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Assessment of methyl thiophanate–Cu (II) induced DNA damage in human lymphocytes

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ABSTRACT

Dimethyl 4,4′-(*O*-phenylene)bis(3-thioallophanate), commonly known as methyl thiophanate (MT), is a category-III acute toxicant and suspected carcinogen to humans. Hence, the ability of this benzimidazole class of fungicide to engender DNA strand breaks was investigated using alkaline single cell gel electrophoresis (SCGE), alkaline unwinding and cytokinesis-blocked micronucleus (CBMN) assays. The SCGE of human lymphocytes treated with 1 mM MT for 3 h at 37 °C showed much higher Olive tail moment (OTM) value of 40.3 ± 2.6 ($p < 0.001$) vis-à-vis 3.3 ± 0.09 in DMSO control. Treatment of cultured lymphocytes for 24 h resulted in significantly increased number of binucleated micronucleated (BNMN) cells with a dose dependent reduction in the nuclear division index (NDI). Stoichiometric data revealed the intrinsic property of MT to bind with Cu (II) and its reduction to Cu (I), which is known to form reactive oxygen species (ROS). We have detected the intracellular ROS generation in MT treated lymphocytes and observed an elevated level of MT-induced strand breaks per unit of calf thymus DNA in presence of Cu (II). Overall the data suggested that the formation of MT–Cu (II)–DNA ternary complex and consequent ROS generation, owing to Cu (II)/Cu (I) redox cycling in DNA proximity, is responsible for MT-induced DNA damage.

1. Introduction

The use of pesticides including the herbicides and fungicides on crops and weeds has been augmented to a significant extent in the last few decades. Large-scale and indiscriminate application of these agrochemicals pose human health risks, specifically in developing countries, where the pesticide users are often ill-trained and devoid of appropriate protective devices. The associated health hazards are further extended to those exposed occupationally or inadvertently. Excessive use of pesticides resulted in prevalence of a variety of cancerous ailments viz. hematopoietic cancers, non-Hodgkin's lymphoma, leukemia and multiple myeloma (Wiklund and Holm, 1986; Morrison et al., 1992; Zham and Blair, 1992). Several immunological abnormalities as well as the nervous, endocrine, reproductive and developmental disorders have also been related to certain pesticides (Koner et al., 1998; Colosio et al., 2003; Gupta, 2004).

Methyl thiophanate (MT), a broad spectrum fungicides widely used for control of some important fungal diseases of crops (Hassall, 1990; Traina et al., 1998) has been chosen in this study for assessment of the nature and extent of MT-induced DNA damage (as promutagenic and pre-carcinogenic lesions) for carcinogenic risk assessment. MT is a benzimidazole class of compound, classified as "likely to be carcinogenic to humans" as per EPA carcinogen risk assessment guidelines (Proposed Guidelines for Carcinogen Risk Assessment, 1996). Being a category-III acute inhalation toxicant, it has been reported to exhibit a dose-related increase in the incidence of follicular and hepatocellular adenomas in male and female F344 rats. It has also been shown to cause skin papilloma at 75 ppm and pituitary adenoma at 200 ppm in male rats, and mammary gland fibroadenoma in female rats at 1200 ppm (Thiophanate Methyl Revised Report of the Hazard Identification Assessment, 2000). Upon oral administration, MT gets absorbed and metabolized into benzimidazole compounds, mainly carbendazim, which is a reproductive toxicant in male and female rats (Goldman et al., 1989; Cummings et al., 1990). At relatively higher doses, MT acts as a weak endocrine disruptor and adversely affects the endocrine tissue development and thyroid–pituitary homeostasis (Maranghi et al., 2003). It is regarded as a potential spindle poison, impairing the polymerization of microtubule formation in

Abbreviations: BNMN, binucleated micronucleated lymphocytes; BCS, bathocuproine disulfonic acid; CBMN, cytokinesis-blocked micronucleus; Cyto B, cytochalesin B; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DMSO, dimethyl sulfoxide; EMS, ethyl methane sulphonate; LMA, low melting temperature agarose; MT, methyl thiophanate; MMS, methyl methane sulphonate; NMA, normal melting temperature agarose; NDI, nuclear division index; OTM, Olive tail moment; PHA-M, phytohemagglutinin-M; ROS, reactive oxygen species; SCGE, single cell gel electrophoresis; TDNA, tail DNA.

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fungal DNA synthesis (Seiler, 1975; Maranghi et al., 2003). Therefore, it is speculated that the actual risk of genotoxicity from this fungicide might be appreciably higher than that predicted from conventional toxicity tests, as also suggested by Bolognesi (2003) for other pesticides. To the best of our understanding, no systematic study has been carried out which has emphatically demonstrated the MT-induced DNA damage, and role of Cu (II) ions in MT–Cu (II) mediated ROS production in vitro system. We have, therefore, conducted apriori model study to investigate the DNA damaging potential of this broad spectrum fungicide using well established sensitive techniques like single cell gel electrophoresis (SCGE or comet), alkaline unwinding, and cytokinesis-blocked micronucleus (CBMN) assay. The data unequivocally demonstrated the MT-induced DNA strand breaks and predicted the plausible role of intracellular ROS being generated in presence of Cu (II) transition metal ions, in triggering DNA damage.

2. Materials and method

2.1. Chemicals

Methyl thiophanate (dimethyl 4,4′-(*O*-phenylene)bis(3-thioallophanate) CAS No. 23564-05-8, 97% pure (Fig. 1) was obtained from Agrochemical Division, (IARI, New Delhi, India). Deoxyribonucleic acid (DNA), sodium salt, highly polymerized (Type I) from calf thymus, low and normal melting temperature agarose (LMA and NMA), Na_2-EDTA, Tris-buffer, ethidium bromide (EtBr), propiodium iodide, methyl methane sulphonate (MMS), ethyl methane sulphonate (EMS), histopaque 1077, cytochalesin B (Cyto B), phytohemagglutinin-M (PHA-M), 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and DMSO were obtained from Sigma Chemical Company (St. Louis, MO, USA). DMSO (1%) was used as solvent control in experiments where specified, unless otherwise stated. RPMI-1640, foetal bovine serum (FBS) were procured from GIBCO BRL Life Technologies Inc. (Gaithersburg, MD, USA). Phosphate buffered saline (PBS, Ca^{2+} Mg^{2+} free), Triton X-100 and bathocuproine disulfonic acid were obtained from Hi-Media Pvt. Ltd. (India). All other chemicals were of analytical grade. The slides for microgel electrophoresis were purchased from Blue Label Scientifics Pvt. Ltd., (Mumbai, India).

2.2. Alkaline single cell gel electrophoresis (comet assay)

Comet assay was performed with human lymphocytes following methods of Singh et al. (1988) as modified by Bajpayee et al. (2002). Lymphocytes were separated from heparinized whole blood of a healthy male volunteer, aged 26 years, with none of the following habits; smoking, consumption of alcohol, chewing of tobacco, not participating in high physical activities and was not on any type of medication during the period of blood sampling. Freshly isolated cells were treated with varying concentrations (0.25, 0.5, 0.75 and 1 mM) of MT for 3 h at 37 °C. Viability of lymphocytes was checked before and after treatment with MT using 0.4% trypan blue dye. The lymphocytes ($\sim 4 \times 10^4$ cells) both untreated and treated were suspended in 100 μl of Ca^{2+} Mg^{2+} free PBS and mixed with 100 μl of 1% LMA. The cell suspension (80 μl) was then layered on one-third frosted slides, pre-coated with NMA (1% in PBS) and kept at 4 °C for 10 min. After gelling, a layer of 90 μl of LMA (0.5% in PBS) was added. The cells were lysed in a lysing solution for overnight. After washing with Milli Q water, the slides were subjected to DNA denaturation in cold electrophoretic buffer at 4 °C for 20 min. Electrophoresis was performed at 0.7 V/cm for 30 min (300 mA, 24 V) at 4 °C. The slides were then washed three times with neutralization buffer. All preparative steps were conducted in dark to prevent secondary DNA damage. Each slide was stained with 75 μl of 20 μg/ml ethidium bromide solution for 5 min. The slides were analyzed at 40X magnification (excitation wavelength of 515–560 nm and emission wavelength of 590 nm) using fluorescence microscope (Leica, Germany) coupled with charge coupled device (CCD) camera. Images from 50 cells (25 from each replicate slide) were randomly selected and subjected to image analysis using software Komet 3.0 (Kinetic Imaging, Liverpool, UK). The data were subjected to one-way analysis of variance (ANOVA). Mean values of the tail length (μm), OTM and % tail DNA (% TDNA) were separately analyzed for statistical significance. The level of statistical significance chosen was $p \leqslant 0.05$, unless otherwise stated.

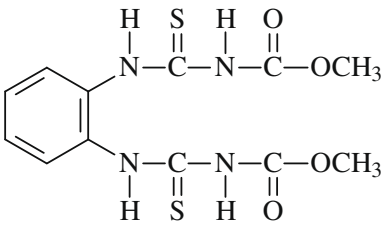


Fig. 1. Structure of methyl thiophanate.

2.3. Alkaline unwinding assay

MT-induced strand breaks in the DNA were quantitated by alkaline unwinding assay using hydroxyapatite batch procedure (Kanter and Schwartz, 1979). In brief, the calf thymus DNA (100 μg) in a volume of 0.5 ml in multiple sterile tubes were treated with MT at 1:2 to 1:10 DNA nucleotide/MT molar ratios in the absence and presence of 100 μM Cu (II). The untreated and EMS treated DNA were taken as negative and positive controls. The treatment was carried out for 30 min at 37 °C. The tubes were immediately placed on ice and subjected to alkaline unwinding by rapid addition of an equal volume of 0.06 N NaOH in 0.01 M Na_2HPO_4, pH 12.5 followed by brief vortexing. Alkaline unwinding was allowed to complete in dark for 30 min. The pH of the reaction mixture was then neutralized to pH 7.0 with the addition of 0.07 N HCl. Subsequently, 20 μM EDTA containing 2% SDS was added and the resultant mixture was transferred to pre-heated stoppered glass tubes containing 0.5 M potassium phosphate buffer, pH 7.0 and 10% formamide. The samples were incubated at 60 °C for 2 h with intermittent vortexing. The relative amount of duplex and single stranded DNA present at the end of alkaline unwinding was quantitated. Single stranded DNA was selectively eluted from the hydroxyapatite matrix with 0.125 M potassium phosphate buffer, pH 7.0 containing 20% formamide. However, duplex DNA was removed with 0.5 M potassium phosphate buffer, pH 7.0 containing 20% formamide. Strand breaks were estimated following the equation $\ln F = -(K/MN)\ t\beta$, where F is the fraction of double stranded DNA remaining after alkali treatment for the time t, MN is the number-average molecular weight between two strand breaks and β is a constant that is less than 1 (Rydberg, 1975). The number of unwinding points (P) per alkaline unwinding unit of DNA were calculated according to the equation, $P = \ln Fx/\ln Fo$ (Kanter and Schwartz, 1979), where, Fx and Fo are the fraction of double stranded DNA remaining after alkaline denaturation of treated and untreated samples, respectively. The number of breaks (n) per unit DNA were then determined using the equation $n = P - 1$.

2.4. Cytokinesis-blocked micronucleus (CBMN) assay

The CBMN assay was performed following the method of Kalantzi et al. (2004). The whole blood (0.5 ml) was cultured in 4.5 ml complete RPMI 1640 medium supplemented with 20% heat-inactivated FBS, L-glutamine (0.02 mM), sodium bicarbonate (2.0 g/L), penicillin

(100 U/ml), streptomycin (100 μg/ml) and 7.5 μg/ml PHA-M. Cells were exposed to MT at final concentrations of 0.05, 0.1 and 0.2 mM and allowed to grow at 37 °C in presence of 5% CO_2 in air in a humidified atmosphere chamber of CO_2 incubator. After 24 h, cells were pelleted by spinning at 1000 rpm for 10 min, washed twice with RPMI 1640 and resuspended in complete medium without MT. Cytokinesis was blocked by adding Cyto B (6 μg/ml) after 44 h of incubation. The binucleated lymphocytes were harvested after 72 h of culture condition at 1000 rpm for 10 min. Cells were then treated with hypotonic solution (0.56% KCl) for 2–3 min to lyse erythrocytes, fixed once with methanol and glacial acetic acid (3:1) for overnight at 4 °C. Finally, the cell solution was dropped onto cold glass slides. Staining was performed by separately immersing the air-dried slides in 6% Giemsa stain and propiodium iodide (6 μg/ml in phosphate buffer) solution. One thousand binucleated cells for each experimental point were counted, following the scoring criteria adopted by the Human Micronucleus Project (Bonassi et al., 2001). The mean binucleated micronucleated lymphocytes (BNMN) were evaluated as the number of binucleated lymphocytes containing one or more micronuclei per 1000 binucleated cells. Moreover, 500 binucleated cells were scored to evaluate the percentage of binucleated cells for expressing the toxicity and/or inhibition of cell proliferation as nuclear division index (NDI) calculated according to the following formula:

$$\mathrm{NDI} = (\mathrm{Mono} + 2\mathrm{BN} + 3\mathrm{Tri} + 4\mathrm{Tetra})/500$$

where Mono, BN, Tri and Tetra are mononuclear, binucleated, trinucleated and tetranucleated lymphocytes, respectively. The data were analyzed by one-way ANOVA (Tukey test) for determining statistical significance.

2.5. Measurement of intracellular reactive oxygen species (ROS) generation

Intracellular ROS production was detected by using a fluorescent probe DCFH-DA, according to the method of Huang et al. (2008) with slight modifications. About 2×10^6 cells of human lymphocytes were exposed to varying concentrations of MT (0.1–0.5 mM) in complete RPMI 1640 medium. Cells were cultured for 24 h at 37 °C in presence of 5% CO_2 in air in a humidified atmosphere chamber of CO_2 incubator (Sheldon Manufacturing Inc., USA). After 24 h, the cells were pelleted by spinning at 3000 rpm for 5 min and washed twice with cold PBS and resuspended in 2 ml of PBS. Cells were incubated with DCFH-DA (5 μM) for 60 min at 37 °C in dark. Cells were immediately washed twice with PBS and finally suspended with 3 ml PBS. Fluorescence measurements were carried out on a Shimadzu spectrofluorophotometer (RF5301PC equipped with RF 530XPC instrument control software) at 37 °C, using a quartz cell of 1 cm path length. The fluorescence intensity was recorded at an excitation wavelength of 485 nm and emission wavelength of 525 nm.

2.6. MT–Cu (II) complexation and stoichiometric titration of Cu (I)

The amount of Cu (I) produced during MT–Cu (II) reaction was determined upon titration with Cu (I) specific chelator bathocuproine disulfonic acid (BCS). Briefly, 10 μM MT in 10 mM Tris–HCl buffer, pH 7.0 were mixed with $CuCl_2$ at varying molar ratios (1:0.25–1:10) in presence of constant amount (0.3 mM) of bathocuproine in a total volume of 1 ml. The extent of bathocuproine–Cu (I) complex formation at increasing Cu (II)–MT molar ratios was determined by measuring absorbance at 480 nm.

3. Results

3.1. Assessment of DNA damage in human lymphocytes by alkaline SCGE assay

MT-induced single strand breaks in human lymphocytic DNA have been observed upon SCGE under alkaline conditions. Fig. 2(Panels D–G) shows dose-dependent increase in the size of comet tails with concomitant reduction in head size. The digitized images of representative comets clearly demonstrate the extent of broken DNA liberated from the heads of the comets during electrophoresis with increasing MT doses. The quantitative data obtained from the comparative analysis of a set of 150 cells at each MT concentration with the untreated and DMSO treated control cells revealed 4.6-fold enhanced DNA migration at 1.0 mM MT. The lymphocytes treated with MT (1.0 mM) showed an OTM value of 40.3 (AU) as compared to 3.29 (AU) and 3.30 (AU) with untreated and DMSO controls, respectively. The MT treated lymphocytes at the highest dose of 1.0 mM showed almost similar extent of DNA damage as observed with 2 mM EMS, as positive control. A significant increase in the values of OTM, % tail DNA and tail length (μm) at concentrations above 0.25 mM was observed (Table 1). The advantage of comet assay is that it is capable of analyzing population of cells with various degrees of DNA damage. Notwithstanding the differences existing in the distribution of damage in cell population. The heterogeneity in the distribution of DNA damage among cells is shown in Fig. 3. The histogram shows that 100% of EMS treated cells (positive control) have OTM > 20 as compared to 60% cells of untreated controls with the OTM value of 4.0. At 0.25 mM concentration, the OTM values of MT-treatment cells vary from 4.0 to 12.0. Around 40% cells at this concentration were observed with OTM value of 8.0 and none were found to have the OTM of >20. The percentage of cells with OTM > 20.0 increased substantially from 10% at 0.5 mM to 90% and100% cells at 0.75 and 1 mM MT doses, respectively.

Table 1
MT-induced DNA damage in human lymphocytes analyzed using different parameters of SCGE assay.

Groups	Olive tail moment (arbitrary unit)	Tail DNA (%)	Tail length (μm)
Control	3.29 ± 0.35	8.52 ± 1.12	45.23 ± 0.54
DMSO (1%)	3.30 ± 0.09^{ns}	8.52 ± 0.45^{ns}	43.78 ± 0.71^{ns}
EMS (2 mM)	48.99 ± 5.29 ***	51.55 ± 3.62 ***	204.41 ± 1.09 ***
MT (mM)			
0.25	6.96 ± 0.85^{ns}	15.46 ± 0.72*	93.94 ± 0.84**
0.50	15.05 ± 2.91**	25.19 ± 3.17***	148.84 ± 1.06***
0.75	30.09 ± 0.45***	37.78 ± 0.40***	171.69 ± 0.75***
1.0	40.30 ± 2.64***	44.39 ± 2.03***	201.22 ± 1.09***

Data represent the mean ± SEM of three independent experiments in duplicate. ns = non significant; EMS: ethyl methanesulphonate; DMSO: dimethysulfoxide.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

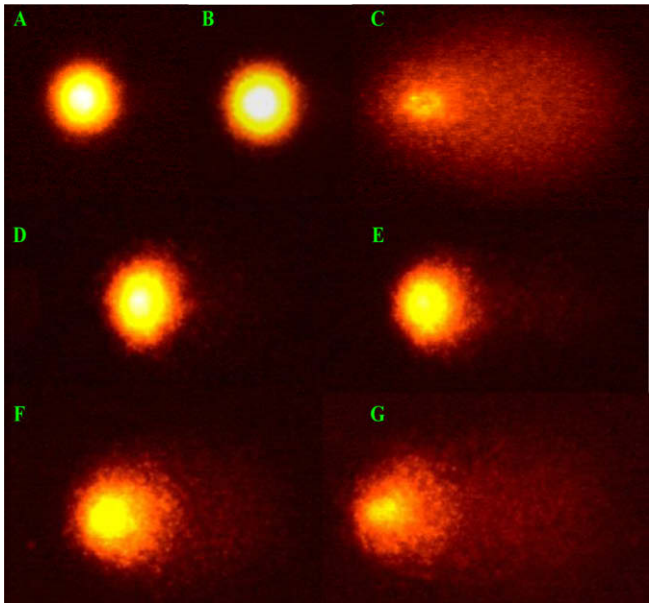


Fig. 2. Epi-fluorescence comet images of MT-induced DNA damage in human lymphocytes. The representative photomicrographs have been acquired through Komet 3.0 software. (A) Untreated control, (B) DMSO (1%), solvent control, (C) EMS (2 mM) treated cells, positive control, (D–G) cells treated with MT at 0.25, 0.5, 0.75 and 1 mM, respectively.

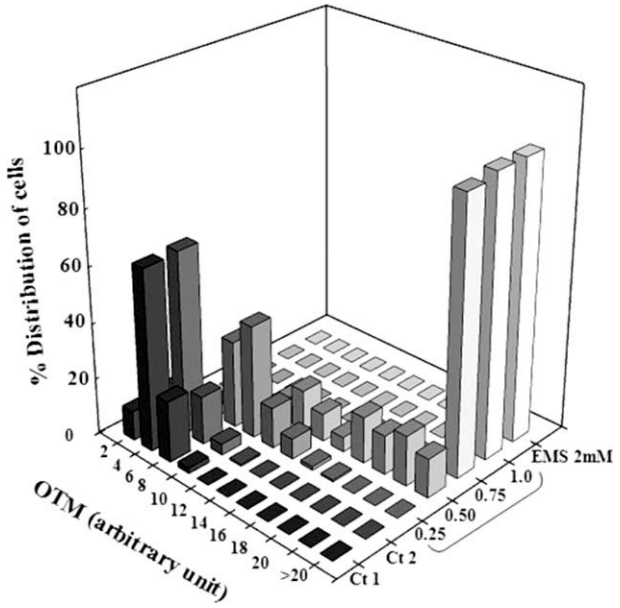


Fig. 3. Percent distribution of lymphocytes based on the extent of DNA damage. The untreated (Ct 1), DMSO (1%) (Ct 2) and EMS (2 mM) treated cells were taken as negative and positive controls. Each histogram represents the mean value of three independent experiments done in duplicate.

3.2. Quantitative assessment of MT-induced strand breaks in DNA

Treatment of calf thymus DNA with MT resulted in a concentration dependent decrease in the fraction of duplex DNA with simultaneous increase in the degree of single strandedness in DNA (Fig. 4). Based on the amount of duplex DNA remaining after alkali treatment for a specified time, the number of strand breaks formed per unit DNA was determined at corresponding MT concentrations. A parallel control does not show any reduction in the amount of duplex DNA. At the highest DNA nucleotide/MT molar ratio of

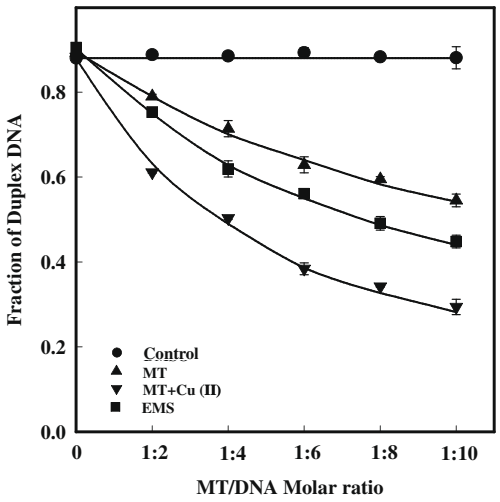


Fig. 4. Alkaline unwinding of MT treated DNA. The data are mean ± SEM of two independent experiments done in duplicate.

Table 2
Strand breaks in calf thymus DNA produced by MT and MT plus 0.1 mM Cu (II).

DNA nucleotide/MT molar ratio	Number of breaks per unit DNA (n)	
	MT	MT + Cu (II)
Control untreated DNA	N.D.	N.D.
Treated DNA (EMS, 1:10)	7.0	–
1:2	1.09	2.96
1:4	2.07	4.53
1:6	3.13	6.68
1:8	3.61	7.63
1:10	4.40	8.82

N.D.: not detectable.

Table 3
Effect of MT on micronuclei formation in human lymphocytes.

Compound	Dose	BNMN/1000 cells	NDI
Control	0	7.0 ± 1.41	1.9
DMSO (%)	1.2	6.0 ± 1.38^{ns}	1.9
MMS (mM)	0.1	16.0 ± 2.82*	1.82
MT (mM)	0.05	13.5 ± 3.53^{ns}	1.87
	0.10	21.0 ± 3.48*	1.82
	0.20	45.0 ± 5.65*	1.74

Data represent the mean ± S.D. of three independent experiments done in duplicate. MMS: methyl methane sulphonate (as positive control); DMSO: solvent control; ns = non significant.
* $p < 0.05$ analyzed by Tukey test.

1:10 in absence and presence of Cu (II), about 4.4 and 8.8 strand breaks per unit of DNA were produced (Table 2).

3.3. Micronucleus formation in MT exposed human lymphocytes

A concentration dependent increase in the total number of BNMN human lymphocytes upon exposure to MT has been noticed. Treatment of cells with MT for 24 h resulted in a significant increase ($p < 0.05$) in the mean BNMN/1000 cells as validated by one-way ANOVA (Table 3). The mean BNMN with 0.05, 0.1 and 0.2 mM of MT concentrations were determined to be 13.5, 21, and 45, respectively. The mean BNMN cells with the untreated, DMSO solvent and MMS as a positive controls were 7, 6 and 16, respectively. In order to better characterize the effect of MT, NDI was also evaluated, which shows a decline in the BN cell formation. The MT treated groups showed the NDI of 1.87, 1.82 and 1.74 with

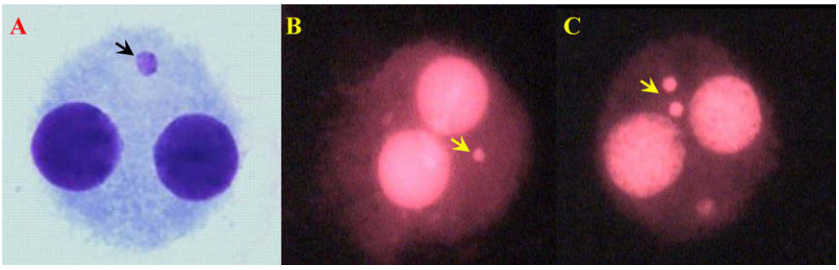


Fig. 5. Representative photomicrographs of the binucleated micronucleated cells appeared after 24 h of MT exposure. Panel (A) represents 6% Giemsa stained cells treated with MT. Panels (B and C) are the propiodium iodide (6 μg/ml) stained cells. Arrows indicate the presence of micronucleus in cells.

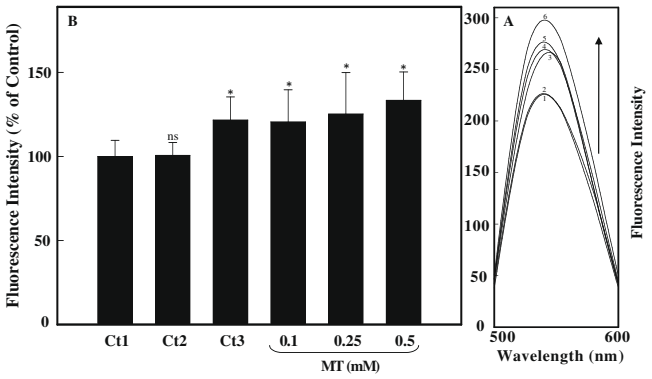


Fig. 6. Fluorescence emission spectra of MT treated cells (Panel A) showing the fluorescence enhancement of DCF with increasing concentrations of MT. From bottom to top, curves 1–6 are: untreated control, DMSO (1%) solvent control, MT 0.1 mM, H_2O_2 (0.4 mM) positive control, MT 0.25 and 0.5 mM, respectively. Panel B shows the corresponding plot indicating the percent fluorescence increase over and above the control. Ct1, untreated control; Ct2, 1% DMSO (solvent control); Ct3, 0.4 mM H_2O_2 (positive control).

Table 4
Production of Cu (I) upon MT–Cu (II) interaction.

Cu (II)/MT molar ratio	Cu (II) added (μM)	Cu (I) produced (μM)[a]
MT alone (10 μM)	0	0
1:0.25	2.5	1.10
1:0.5	5.0	2.12
1:1.0	10.0	4.31
1:1.5	15.0	6.88
1:2.0	20.0	9.47
1:2.5	25.0	12.74
1:3.0	30.0	13.89
1:3.5	35.0	15.67
1:4.0	40.0	18.64
1:4.5	45.0	21.62
1:5.0	50.0	26.21
1:5.5	55.0	29.11
1:6.0	60.0	32.26
1:6.5	65.0	33.65
1:7.0	70.0	35.26
1:7.5	75.0	35.29
1:8.0	80.0	35.30
1:8.5	85.0	35.28
1:9.0	90.0	35.13
1:9.5	95.0	35.25
1:10.0	100.0	35.30

[a] Concentration of Cu (I) were calculated by using the equation $A = \varepsilon cl$ in which molar absorptivity (ε) of Cu (bathocuproine)$_2$ + complex = 13,500 and path length (l) = 1 cm, ΔA_{480} was obtained from A_{480} of the sample with and without Cu (II) addition.

0.05, 0.1 and 0.2 mM of MT vis-à-vis the untreated control showing an NDI of 1.9 (Table 3). The representative photographs of MT treated human lymphocytes in Fig. 5A–C stained with Giemsa and propiodium iodide, clearly indicate the presence of micronucleus.

3.4. ROS generation in MT treated human lymphocytes

Intracellular ROS generation was detected in lymphocytes treated with MT in concentration range of 0.1–0.5 mM. Fig. 6A shows enhancement of the fluorescence intensity at λ_{em} 525 nm of DCFH-DA stained MT treated human lymphocytes, after 24 h of treatment. The dye in cells undergoes oxidation to yield substantial amount of fluorescent probe DFC, as compared to the untreated control. Considering the fluorescence intensity of untreated control cells as 100%, the MT treated cells in concentration range of 0.1, 0.25 and 0.5 mM exhibited 20.5%, 25.24% and 33.41% higher fluorescence intensity of DCF. Cells treated separately with H_2O_2 (0.4 mM) and DMSO (1%) were taken as positive and solvent controls, and they exhibited 21% and 0.61% higher fluorescence intensity as compared to the untreated control cells (Fig. 6 B).

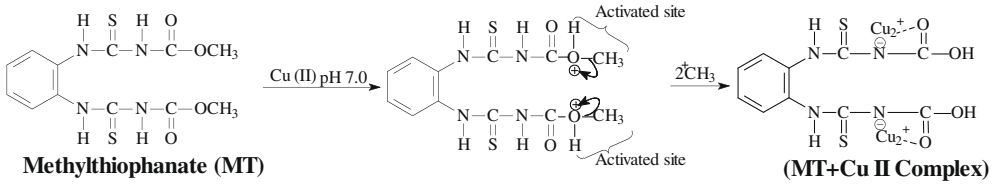
3.5. Stoichiometry of MT–Cu (II) interactions and quantitation of Cu (I) production

The spectroscopic data with MT–Cu (II) in combination with the bathocuproine clearly indicate the production of Cu (I) upon MT–Cu (II) interaction. The results in Table 4 revealed 35.3 μM of Cu (I) produced from 100 μM of Cu (II) added. The amount of Cu (I) produced in the reaction mixture forms complex with bathocuproine and absorbs maximally at 480 nm. An increase in absorbance at this wavelength was noticed with increasing Cu (II)–MT ratios. The Cu (II) or MT alone does not interfere at the absorption maxima.

4. Discussion

It is well known that the genotoxicity and carcinogenicity risk associated with the exposure to certain xenobiotics including the agrochemicals such as fungicides, herbicides and insecticides etc. depends on the extent of their interactions and consequent cellular and genetic damages. One of the widely used and less studied fungicides in terms of its reactivity with DNA is MT, a known spindle poison in both fungal and mammalian cells (Cummings et al., 1990; Maranghi et al., 2003). Several in vitro studies have reported the chromosomal aberrations (Šaram et al., 1998; Ribas et al., 1996), MN in peripheral blood lymphocytes (Šaram et al., 1998; Gebel et al., 1997) and sister chromatid exchanges (Ribas et al., 1996; Kevekordes et al., 1996) with certain pesticides. However, published information on MT genotoxicity is very scanty and inconclusive. This has led us to further probe and establish the DNA damaging ability of MT employing more sensitive and quantitative assays. To the best of our knowledge, this is the first report explicitly demonstrating the MT-induced DNA damage, and plausible involvement of ROS in MT engendered DNA breaks.

In this study, the SCGE (comet) data revealed a significant increase in the OTM and comet tail length, as an index of MT-induced DNA damage in human lymphocytes. Treatment of lymphocytes



Scheme 1.

with MT for 24 h with subsequent addition of Cyto B also resulted in formation of binucleated cells with variable number of micronuclei as a function of MT concentration, which is suggestive of the clastogenicity of this fungicide. This is in concurrence with the earlier reports indicating the clastogenic potential of MT (Hrelia et al., 1996; Fimognari et al., 1999). The significant increase ($p < 0.05$) in the numbers of MN formation in binucleated cells at the MT doses of 0.1 and 0.2 mM vis-à-vis untreated control, unequivocally demonstrated the DNA damaging potential of the MT in human cells. The observed damage in treated lymphocytes possibly occurred due to direct interaction of MT or its metabolites with cellular DNA involving ROS in the development of frank strand breaks in DNA.

The mechanism proposed in Scheme 1 suggests a stable MT–Cu (II) complex formation due to electron sharing with nitrogen and oxygen atoms present on bilaterally symmetrical arms of MT. Copper ions are known to exhibit high affinity for DNA and the DNA-bound Cu (II) can undergo Cu (II)/Cu (I) redox cycling in a reducing environment. Our studies with Cu (I) specific chelating agent bathocuproine demonstrated the MT–Cu (II) interaction and consequent reduction of Cu (II) to Cu (I). Copper is considered as one of the major metals present in the nucleus (Bryan, 1979). Its concentration in different tissues ranges from 10 to >100 μM with 20% found in the nucleus (Linder, 1991). Mobilization of endogenous copper ions possibly the copper bound to chromatin (Hanif et al., 2008) has been implicated in the formation of ROS such as the hydroxyl radicals, close to the site of DNA cleavage (Pryor, 1988; Chevion, 1988,). Thus, the generation of ROS in MT treated lymphocytes has been assessed using fluorescent dye DCFH-DA. MT concentration dependent oxidation of the dye producing substantial amount of fluorescent probe DFC, as measured at an emission wavelength of 525 nm, confirmed the MT-induced ROS formation in lymphocytes. It is well established that due to high electrophilicity, thermo-chemical reactivity and small diffusion radius, the ROS generated in the proximity of DNA are responsible for strand scission (Pryor, 1988; Chevion, 1988).

The quantitative assessment of strand breaks in MT and MT + Cu (II) treated calf thymus DNA using alkaline unwinding assay revealed the formation of about 4.4 and 8.8 strand breaks per unit of DNA molecule, respectively. These results reaffirmed that the MT–Cu (II) complex formation and the Cu (II)/Cu (I) redox cycling in vicinity of the DNA molecule is causal for DNA damage. Indeed, the level of copper in tissue (Yoshida et al., 1993; Nasulewis et al., 2004) and serum (Ebadi and Swanson, 1998) is reported to be substantially enhanced under clinical and oncological conditions. It has also been suggested that in case of women bearing a copper containing intrauterine pessar (IUP), ~50 μg copper is being released per day (Hagenfeldt, 1972). Thus, the increased strand breaks in presence of Cu (II) strongly suggest the likelihood of elevated MT-induced DNA damage under the conditions of higher intracellular Cu (II) particularly in MT exposed individuals with impaired copper metabolism and female agricultural workers. The patients suffering from copper metabolism disorders such as Menkes syndrome, Wilson's disease and certain neurodegenerative ailments including amyotropic lateral sclerosis (ALS) and Alzheimer's disease possibly could be the vulnerable targets. Therefore, cellular exposure to MT resulting in accumulation of MT–Cu (II) induced damage in genetic material may ultimately triggers mutagenecity and carcinogenecity, if the lesions are not accurately processed and repaired by the cellular DNA repair machinery.

Conflict of Interest

There are no conflicts of interest in this study.

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