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Effect of Curcumin On the Genotoxicity Induced By Alkylating Agents

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Abstract

DNA damage due to genotoxic stress is an important type of stress which organisms are exposed during their life. The direct-acting alkylating agent methyl methanesulfonate (MMS), is covalently linked to DNA and cause DNA damage, creating an indirect effect of the alkylating agent cyclophosphamide (CP) cause to DNA damage by changing the function of cellular proteins. In this study, the effects of curcumin on MMS and CP treated mice DNA damage, total antioxidant capacity, total oxidant capacity (oxidative stress index) and genotoxicity markers such as micronucleus index, cell growth arrest and induced DNA damage, which is Gadd45 and Gadd153 genes expression levels were investigated. Curcumin reduce DNA damage, the number of micronucleus and oxidative stress occurred by both MMS and CP induction. Curcumin's protective effect on DNA damage was provided by inducing Gadd45 and Gadd153 mRNA expression levels. We could state that curcumin, a phenolic compound show protective effects before the damage. In brief, curcumin has both antioxidant and antigenotoxic effects.

Keywords

Curcumin,
Alkylating agents,
Genotoxicity,
Comet assay,
Gene expression.

Alkilleyici Ajanlar Tarafından Uyarılan Genotoksisite Üzerine Curcumin'in Etkisi

Özet

DNA hasarı organizmaların yaşamı boyunca maruz kaldığı önemli bir genotoksik stres türüdür. Doğrudan etkili alkilleyici ajanlardan olan metil metansülfonat (MMS), DNA'ya kovalent olarak bağlanarak DNA'da hasar oluştururken, dolaylı yoldan etkili alkilleyici ajanlardan olan siklofosfamid (SP) ise hücre proteinlerinin fonksiyonlarını değiştirerek DNA hasarına sebep olur. Bu çalışmada, deneysel olarak MMS ve SP uygulanan farelerde DNA hasarı, total antioksidan kapasite, total oksidan kapasite (oksidatif stres indeksi) ile genotoksisite belirteci mikronukleus indeksi ve hücre büyümesini durduran ve indüklenmiş DNA hasarının belirteci olan genlerden Gadd45 ve Gadd153'ün ifadelerindeki değişiklikler ile bunlara curcumin'in etkileri araştırıldı. Önce curcumin, sonra MMS ve SP'in uygulandığı gruplarda, curcumin'in DNA hasar derecesini mononükleer lökositlerde azalttığı, kemik iliğinde oluşan mikronukleus sayısını azalttığı, plazmada oksidatif stresi azalttığı ve indüklenmiş DNA hasarının belirteci olan Gadd45 ve Gadd153'ün mRNA ekspresyon düzeylerinin uyarılmasını sağlayarak koruyucu özellik gösterdiği bulundu. Sonuç olarak, fenolik bir bileşik olan curcumin'in, hasar oluştuktan sonra değil de, hasar oluşmadan önce koruyucu özellik göstererek antioksidan ve antigenotoksik özelliğe sahip olduğu bulundu.

Anahtar kelimeler

Curcumin,
Alkilleyici ajanlar,
Genotoksisite,
Comet assay,
Gen ekspresyonu.

1. Introduction

DNA damage due to endogenous and exogenous factors lead to the deterioration of genetic information. It leads to the transfer of exact genetic information from generation to generation. Different environmental toxins and chemicals can lead to oxidative stress and ultimately DNA damage. These agents cause mutations, chromosomal effects, unscheduled DNA synthesis and sister chromatid exchange. Moreover, these changes also responsible in the different expressions of genes (Junk *et al.* 2000). In order to overcome the genotoxic effects, different dietary constituents are being consumed daily by most of the world population. Many of them have been identified as potential chemopreventive agents. Among the species, curcumin is widely used. Curcumin is major component in *Curcuma longa L.* (*Zingiberaceae*), which has been shown to possess many medicinal properties (Agarwal 1996, Araújo and Leon 2001). Still research is going on to discover the remarkable properties of curcumin at the molecular level. So, current study was conducted to find the effects of curcumin on the genotoxicity induced by alkylating agents. Alkylating exogenous agents are capable of adding bases to ethyl or methyl groups. The direct-acting alkylating agent, methyl methanesulfonate (MMS), is covalently linked to DNA and cause DNA damage, creating an indirect effect of the alkylating agent (Franke *et al.* 2005). Cyclophosphamide (CP) causes the DNA damage by changing the function of cellular proteins (Matalon *et al.* 2004, Franke *et al.* 2005).

In present study, different parameters like DNA damage, total antioxidant capacity, total oxidant capacity (oxidative stress index) and genotoxicity markers such as micronucleus index, cell growth arrest and genes expression levels of Gadd45 and Gadd153 were studied to uncover the molecular aspect of this phytochemical against alkylating agents.

2. Material and Methods

2.1. Chemicals

Methyl methanesulphonate (MMS) cyclophosphamide (CP), high melting agarose (NMPA), low melting agarose (LMPA), chloroform, may grunwald, giemsa and entellan and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animals, experimental designs and sampling

In this study, the effects of curcumin were investigated on mice treated with MMS and CP. A total of 54 Swiss Webster mice of age 8-10 weeks and weighting 20-40 g were used. These were further divided into 9 treatment groups, each containing 6 mice. All protocols were performed according to the International Practices for animal use and care under the control of an Internal Committee. (AKÜ.0.A2.00.00/183). Different treatments were given to all 9 groups and blood samples were collected after 24 hours and 48 hours of each treatment. Total nine groups were divided as:

Group 1; (Control group), simple water and normal feed were given to mice.

Group 2; (Curcumin group), only 100 mg/kg curcumin was given.

Group 3; (Solvent group), curcumin was solved in dimethyl sulfoxide (DMSO) and given to mice.

Group 4; (Curcumin + MMS group), first 100 mg/kg curcumin was given for 24 hours then 40 mg/kg MMS for 48 hours.

Group 5; (Curcumin + CP), first 100 mg/kg curcumin was given for 24 h and then 25 mg/kg CP given for 48 hours.

Group 6; (MMS + Curcumin), first 40 mg/kg MMS was given for 24 hours and blood sample was collected, then 100 mg/kg curcumin given for 48 hours.

Group 7 (CP + Curcumin), first 25 mg/kg CP was given for 24 hours and blood sample was collected, then 100 mg/kg curcumin given for 48 hours.

Group 8; (MMS group), only 40 mg/kg MMS for 24 and 48 hours was given.

Group 9; (CP group), only 25mg/kg CP was given for 24 and 48 hours.

Curcumin dose of 100 mg/kg Kürkçü (2008) was given a 20 ml DMSO dissolving intragastric (i.g) by gavage way.

40 mg/kg MMS and 25 mg/kg CP dose Franke *et al.* (2005), was administered by intraperitoneally (i.p.) dissolved in 500 µl dH₂O.

After blood samples collection, the rats were anesthetized with intramuscular 5-10 mg/kg of xylazine hydrochloride (Rompun^R) for liver tissue collection. Liver tissue was kept in RNAlater[®] to inhibit the activity of RNase enzyme. Cervical dislocation was done to take bone marrow from femur. Bone marrow was kept in 1 ml bovine serum. The samples were stored at -20 °C. for further examination.

2.3. Comet assay

From each mouse, 2-3 ml of blood was taken and alkaline comet assay was performed following Singh *et al.* (1988) method with slight modifications. Blood was mixed with histopaque in ependorf tube with ratio of 1:1 and centrifuged at 2000 rpm for 20 min. Supernatant was discarded and pellet was washed with phosphate buffered saline (1 ml) at 2300 rpm for 10 min. 25 µL cell suspension was mixed with 75 µL LMPA and spreaded over the pre-coated normal agrose gel slides and kept over cold ice slabs for 5 min. Subsequently, lysis was performed for one hour at 4 °C. The slides were removed from the lysis buffer, drained and placed in a horizontal electrophoresis unit filled with fresh alkaline electrophoresis solution, containing 300 mM NaOH and 1 mM EDTA, (pH 13), for 20 min to allow the DNA to unwind. Electrophoresis was performed for 20 min at room temperature at 25 V and was adjusted to 300 mA. Subsequently, the slides were washed with a neutralizing solution of 0.4 M Tris, pH (7.5), in order to remove alkali and detergents and stained with 60 µL ethidium bromide (20 µg/mL). One hundred cells per sample (two slides of 50 cells each) were analyzed at 400x using a

fluorescence microscope (BX 50 Olympus, Japan) to investigate the level of DNA damage.

2.4. Micronucleus test

Mice were sacrificed by cervical dislocation 48 hours after the last treatment. The frequency of micronucleated cells in femoral bone marrow was evaluated according to Agarwal and Chauhan (1993). Micronucleus assay was performed on bone marrow, which was taken from each group and centrifuged at 1000 rpm for 10 min. Supernatant was discarded and fetal calf serum was added to pellet. One drop of suspension was taken, spreaded on slide and dried for 24 hours. Fixation was done with 100 % methanol for 10 min. Then the slides were kept for 3 min in May-Grunwald solution. After that, slides were passed through a solution of May-Grunwald and Sorensen phosphate solutions (1:1) for 5 min. Slides were then stained with giemsa stain for 10 min. Finally, slides were dried for 1 day and closed with entellan. Randomly 2000 PCE were counted from each slide. Moreover, from each animal PCE/NCE ratios determined by counting 1.000 erythrocytes. Slides were observed under microscope, 1000 cells per slide were counted to find the micronucleus.

2.5. Oxidative stress index analysis

Oxidative stress index (OSI) was calculated by the Total Antioxidant Status (TAS) Kit (Rel Assay Diagnostics) and Total Oxidant Status (TOS) Kit (Rel Assay Diagnostics). TOS and TAS were analysed spectrophotometrically by reading at 530 nm and 660 nm, respectively. TOS and TAS were calculated by using following formula,

$$OSI = [(TOS, \mu\text{mol/L}) / (TAS, (\text{mmolTroloxEquiv/L}) \times 100)]$$

2.5. RNA isolation, cDNA synthesis and Real Time QRT-PCR

Liver tissue samples were taken out from RNAlater to do RNA expression analysis for Gadd45 and

Gadd153 genes. RNA isolation was carried out by Trizol reagent according to the manufacturer's protocol and mixed in 50 µl deionised DEPC-treated water. 5 µg of RNA was used for cDNA synthesis by the Thermo Script™ Reverse Transcriptase Enzyme (Invitrogen) as per manufacturer's instructions and real time PCR was done (MX3005p, Stratagene, United States). The PCRs were run by mixing the 10X SYBR green (10

µl) PCR mix, diluted cDNA templates (1µl), Gadd45 and Gadd153 primers (1µl) (Table 1) and distilled water (7µl). This reaction mixture (20 µl) was amplified by; denaturation at 95 °C for 10 min, denaturation at 94°C for 1 min, annealing at 57°C for 1 min and finally extension at 72 °C for 1 min was carried out. The threshold values (CT) values were attained for gene expression level analysis (Pfaffl,2001).

The expression analysis was normalized relative to housekeeping gene (GAPDH).

Primers used against specific genes are presented (Table 1).

Table 1. The primers sequences, degree and melting temperatures (Tm) of different genes used in this study

Genes	Accession number	Oligonucleotide sequence	E ⁺	Tm	bp	References
Gadd45	NM_007836.1	F GCTGCCAAGCTGCTCAA	2	57.6	71	Kaufmann <i>et al.</i> 2011
		R TGCTGACGAAGACGA	3	56.2		
Gadd153	NM_007837.3	F AATAACAGCCGGAACCTGAGGA	1	58.2	200	Moriya <i>et al.</i> 2011
		R CCCAATTCATCTGAGGACAGGA	3	56.6		
GAPDH	NM_008084.2	F TGTGTCCGTCGTGGATCTGA		58.1	150	Moriya <i>et al.</i> 2011
		R TTGCTGTTGAAGTCGCAGGAG	1	57.8		

E⁺: exon location of primers; F: forward primer; R: reverse primer; Tm: melting temperature; bp: amplicon size.

2.6. Statistical analysis

Analysis of data was performed by PASW Statistics 18.0 software package. One-way analysis of variance was done for determining the difference between groups. Tukey's multiple comparison test was used to find average difference among the groups. Significance level, $p \leq 0.05$ was taken.

3. Results

3.1. Comet assay

No significant difference was observed in peripheral white blood cells DNA damage at 24 hours for the animals that received only curcumin, DMSO and water as shown (Table 2). MMS and CP showed genotoxicity at 24 hours compared to water, DMSO and curcumin treatments and;

reduction in DNA damage was observed for both MMS and CP at 48 hours (Table 3).

But this reduction in DNA damage, both for MMS and CP at 48 hours, was still higher in relation to water, DMSO and curcumin treatments ($p \leq 0.05$). Pre- and post-treatment with curcumin induced significant reduction ($p \leq 0.05$) in DNA damage in MMS and CP treated groups.

3.2. Micronucleus test

Effects of curcumin on the MMS and CP induced genotoxicity in bone marrow is shown in the form of Micronucleated Polychromatic Erythrocytes (mn-PCEs) and ratio of PCEs/NCEs (Polychromatic Erythrocytes/Normochromatic Erythrocytes) is represented (Table 4). MMS and CP induced a significant increase in the frequency of mn-PCEs, while with curcumin caused a reduction in the frequency of mn-PCEs. Pre-treated curcumin groups, MMS and CP, showed significant decrease

in the frequency of mn-PCEs; 70% and 65% respectively. Moreover, no significant increase in mn-PCEs was observed between post-treatment curcumin groups. The ratio of PCEs/NCEs signified the bone marrow's proliferation rate. The ratio of

PCEs/NCEs in MMS and CP treated mice was lower as compared to control, curcumin and DMSO treated groups. Whereas pre-treatment groups showed higher ratio of PCEs/NCEs.

Table 2. Effect of Curcumin 24 hour on mononuclear leukocyte DNA damage induced by MMS and CP

Experimental groups	n	DNA damage score
		AU Mean ± SE
1) Control	6	10,67±1,12 ^a
2) Curcumin	6	9,67±0,80 ^a
3) DMSO	6	12,33±0,95 ^a
4) Curcumin + MMS	6	15,67±1,23 ^a
5) Curcumin + CP	6	42,67±6,61 ^a
6) MMS + Curcumin	6	71,83±6,74 ^{bc}
7) CP + Curcumin	6	75,33±6,34 ^{bc}
8) MMS	6	73,00±10,54 ^{bc}
9) CP	6	84,33±15,57 ^c

Different letter within a given column are significantly different at $P \leq 0.05$, Tukey's test.

Values are expressed as mean ± SE; AU: arbitrary unite.

MMS: methyl methanesulphonate; CP: cyclophosphamide; DMSO: dimethyl sulphoxide; SE: standart error.

Table 3. Effect of Curcumin 48 hour on mononuclear leukocyte DNA damage induced by MMS and CP

Experimental groups	n	DNA damage score
		AU Mean ± SE
1) Control	6	6,67±0,61 ^a
2) Curcumin	6	9,33±1,26 ^a
3) DMSO	6	10,33±1,89 ^a
4) Curcumin + MMS	6	32,33±6,25 ^{ab}
5) Curcumin + CP	6	18,17±2,63 ^a
6) MMS + Curcumin	6	30,00±2,38 ^{ab}
7) CP + Curcumin	6	69,67±6,64 ^c
8) MMS	6	38,33±5,6 ^{abc}
9) CP	6	64,67±19,67 ^{bc}

Different letter within a given column are significantly different at $P \leq 0.05$, Tukey's test.

Values are expressed as mean ± SE; AU: arbitrary unite.

MMS: methyl methanesulphonate; CP: cyclophosphamide; DMSO: dimethyl sulphoxide; SE: standart error.

Table 4. Effects of different treatments in mice bone marrow in the form of mn-PCEs and ratio of PCEs/NCEs

Experimental groups	(%) mn-PCEs Mean \pm SE	PCEs/NCEs ratio Mean \pm SE
1) Control	4.17 \pm 0.60 ^a	1.80 \pm 0.04 ^a
2) Curcumin	4.33 \pm 0.67 ^a	1.72 \pm 0.02 ^a
3) DMSO	3.50 \pm 0.43 ^a	1.75 \pm 0.02 ^a
4) Curcumin + MMS	10.00 \pm 0.58 ^b	1.25 \pm 0.01 ^{ab}
5) Curcumin + CP	10.50 \pm 0.67 ^{bc}	1.39 \pm 0.02 ^{ab}
6) MMS + Curcumin	10.17 \pm 0.60 ^{bc}	1.18 \pm 0.03 ^{bc}
7) CP + Curcumin	12.83 \pm 0.48 ^c	1.06 \pm 0.06 ^b
8) MMS	14.33 \pm 0.72 ^{cd}	1.08 \pm 0.03 ^{bc}
9) CP	16.33 \pm 0.62 ^{de}	0.98 \pm 0.03 ^c

Different letter within a given column are significantly different at $P \leq 0.05$, Tukey's test.

Values are expressed as mean \pm SE.

MMS: methyl methanesulfonate; CP: cyclophosphamide; DMSO: dimethyl sulphoxide, SE: standart error.

mn-PCEs: micronucleated polychromatic erythrocytes; NCEs: normochromatic erythrocytes.

3.3. Effects of curcumin on the oxidative stress induced by MMS and CP

Results of different treatment groups on the OSI level are shown (Table 5). MMS and CP treated

groups led to the statically significant increase OSI level, when compared to the control, DMSO and curcumin groups (Table 5). Pre-treated curcumin groups significantly reduced the OSI level ($p \leq 0.05$).

Table 5. Effect of different treatment groups on the level of oxidative stress index (OSI)

Experimental groups	OSI levels (%) Mean \pm SE
1) Control	9.88 \pm 0.26 ^a
2) Curcumin	8.55 \pm 0.24 ^a
3) DMSO	9.40 \pm 0.18 ^a
4) Curcumin + MMS	10.58 \pm 0.63 ^a
5) Curcumin + CP	9.18 \pm 0.52 ^a
6) MMS + Curcumin	21.50 \pm 1.23 ^b
7) CP + Curcumin	19.55 \pm 1.31 ^b
8) MMS	22.17 \pm 1.09 ^{bc}
9) CP	25.45 \pm 0.80 ^c

Different letter within a given column are significantly different at $P \leq 0.05$, Tukey's test.

Values are expressed as mean \pm SE.

MMS: methyl methanesulfonate; CP: cyclophosphamide; DMSO: dimethyl sulphoxide; OSI; oxidative stres index; SE: standart error.

3.4. mRNA expression analysis of Gadd45 and Gadd153 genes in hepatic tissue

The real time PCR analysis of Gadd45 and Gadd153 genes expression in different groups is presented (Figure 1). The results showed that mice treated

with MMS and CP led to substantial increase in the expression of Gadd45 and Gadd153 as compared to control group. Post-treated group with curcumin led to the significant expression of Gadd153 and Gadd45 in MMS + Curcumin and CP + Curcumin groups as shown (Figure 1).

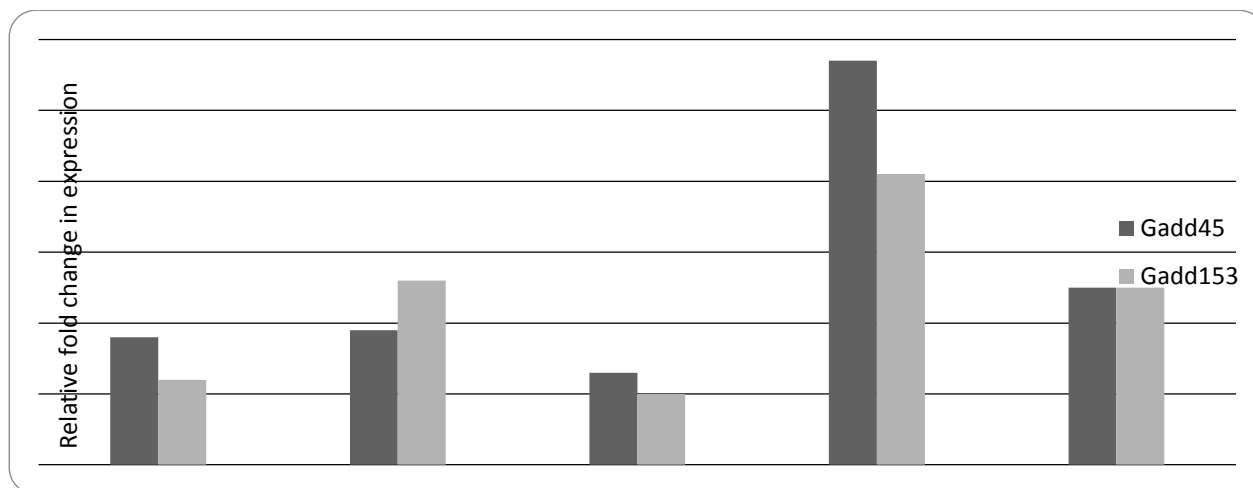


Figure 1. Effect of Curcumin post-treatment on mRNA expressions of Gadd45 and Gadd153 genes in hepatic tissue after MMS and CP as estimated by real-time PCR. The expressions of above genes in different treatment groups were normalized against the sham control group and relative expression changes have been plotted. GAPDH mRNA expression was used in internal control.

4. Discussion and Conclusion

It has been demonstrated by the epidemiological, clinical and animal studies that curcumin has antimicrobial, antioxidant, antiinflammatory, antifungal, antiinfectious, antiviral, antimutagenic, anticarcinogenic, antimetastatic, antigrowth, antiarthritic, antiatherosclerotic, antidepressant, antiaging, antidiabetic, wound healing memory-enhancing activities and neuroprotective properties (Priyadarsini 1997, Limtrakul *et al.* 1997, Pandya *et al.* 2000, Anand *et al.* 2008, Aggarwal *et al.* 2013a, Gopal *et al.* 2014, Kunnumakkara *et al.* 2016). Many studies have demonstrated the anticancer effect of curcumin in a variety of tumors as gastrointestinal, breast, pancreatic and hepatic cancer (Anand *et al.* 2008). No extensive study has been observed to show the antigenotoxicity, antioxidant capacity and expression levels of Gadd45 and Gadd153 genes, against alkylating agents-induced genotoxicity. Both substances (MMS and CP) have been shown to induce gene mutations in prokaryotes and eukaryotes. In current study, MMS and CP showed clear genotoxic effects. The direct-acting alkylating agent, MMS, gets covalently linked to DNA and causes DNA damage, creating an indirect effect of the alkylating agent.

MMS does the methylation of DNA nucleophilic regions, especially at the nitrogen atoms of amino acid molecules. Phosphate methylation by the MMS accounts almost 51 % of total methylation. MMS induces genotoxicity by changing base modifications which further weakens the N-glycosidic bond, causing the depyrimidination/depurination of DNA strands, which leads to the formation of alkali-labile abasic (AP) sites. Cleavage of these AP sites occurs by the AP endonucleases enzyme which finally breaks the DNA strands (Loeb *et al.* 1986, Franke *et al.* 2005). CP causes DNA damage by changing the function of cellular proteins. CP is easily absorbed in body through oral administration and its half-life is almost 6-9 hours. Parent compound of CP has low protein affinity (20 %) inside the body (Boiteux and Guillet 2004). CP in its activated form leads to the formation of DNA-DNA and DNA-protein cross linking. It also produces free radicals that lead to epithelial and endothelial damage (Matalon *et al.* 2004). In current study, curcumin showed antigenotoxic effect against both alkylating agents. This dietary agent (curcumin) has been used traditionally in medicine in India and other Asian countries. Curcumin has phenolic components, which provide protective effects against alkylating agents. Phenolic components in curcumin play a diverse role against DNA damage; either by

stimulating detoxifying enzymes or by stimulating DNA repair pathways through transcription regulation or mRNA stabilization (Abalea *et al.* 1999). Previous studies also showed the protective effect of phenolic components against the alkylating agents (Franke *et al.* 2005, Corona *et al.* 2007).

Higher mn-PCEs and PCEs/NCEs ratios in curcumin-treated groups was indicative of its protective effects against alkylating compounds. PCEs are immature erythrocytes in intermediate stages of development, have ribosomes; while, NCEs are mature, ribosome-free and can remain about 1 month in the peripheral loop (Mavourn *et al.* 1990). It clearly shows the arrest of mature erythrocyte formation by damaging DNA (Cicchetti *et al.* 1999, Yener and Dikmenli 2009). It has been shown that herbal treatment by the curcumin effectively leads to the formation of mature erythrocytes in sodium arsenide- and copper-treated mice (Corona *et al.* 2007, Biswas *et al.* 2010). The observed protective effect in current study against alkylating agents-induced genotoxicity may be due to one or more of the following: antioxidant action, formation of complex with mutagen, trapping of free radicals, adsorbing the xenobiotic modulation of mutagen metabolism. This is feasible because many naturally occurring compounds are known to exhibit discrete mechanisms of protection (Wattenberg 1985; Morse and Stoner 1993).

Positive effects were observed by curcumin in reducing the oxidative stress induced by MMS and CP in present study. Previously, MMS has been observed as oxidative-stress inducer (Horvaathovaa *et al.* 1998). Curcumin due to its free radical scavenging property, by acting as electron donor and inhibition of lipid peroxidation acts as an antioxidant (Reddy and Lokesh 1994, Sreejayan and Rao 1994). Oxidative stress is the cause of many diseases, while curcumin has the ability to cure many diseases like diabetes and cancer due to its antioxidant capacity (Sajithlal *et al.* 1998, Majithi *et al.* 2005, Saha *et al.* 2010). In another study,

curcumin's has been shown to reduce the harmful effects of MMS induced effects on kidneys of mice (Cuce *et al.* 2016).

In our study, higher mRNA genes expression of Gadd45 and Gadd153 were induced by alkylating agents whereas post treated curcumin groups showed substantial increased expression of these genes. Previously, studies clearly stated the anti-apoptotic and anti-proliferative activity of curcumin by arresting the cell cycle in G₁ phase in different cell lines (Aggarwal *et al.* 2007, Srivastava *et al.* 2007). Curcumin arrests the cell cycle by down regulating the various genes like cyclin D1 and the cyclin dependent kinases CDK2, CDK4 and CDK6. This suggested that curcumin induced the more expression of Gadd45 and Gadd153 and stopped the cell cycle to avoid further damage of cells by the alkylating agents (Saha *et al.* 2010).

Curcumin is found one of the herbal dietary supplements in daily life which is easy to apply, cheap, and can be used as a reliable drug in the treatment of several major genetic diseases including cancer. So, this study could contribute an important contribution to open molecular perspective of curcumin's protective efficiency against genotoxic chemicals.

In conclusion, the findings from the present investigations highlight the importance of curcumin and present its potential antigenotoxic effects against alkylating agents.

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