horizontal gel electrophoresis tank. The tank was filled with fresh electrophoresis solution (1 mM NaEDTA, and 300 mM NaOH, pH 13). Slides were left in the electrophoresis solution for 20 min to allow the unwinding of the DNA and expression of alkaline labile damage before electrophoresis. Electrophoresis was conducted at a low temperature for 20 min using 25 volts and adjusting the current to 300 miliamperes. All of the steps were carried out under dimmed light to prevent the occurrence of additional DNA damage. The slides were washed with neutralysing buffer, three times after electrophoresis. Following the slides were washed in distilled water, 50%, 75% and 99% ethanol (for 5 min each), they were allowed to dry at room temperature. 30 mL of EtBr solution (20 mg/mL) was added to each slide for staining. The slides were investigated thoroughly by the use of a Leica fluorescence microscope (Leica, Wetzlar, Germany) with 400-fold magnification. The tail length, intensity, moment and migration of 'comets' were measured with a computerised image analysis system-comet Assay III Perceptive Instruments (Suffolk, England) for randomly selected 100 cells, i.e. 50 cells from each of two replicate slides from each sample. And the mean value of these parameters was used for the evaluation of the DNA damage by repeating the experiments for three times.

Statistical analysis

The statistical analysis was performed by SPSS for Windows 11.5 software (SPSS Software, Chicago, IL, USA). The results from the DMSO+olive oil-treated negative control, H_2O_2-treated positive control, and test groups treated with different concentrations of PM were statistically compared by the use of one-way ANOVA and the post hoc analysis of the group differences was evaluated by the least significant difference test and finally the results were expressed as means ± standard error of the mean (SEM).

RESULTS

CHO cells are exposed to PM (1-10000 mM) for 30 min. Cell viability was greater than 90%. DNA damage is determined with comet assay in this study. In comet assay, the amount of DNA breakage in a cell is estimated from the migration extent (tail length) of the genetic material in the direction of anode.[20] Also, the DNA percentage in the tail (tail intensity) has been shown to be proportional to the frequency of DNA strand breaks.[24] Tail moment is a simple identifier calculated by the computerized image analysis system considering both the migration tail length as well as the fraction of DNA migrated in the tail.[25] The DNA damage in CHO cells following *in vitro* 30 min treatment with 1-10000 mM concentrations of PM and post treatment with 50 mM H_2O_2 and 50 mM H_2O_2+FPG protein after PM treatment are shown in Table 1, 2 and Figure 1a-d. Results are given as

Table 1. DNA damage as tail length and tail intensity in CHO cells treated with pendimetalin

		PM treated CHO cells	PM+H_2O_2 treated CHO cells	PM+H_2O_2+FPG treated CHO cells
	Comet parameter	Tail length		
1	(-) Control	17.25±0.35	28.17±0.45***	60.56±0.88$^{\#\#\#}$
2	1 µM PM	18.41±0.40^{+}	32.23±0.41***	61.55±0.72$^{\#\#\#}$
3	10 µM PM	15.66±0.36^{++}	31.39±0.35***	71.21±0.62$^{\#\#\#}$
4	100 µM PM	19.93±0.42^{+++}	29.88±0.32***	66.43±0.61$^{\#\#\#}$
5	1000 µM PM	19.13±0.39^{++}	29.18±0.37***	65.49±0.57$^{\#\#\#}$
6	10000 µM PM	20.65±0.50^{+++}	34.64±0.36***	65.60±0.59$^{\#\#\#}$
		Tail intensity		
1	(-) Control	7.89±0.54	15.23±0.99***	64.46±1.41$^{\#\#\#}$
2	1 µM PM	7.46±0.62	24.34±1.53***	56.79±1.32$^{\#\#\#}$
3	10 µM PM	6.06±0.54^{+}	34.81±1.37***	62.28±1.32$^{\#\#\#}$
4	100 µM PM	8.40±0.65	38.85±1.32***	62.52±1.33$^{\#\#\#}$
5	1000 µM PM	8.48±0.62	33.33±1.32***	59.01±1.25$^{\#\#\#}$
6	10000 µM PM	9.83±0.86	51.03±1.36***	57.75±1.33$^{\#\#\#}$

Data represent mean values (±standard error of the mean) of tail length, tail intensity, tail moment and tail migration of the alkaline comet assay and refers to 300 scores/concentration (100 scores/experiment, three experiments)

PM: Pendimetalin, CHO: Chinese hamster over, $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$ significance of DNA damage in PM treated CHO cells compared with DMSO (0.5%) + olive oil (0.02%) treated negative control cells; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ significance of DNA damage in PM treated CHO cells compared with PM+H_2O_2 treated CHO cells; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ significance of DNA damage in PM+H_2O_2 treated CHO cells with PM+H_2O_2+FPG treated CHO cells

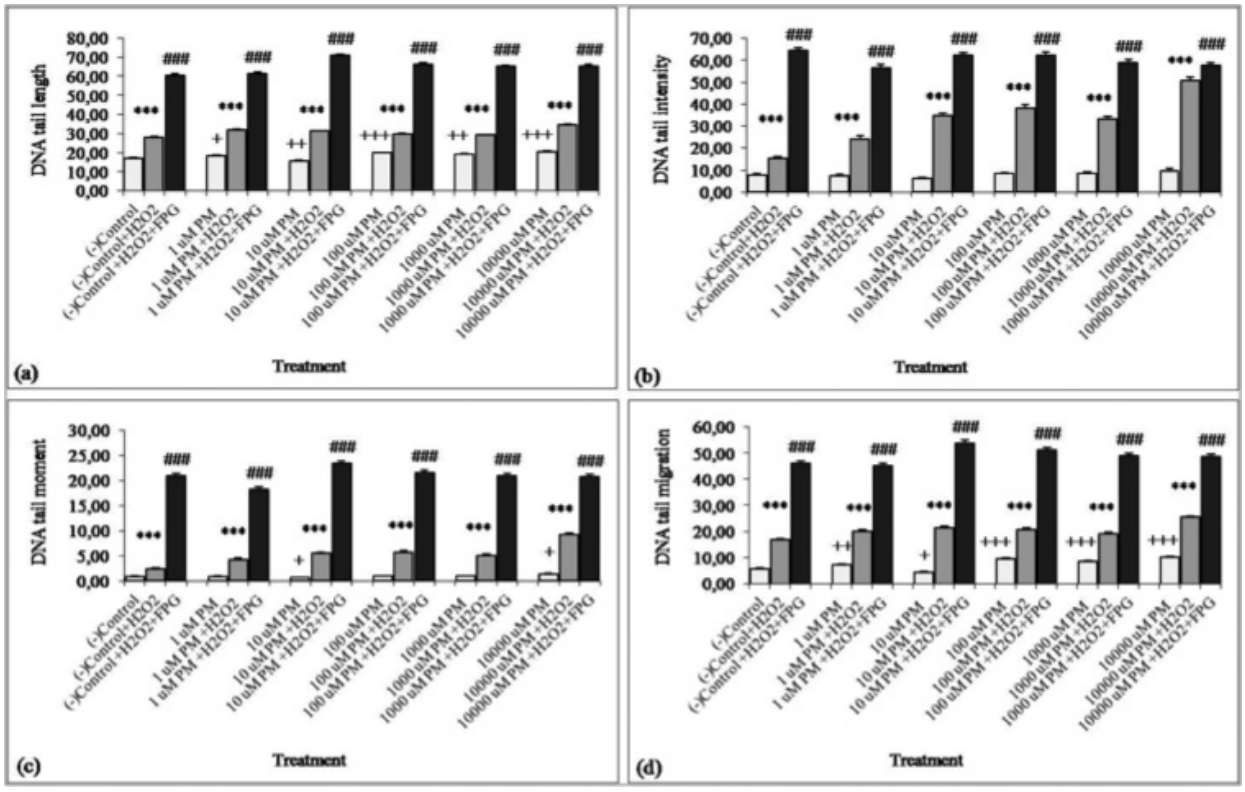

Figure 1. Genotoxic effects of pendimethalin for 30 min with or without Fpg in CHO cells, a) DNA damage was expressed as DNA tail length, b) DNA tail intensity (% DNA tail), c) DNA tail moment and d) DNA tail migration, results were given as the mean ± standard deviation

PM: Pendimethalin, FPG: Formamidopyrimidine *N*-glycosylase, $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$, PM treatment compared to negative control [DMSO (0.5%) + olive oil (0.2%)], $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, PM treatment compared to PM+50 µM H_2O_2, $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, PM+50 µM H_2O_2 treatment compared to PM+50 µM H_2O_2+FPG

Table 2. DNA damage as tail moment and tail migration in CHO cells treated with pendimetalin

		PM treated CHO cells	PM+H_2O_2 treated CHO cells	PM+H_2O_2+FPG treated CHO cells
	Comet parameter	Tail moment		
1	(-) Control	0.91±0.07	2.40±0.18**	20.85±0.53$^{\#\#\#}$
2	1 µM PM	0.90±0.08	4.24±0.28***	18.22±0.47$^{\#\#\#}$
3	10 µM PM	0.64±0.06^{++}	5.53±0.23***	23.34±0.54$^{\#\#\#}$
4	100 µM PM	0.98±0.08	5.78±0.22***	21.49±0.53$^{\#\#\#}$
5	1000 µM PM	1.00±0.08	5.11±0.24***	20.86±0.50$^{\#\#\#}$
6	10000 µM PM	1.37±0.15^{++}	9.20±0.27***	20.59±0.54$^{\#\#\#}$
		Tail migration		
1	(-) Control	5.67±0.35	16.75±0.46***	46.11±1.04$^{\#\#\#}$
2	1 µM PM	7.24±0.39^{++}	20.26±0.53***	45.33±0.92$^{\#\#\#}$
3	10 µM PM	4.36±0.35^{+}	21.41±0.47***	54.05±1.05$^{\#\#\#}$
4	100 µM PM	9.34±0.43^{+++}	20.78±0.44***	51.39±0.91$^{\#\#\#}$
5	1000 µM PM	8.57±0.39^{+++}	19.29±0.49***	49.04±0.90$^{\#\#\#}$
6	10000 µM PM	10.04±0.52^{+++}	25.42±0.53***	48.85±0.91$^{\#\#\#}$

Data represent mean values (±standard error of the mean) of tail length, tail intensity, tail moment and tail migration of the alkaline comet assay and refers to 300 scores/concentration (100 scores/experiment, three experiments)

PM: Pendimetalin, CHO: Chinese hamster over, $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$ significance of DNA damage in PM treated CHO cells compared with DMSO (0.5%) + olive oil (0.02%)-treated negative control cells; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ significance of DNA damage in PM treated CHO cells compared with PM+H_2O_2 treated CHO cells; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ significance of DNA damage in PM+H_2O_2 treated CHO cells with PM+H_2O_2+FPG treated CHO cells

the mean values (±SEM) of tail length, tail intensity, tail moment and tail migration.

According to the data obtained from three separate experiments, tail length and tail migration are significantly increased at all concentrations of the PM tested (1, 100, 1000 and 10000 µg/mL) when compared with those of untreated cells (Figure 1a, 1d, respectively). The tail length, tail intensity, tail moment and tail migration are significantly decreased at PM concentrations of 10 mM above the control values (Figure 1a-d). Moreover, the tail moment is significantly increased at PM concentrations of 10000 mM above the control values (Figure 1c).

Some inconsistencies among tail length, tail intensity, tail moment, tail migration and concentration are found (Figure 1a-d). Significant tail length and tail moment increases are found in CHO cells with 1, 100, 1000 and 10000 mM PM concentrations (Figure 1a, 1c, respectively). On the other hand, significant tail length, tail intensity, tail moment and tail migration decreases are found in these cells with only 10 mM PM concentration (Figure 1a-d). Generally, the genotoxic effects of the PM in CHO cells are appeared to be better reflected by tail length and tail migration than tail intensity and tail moment in this study. Post-treatment with H_2O_2+FPG protein reveals increases in all the investigated comet variables when compared to post-treatment with just H_2O_2, in previously 10, 100 and 1000 mM concentrations of PM treated CHO cells (Figure 1a-d). This finding shows that, DNA damage performed with 10, 100 and 1000 mM concentrations of PM+H_2O_2 treatment is an oxidative DNA damage which may be repaired by FPG protein.

DISCUSSION

DNA oxidative damage is a common type of damage of cells from reactive oxygen species (ROS) and it can lead to many different mutations and problems in DNA. Reactive oxygen species include hydrogen peroxide, superoxide anion radical, singlet oxygen, hydroxyl radical and nitric oxide. They are the consequence of normal body processes such as metabolism and also the consequence of interactions with toxic chemicals, certain drugs and radiation. ROS is not the only reason of oxidative DNA damage. Decreases in antioxidant defence and inhibition of repair of oxidative damage can also cause oxidation of DNA.[26] It is clear that oxidative DNA damage may lead to mutations upon cells. Mutations are very important step in carcinogenesis, and so elevated levels of oxidative DNA damage plays a critical role in the initiation, promotion and progression stages of carcinogenesis.[26,27] Oxidative DNA damage has also been associated with other diseases such as Alzheimer's disease, Huntington's disease, Parkinson's disease, hepatitis, atopic dermatitis, autoimmune diseases, atherosclerosis.[26-28] ROS may also cause sperm disfunction and plays a significant role in male infertility.[29] Additionally, it is suggested that the gradual accumulation of free-radical damage to biomolecules occurs aging.[26,30]

PM is widely used dinitroaniline herbicide in all continents of the world. On the other hand, scanty number of studies have been conducted on the genotoxic effects of PM. In this study, the DNA damage in all variables examined in CHO cells at various concentrations with PM application for 30 min, is created hormetic concentration-response (U shape concentration-response curve). Tail length and tail migration with 1 mM concentration of PM significantly increases as compared to negative controls ($p<0.05$ and $p<0.01$, respectively); while tail intensity and tail moment are decreased at 1 mM concentration of PM, this decrease is statistically insignificant. However, DNA tail length ($p<0.01$), tail intensity, tail moment and tail migration ($p<0.05$) values of CHO cells at 10 mM concentrations of PM application, significantly decreases compared to negative controls. In all the examined parameters, the DNA damage is increased with 100, 1000 and 10000 mM concentrations of PM applications when compared to negative controls. The increases in the DNA tail length and tail migration at three concentrations ($p<0.001$) and in the DNA tail moment for 1000 mM concentration ($p<0.05$) are statistically significant.

Our findings are in agreement with the results of the study conducted by Patel et al.[8] This study is the only other comet study in the literature performed in various concentrations of PM applied CHO cells. Patel et al.[8] were evaluated the cytotoxic effects of PM in CHO cells after application of PM for 3 h at 1, 10 , 100, 1000 and 10000 mM concentrations by MTT (*3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)* method, then they determined the DNA damage by comet assay. The results of this study show that, the IC_{50} value of PM is close to the 10000 mM. Moreover mitochondrial activity of CHO cells exposed to 10000 mM concentrations of PM for 3 h, shows a decrease of 54% compared to controls. The 90% cell viability after 3 h of exposure of PM is determined in 10-100 mM. Patel et al.[8] also report over 90% of cell survival by trypan blue staining method in CHO cells which are exposed to 0.01, 0.1, 1, 10 and 100 mM concentrations of PM for 3 h. The DNA damage is investigated by comet assay in terms of the arbitrary units, % tail DNA and tail length. PM is found to cause a significant concentration dependent increase in DNA damage in the studied variables, at the concentrations of 0.1 to 100 mM ($p<0.05$). In our study, unlike the work of Patel et al.[8], CHO cells are exposed to PM for 30 min and comet assay is performed by using FPG enzyme additionally. According to the results of our study, after 30 min application of PM in 1-10000 mM concentrations and following 50 mM H_2O_2 application for 5 min, the oxidative DNA damage which is formed in CHO cells can be repaired by FPG enzyme.

CONCLUSION

The data in the literature combined with our results show that commonly used dinitroaniline herbicides PM induced DNA damage in 1-10000 mM concentration in CHO cells can be detected by comet assay. Patel et al.[8] also conclude that PM may possess clastogenic effects such as some compounds having the same chemical structure of dinitroaniline herbicides. In this study, CHO cells are incubated with PM for 30 min. For more information about DNA damaging effects of PM, it will be useful to perform similar studies using longer incubation periods more than 30 min. In further studies, to refine the

data obtained in our study in the concentration range from 1 to 10000 mM and to be informed about the possible effects on close to human exposure concentrations, the DNA damage should be detected in concentrations below 10 mM. In addition, chromosomal aberration studies must be carried out for the detection of claimed clastogenic effects of PM.

Conflict of Interest: No conflict of interest was declared by the authors.

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