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Butachlor induced dissipation of mitochondrial membrane potential, oxidative DNA damage and necrosis in human peripheral blood mononuclear cells

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ABSTRACT

Butachlor is a systemic herbicide widely applied on rice, tea, wheat, beans and other crops; however, it concurrently exerts toxic effects on beneficial organisms like earthworms, aquatic invertebrates and other non-target animals including humans. Owing to the associated risk to humans, this chloroacetanilide class of herbicide was investigated with the aim to assess its potential for the (i) interaction with DNA, (ii) mitochondria membrane damage and DNA strand breaks and (iii) cell cycle arrest and necrosis in butachlor treated human peripheral blood mononuclear (PBMN) cells. Fluorescence quenching data revealed the binding constant ($Ka = 1.2 \times 10^4\ M^{-1}$) and binding capacity ($n = 1.02$) of butachlor with ctDNA. The oxidative potential of butachlor was ascertained based on its capacity of inducing reactive oxygen species (ROS) and substantial amounts of promutagenic 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) adducts in DNA. Also, the discernible butachlor dose-dependent reduction in fluorescence intensity of a cationic dye rhodamine (Rh-123) and increased fluorescence intensity of 2′,7′-dichlorodihydro fluorescein diacetate (DCFH-DA) in treated cells signifies decreased mitochondrial membrane potential ($\Delta\Psi m$) due to intracellular ROS generation. The comet data revealed significantly greater Olive tail moment (OTM) values in butachlor treated PBMN cells vs untreated and DMSO controls. Treatment of cultured PBMN cells for 24 h resulted in significantly increased number of binucleated micronucleated (BNMN) cells with a dose dependent reduction in the nuclear division index (NDI). The flow cytometry analysis of annexin V^-/7-AAD^+ stained cells demonstrated substantial reduction in live population due to complete loss of cell membrane integrity. Overall the data suggested the formation of butachlor–DNA complex, as an initiating event in butachlor-induced DNA damage. The results elucidated the oxidative role of butachlor in intracellular ROS production, and consequent mitochondrial dysfunction, oxidative DNA damage, and chromosomal breakage, which eventually triggers necrosis in human PBMN cells.

1. Introduction

Butachlor [N-(butoxymethyl)-2-chloro-2′,6′-diethyl acetanilide] is a selective pre-emergent systemic herbicide widely used for control of a range of annual grass and broad leaf weeds (Chang, 1971). This class of herbicide inhibits the biosynthesis of lipids, alcohols, fatty acids, proteins, isoprenoids and flavonoids (Ecobichon, 2001; Heydens et al., 2002). It is widely recommended herbicides for rice cultivation, which affects soil reduction processes including acetylene reduction activity (ARA) in flooded rice soils (Jena et al., 1987). The increased application of herbicides on rice, tea, wheat, beans and other crops, reportedly exerts detrimental effects on beneficial organisms like earthworms (Muthukaruppan and Gunasekaran, 2010) and other non-target animals (Kumari et al., 2009). Ecotoxicological studies suggested that butachlor and their metabolites may be harmful to aquatic invertebrates (Ateeq et al., 2002, 2006; Vallotton et al., 2009), microbial communities (Min et al., 2002; Widenfalk et al., 2008) and possibly carcinogenic in animals and humans (Panneerselvam et al., 1999; Geng et al., 2005a,b). Butachlor has been suggested to be mutagenic in primary rat tracheal epithelial cells and Chinese hamster ovarian cells (Wang et al., 1987; Hill et al., 1997), and causes stomach tumors in rats. The mutagenicity and carcinogenicity of butachlor and other chloracetamide herbicides like acetochlor, alachlor and metolachlor have been thoroughly reviewed (Dearfield et al., 1999). Coleman et al. (2000) have determined the metabolism of butachlor to 2-chloro-N-(2,6-diethylphenyl) acetamide (CDEPA) and 2,6-diethylaniline (DEA) both in rat and human livers. The common metabolic activation pathway of chloracetamide compounds leading to the formation

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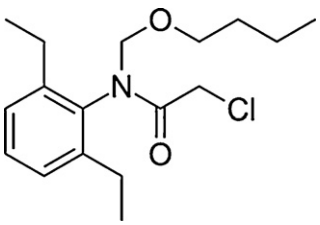

Fig. 1. Chemical structure of butachlor.

of a putative ultimate carcinogenic metabolite, suggests that this class of herbicides have a common mechanism of carcinogenicity. Prolonged exposure to butachlor has also been found to be toxic to spotted snakehead fish (*Channa punctata*), and accumulates through the food chain (Tilak et al., 2007). It has been reported to be neurotoxic to land snails (Rajyalakshmi et al., 1996), genotoxic to toad and frog tadpoles, flounder, and catfish (Ateeq et al., 2005; Geng et al., 2005b; Yin et al., 2007, 2008), and causes DNA strand breaks and chromosomal aberrations in cultured mammalian cells (Sinha et al., 1995; Panneerselvam et al., 1999).

Earlier studies have suggested the possible links between pesticides induced genotoxicity and damage to biological macromolecules in human population exposed to either single or mixture of pesticides (Garaj-Vrhovac and Zeljezic, 2001; Zeljezic and Garaj-Vrhovac, 2002; Padmavathi et al., 2000). Such pesticides when intercalate or covalently bind with DNA molecule may form DNA adducts, which can lead to gene mutations and initiate carcinogenesis, if the adducts are not repaired or misrepaired before DNA replication occurs (Saquib et al., 2010a). Increased DNA damage enhances the probability of mutations occurring in critical target genes and cells, which may trigger the process of carcinogenesis (Eisenbrand et al., 2002). The genotoxicity of butachlor in mammals and invertebrates has been extensively demonstrated (Simpson et al., 1994; Panneerselvam et al., 1999; Geng et al., 2005a,b; Ateeq et al., 2005, 2006). However, no systematic studies have been carried out on the nature and extent of physical interaction of butachlor with DNA, and/or its role as an oxidative genotoxicant in human peripheral blood mononuclear (PBMN) cells. To the best of our understanding, this study provides the first evidence that butachlor as a ligand can bind to DNA with high affinity and have a potential of producing intracellular reactive oxygen species (ROS) leading to oxidative stress, DNA damage and necrotic effects in human PBMN cells. For this investigation, several sensitive techniques such as fluorescence spectroscopy (Zhang et al., 2005; Kashanian et al., 2008; Khan and Musarrat, 2003; Saquib et al., 2010a); single cell gel electrophoresis (SCGE) assay (Singh et al., 1988; Saquib et al., 2009); Cytokinesis blocked micronucleus (CBMN) assay (Kalantzi et al., 2004; Saquib et al., 2009) and flow cytometry (Saquib et al., 2010a, 2012) have been exploited. This study has elucidated some important and relatively unattended issues of toxicological significance, such as the (i) nature of butachlor–DNA interaction, (ii) extent of DNA strand breaks, (iii) induced ROS production and cytotoxicity and (iv) impact on cell cycle progression, eventually leading to cell apoptosis and/or necrosis.

2. Materials and methods

2.1. Chemicals

Butachlor [N-(butoxymethyl)-2-chloro-2′,6′-diethyl acetanilide] CAS No. 23184-66-9, 95% TC (Fig. 1) was a kind gift from the 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), 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) was procured from GIBCO BRL Life Technologies Inc. (Gaithersburg, MD, USA). Phosphate buffered saline (PBS, Ca^{2+} Mg^{2+} free) and Triton X-100 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. Butachlor–DNA binding analysis by fluorescence spectroscopy

Binding of butachlor to DNA was determined by use of fluorescence spectroscopy. Briefly, to a fixed concentration of butachlor (50 μM), increasing concentrations of ctDNA (5–100 μM) were added to obtain ctDNA to butachlor molar ratios ranging from 0.1 to 2.0 in 10 mM Tris–HCl buffer at ambient temperature. Spectra were recorded under subdued light to prevent undesired photodegradation. Fluorescence was determined by use of a Shimadzu spectrofluorophotometer, model RF5301PC equipped with RF 530XPC instrument control software, Kyoto (Japan). The path length was 1 cm in a quartz cell. Excitation and emission slits were set at 3 and 10 nm, respectively. The excitation and emission wavelengths were 225 and 360 nm, respectively. ctDNA alone does not fluoresce at this wavelength. The fluorescence quenching constant was determined by use of the Stern–Volmer relationship (Eq. (1)), as described previously (Lakowicz, 2006).

$$\frac{F_0}{F} = 1 + \mathrm{Ksv}\,[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of the quencher (ctDNA), respectively, Ksv is the Stern–Volmer quenching constant and [Q] the quencher concentration. The quenching constant was obtained from the slope of the Stern–Volmer plot (F_0/F vs [Q]). The binding constant (Ka) and number of binding sites (n) were estimated, following previously published methods (Lehrer and Fasman, 1996; Chipman et al., 1967) (Eq. (2)) and assuming a 1:1 complex between butachlor and ctDNA, as described previously (Saquib et al., 2010a, 2011).

$$\frac{F_0 - F}{F - F\infty} = \mathrm{Ka} \times [\mathrm{DNA}] \quad (2)$$

where F_0 and $F\infty$ are the relative fluorescence intensities of butachlor alone and butachlor saturated with ctDNA, expressed as the relative fluorescence intensity of ctDNA to butachlor molar ratio of 1:2, respectively. The slope of the linear portion of the double-logarithm plot ($\mathrm{Log}[(F_0 - F)/(F - F\infty)]$ vs Log [ctDNA] provided the number of equivalent binding sites (n). However, the value of Log [ctDNA] at $\mathrm{Log}[(F_0 - F)/(F - F\infty)] = 0$ is equal to the negative logarithm of the binding constant (Ka) (Lakowicz, 2006).

2.3. Measurement of DNA strand breaks in human PBMN cells by Comet assay

Comet assay was performed with human PBMN cells following the methods of Singh et al. (1988) as described by Saquib et al. (2009). Freshly isolated cells were treated separately with varying concentrations (50, 100, 250 and 500 μM) of butachlor for 3 h at 37 °C. The cells (~4×10^4) 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 without Ca^{2+} and Mg^{2+}) 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 electrophoresis 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. The slides were stained with ethidium bromide for 5 min and analyzed at 40× magnification using fluorescence microscope (Olympus, Japan) 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), Olive tail moment (OTM) and % tail DNA (% TDNA) were separately analyzed for statistical significance, level of statistical significance chosen was $p \leq 0.05$, unless otherwise stated.

2.4. Butachlor induced 8-oxo-2′-deoxyguanosine (8-oxodG) formation in ctDNA

Varying amounts (500, 1000, 1500 and 2000 ng) of untreated, butachlor (1000 μM) and methylene blue (100 μM) treated ctDNA samples were immobilized on 96 well microtiter plates by overnight absorption at 40 °C following the procedure of Hirayama et al. (1996). Methylene blue was used as positive control. The non-specific sites were blocked with 300 μl of blocking solution containing 3% BSA in PBS (Ca^{++} and Mg^{++} free). The plates were further incubated at 37 °C for 3 h. Polyclonal goat anti 8-oxodG antibodies (Cat # AHP592; AbDSerotech, UK), 100 μl/well at dilutions of 1:1,00,000 in blocking solution were added and incubated at 37 °C for 2 h. The solution was discarded and the plates washed twice with (300 μl/well) with PBS containing 0.05%Tween-20. Subsequently, the secondary antibody (Donkey anti-goat IgG:HRP; Cat # STAR88P, AbDSerotech, UK) diluted at 1:25,000 in

blocking solution (100 μl/well) was added and incubated for 2 h at 37 °C. After thorough washing, the 50 μl/well of 1× substrate TMB (3,3′,5,5′-tetramethylbenzidine) was added. The reaction was stopped by the addition of 2 M H_2SO_4 (50 μl/well). Absorbance was read at 450 nm on Mutiscan EX reader (Thermo Scientific, USA). The 8-oxodG was quantified using a calibration curve prepared with the commercially available 8-oxodG standard.

2.5. Cytokinesis blocked micronucleus (CBMN) assay

The CBMN assay was preformed following the method of Kalantzi et al. (2004) as described by Saquib et al. (2009). 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 butachlor at final concentrations of 25, 50 and 100 μM 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 butachlor. 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. The nuclear division indexes (NDI) were determined following the method of Saquib et al. (2009). Binucleated cells were scored to evaluate the percentage of binucleated cells for expressing the toxicity and/or inhibition of cell proliferation as NDI calculated according to the following formula:

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

where Mono, Bi, 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.

2.6. Detection of intracellular ROS by fluorescence microscope and flow cytometry analysis

Intracellular reactive oxygen species were analyzed by use of fluorescence microscope and flow cytometry using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma–Aldrich) as a specific dye probe, which fluoresces on oxidation by ROS to 2′,7′-dichlorofluorescein (DCF). In brief, human PBMN cells treated with 25, 50, 100, 250 and 500 μM of butachlor for 1 h were harvested and centrifuged at 1000 rpm for 4 min. Pellets were resuspended in 500 μl of PBS containing DCFH-DA (5 μM in DMSO) for 1 h at 37 °C in dark. Cells were then washed twice with PBS and finally suspended with 500 μl PBS. The cells were then visualized under fluorescence microscope (Nikon, Eclipse 80i, Japan) at the excitation and emission wavelengths of 485 and 530 nm, respectively. For flow analysis, the fluorescence of cells (10,000 cells each) was recorded under 488 nm excitation. Green fluorescence from DCF was measured in the FL1 Log channel through a 525-nm band-pass filter on the Coulter EPICS XL/X1-MCL (Beckman Coulter Company, Miami, FL, USA).

2.7. Assessment of mitochondrial membrane potential ($\Delta\Psi m$) in PBMN cells

Mitochondrial damage was monitored by observing the changes in the fluorescence intensity of mitochondria specific dye rhodamine (Rh123) in PBMN cells, as described by Saquib et al. (2010b). The untreated control cells and those treated with butachlor in concentration range of (25, 50, 100, 250 and 500 μM) for 1 h at 37 °C were stained with 20 μM Rh123 for 1 h at 37 °C, and visualized under fluorescence microscope (Nikon, Eclipse 80i, Japan) at the excitation wavelength of 520 nm and emission wavelength of 590 nm. Flow cytometric measurements of ΔΨm of butachlor treated human PBMN cells were performed by use of a fluorescent probe Rh123. In brief, the cells treated with 25, 50, 100, 250 and 500 μM of butachlor for 1 h were harvested and centrifuged at 1000 rpm for 4 min. Pellets were resuspended in 500 μl of PBS containing mitochondrial specific fluorescence dye Rh123 (5 μg/ml) for 1 h at 37 °C in dark. Cells were then washed twice with PBS and finally suspended with 500 μl PBS. ΔΨm was measured by use of flow cytometry and expressed as the mean fluorescence intensity (MnXI) of 10,000 cells.

2.8. Flow cytometric analysis of cell cycle progression and apoptosis/necrosis

Human PBMN cells treated with 0.1% DMSO as a solvent control or cells treated with 25, 50, 100, 250 and 500 μM of butachlor for 1 h were harvested and centrifuged at 1000 rpm for 4 min. Pellets were resuspended in 500 μl of PBS. Cells were fixed with equal volume of chilled 70% ice-cold ethanol, and incubated at 4 °C for 1 h. After two successive washes with PBS at 1000 rpm for 4 min, cell pellets were resuspended in PBS and stained with 50 μg/ml propiodium iodide (PI) containing 0.1% Triton X-100 and 0.5 mg/ml RNAase A for 1 h at 30 °C in dark. Fluorescence of the PI was measured by flow cytometry by use of a Beckman Coulter flow cytometer (Coulter Epics XL/Xl-MCL, Miami, USA) through a FL-4 filter (585 nm) and 10′000 events were acquired. The data were analyzed by Coulter Epics XL/XL-MCL, System II Software, Version 3.0. Cell debris characterized by a low FSC/SSC was excluded from the analysis.

2.9. Evaluation of apoptosis/necrosis with annexin V-PE and 7-AAD

Apoptosis and/or necrosis in butachlor treated PBMN cells were investigated by flow cytometry by use of the two-color variation of phycoerythrin-labeled annexin V (Annexin V-PE) and cell viability dye 7-amino-actinomycin D (7-AAD) (Cell Lab ApoScreenTM Annexin V-PE Apoptosis Kit, Cat No. 736518, Beckman Coulter, USA). Positioning of quadrants along the axes of Annexin V-PE vs.7-AAD was used to display the relationships between the number of live cells (Annexin V^-/7-AAD^-), early apoptotic cells (Annexin V^+/7-AAD^-), late apoptotic cells (Annexin V^+/7-AAD^+) and necrotic cells (Annexin V^-/7-AAD^+) (Vermes et al., 1995). PBMN cells were exposed to 25, 50, 100, 250 and 500 μM butachlor for 1 h at 37 °C with 5% CO_2, and the harvested cells were processed further as per manufacturer's protocol. In brief, the cell suspension was washed twice with cold PBS and the cell pellets were resuspended to a concentration of 1×10^6 cells/ml in cold 1× binding buffer. An aliquot of 100 μl of cell suspension from each treatment was incubated with 10 μl of annexin V-PE for 15 min in dark on ice followed by the addition of 10 μl of 7-AAD and 380 μl of cold 1× binding buffer to each tube and analyzed within 30 min after staining. The fluorescence of 10,000 cells was recorded by use of a 488 nm excitation wavelength and the fluorescence of annexin V-PE and 7-AAD were expressed on a log scale after enumeration through FL-1 filter (525 nm) and FL-2 filter (570 nm) on the Beckman Coulter flow cytometer (Coulter Epics XL/Xl-MCL, USA). The necrosis index was calculated using the equation: Necrosis Index = % necrotic cells in test − % necrotic cells in control/100 − % necrotic cells in control × 100%, as described by Hoorens et al. (2001).

3. Results

3.1. Fluorescence quench titration of butachlor upon binding with ctDNA

The fluorescence emission spectra of butachlor and butachlor–ctDNA complex at an excitation wavelength of 225 nm are shown in Fig. 2a. A significant decrease in the intrinsic fluorescence of butachlor with increasing ctDNA to butachlor molar ratios has been noticed. The data exhibited progressive quenching effect upon addition of ctDNA in increasing concentrations to a constant amount of butachlor, resulting in 50.1% fluorescence quenching at the highest ctDNA to butachlor molar ratios of 1:2. The plot of F_0/F vs [Q] (Fig. 2b) provided the quenching constant (Ksv) using Stern–Volmer algorithm as $1.19 \times 10^4\ M^{-1}$ ($r^2 = 0.99$). The fluorescence data plotted as $\mathrm{Log}\,[(F_0 - F)/(F - F\infty)]$ vs Log [ctDNA] in Fig. 2c revealed the binding constant (Ka) as $1.2 \times 10^4\ M^{-1}$ and (n) as 1.02. The free energy (ΔG), entropy (ΔS) and enthalpy (ΔH) changes for the formation of the butachlor–ctDNA complex were estimated to −5.46 kcal/mole, 0.156 J/mole and 5.487 kcal/mole, respectively.

3.2. Assessment of butachlor induced DNA damage in human PBMN cells by alkaline comet assay

Butachlor induced single strand breaks in human PBMN DNA have been observed upon single cell gel electrophoresis (SCGE) under alkaline conditions. Fig. 3 (Panels D–F) 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 at increasing butachlor doses. The quantitative data obtained from the comparative analysis of a set of 150 cells at 500 μM butachlor concentration vs DMSO control revealed 26-fold enhanced DNA migration. A significant increase in the values of OTM, % tail DNA and tail length (μm) were observed at all concentrations (Table 1). The cells treated with butachlor (500 μM) showed an OTM value of 20.26 ± 1.12 (AU) as compared to 0.32 ± 0.02 (AU) and 0.35 ± 0.82 (AU) with untreated and DMSO controls, respectively.

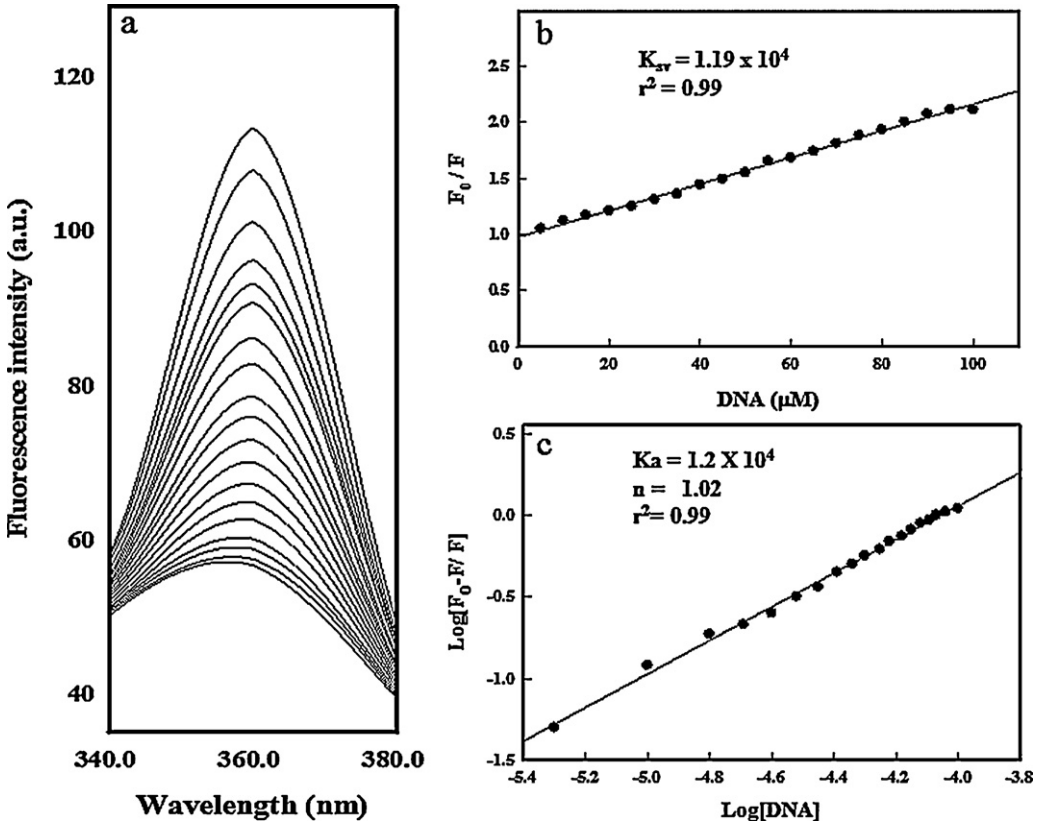

Fig. 2. Fluorescence quench titration of butachlor with ctDNA. Panel (a) depicts fluorescence emission spectra of butachlor in the absence (uppermost curve) and presence of increasing amounts of ctDNA at excitation wavelength of 225 nm. The molar ratio of ctDNA to butachlor varies from 0.1 to 2 (top to bottom). Panel (b) represents the Stern-Volmer plot based on butachlor–ctDNA fluorescence quenching data. Panel (c) shows the double-logarithmic plot of butachlor–ctDNA interaction to determine the binding constant (Ka) and number of binding sites.

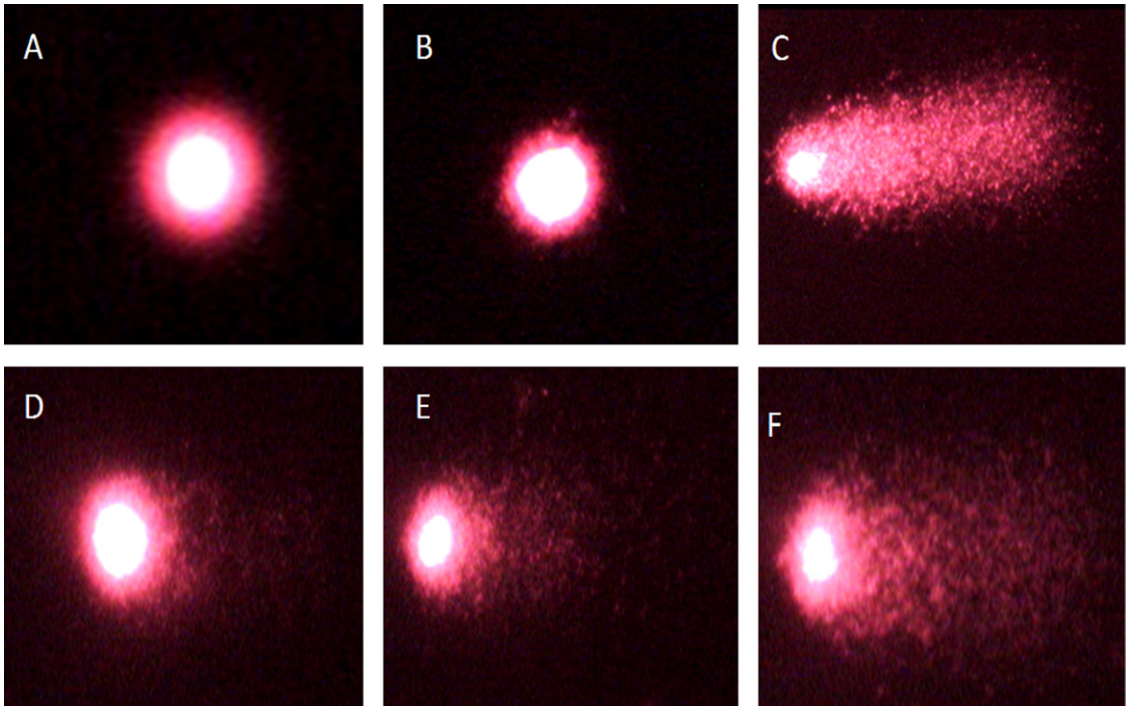

Fig. 3. Comet images of butachlor-induced DNA damage in human PBMN cells. The representative photomicrographs have been acquired through KOMET 3.0 software. (A) Untreated control, (B) DMSO (1%) as solvent control, (C) MMS (2 mM) treated cells, as positive control: (D–F) cells treated with butachlor at 0, 250 and 500 μM, respectively.

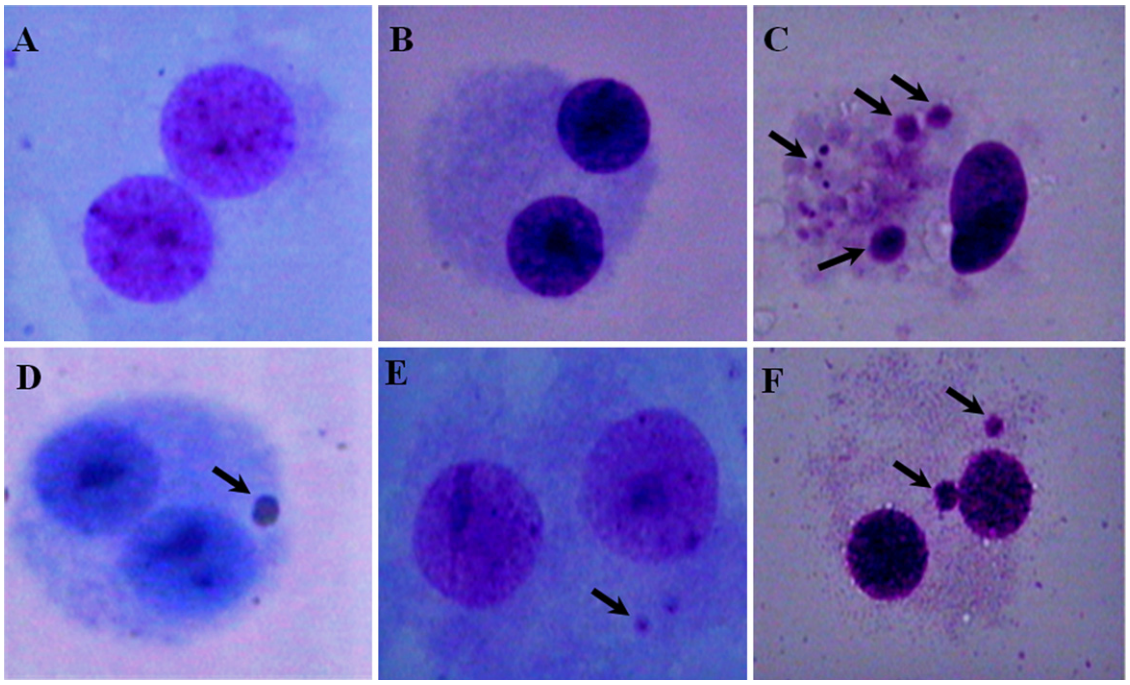

Fig. 4. Representative photomicrographs of the typical binucleated human PBMN cells appeared after 24 h in CBMN assay. Panels (A–C) represent 6% Giemsa stained cells of (A) untreated, (B) DMSO (1%) as solvent and (C) MMS (100 μM) as positive controls. Panels (D–F) show the binucleated cells treated with 25, 50 and 100 μM butachlor, respectively.

3.3. Micronucleus formation in butachlor treated human PBMN cells

A concentration dependent increase in the total number of binucleate micronucleated (BNMN) human lymphocytes has been observed upon exposure to butachlor. Treatment of cells with butachlor for 24 h resulted in a significant increase ($p < 0.05$) in the mean BNMN/1000 cells, as validated by one-way ANOVA (Table 2). The mean BNMN at 25, 50 and 100 μM of butachlor concentrations were determined to be 19, 29 and 34, respectively. Compared to untreated control, the butachlor treated cells have exhibited 3.77 folds higher BNMN formation. Giemsa stain cells in Fig. 4, explicitly indicate the presence of micronucleus in the butachlor treated human lymphocytes (panels D–F). Panels (A–C) show the binucleated cells in a parallel set of untreated, DMSO (solvent) and MMS (positive) control cells. In order to better characterize the effect of butachlor, nuclear division index (NDI) was also evaluated, which shows a decline in the BN cell formation. The butachlor treated cells showed the NDI of 1.90, 1.85 and 1.80 with 25, 50 and 100 μM of butachlor as compared the NDI of 1.92 in case of untreated cells (Table 2).

Table 1
Assessment of butachlor induced DNA damage in human PBMN cells analyzed using different parameters of comet assay.

Groups	Olive tail moment (Arbitrary unit)	Tail DNA (%)	Tail length (μm)
Control	0.32 ± 0.02	0.91 ± 0.50	9.6 ± 1.08
DMSO (1%)	0.35 ± 0.82*	0.74 ± 0.11	11.38 ± 2.54*
MMS (2 mM)	26.27 ± 0.19*	33.94 ± 0.46*	496.36 ± 0.48*
Butachlor (μM)			
50	3.07 ± 0.49*	8.53 ± 0.69	102.02 ± 1.68*
100	10.09 ± 1.2*	19.90 ± 1.14*	180.24 ± 1.69*
250	16.65 ± 0.85*	24.36 ± 1.34*	224.57 ± 0.91*
500	20.26 ± 1.12*	31.31 ± 1.54*	296.49 ± 0.90*

Data represent the mean ± SEM of three experiments. MMS: Methyl methanesulfonate; DMSO: Dimethysulfoxide.
* $p < 0.05$.

3.4. Quantification of butachlor induced 8-oxodG formation in DNA

The extent of butachlor induced 8-oxodG formation in ctDNA is shown in Fig. 5.The amount of 8-oxodG in ct-DNA after treatment with 1000 μM butachlor was found to increase linearly with the increasing amount of DNA. The levels of 8-oxodG was estimated to be 120, 181, 220, 398, 548, 688 and 821 ng/ml in 50, 100, 250, 500, 1000, 1500 and 2000 ng/ml ctDNA, respectively. The induced level of 8-oxodG at the highest concentration (2000 ng/ml) of butachlor treated ctDNA was determined to be 3.16- and 1.68-fold greater as compared to the methylene blue treated ct DNA (positive control) and untreated control DNA.

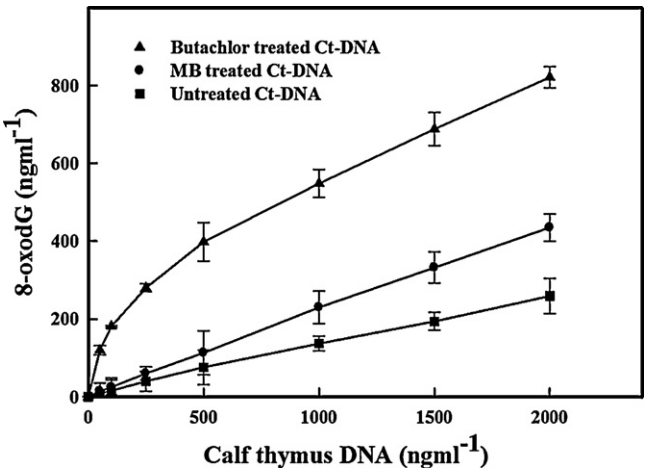

Fig. 5. ELISA based quantitative analysis of 8-oxodG DNA adducts in butachlor treated ctDNA. Each point represents the mean ± S.D. of three independent experiments done in triplicate.

3.5. ROS production and alterations in mitochondrial membrane damage in butachlor treated PBMN cells

The intracellular ROS have been assessed based on detection of peroxide-dependent oxidation of DCFH-DA to fluorescent 2′,7′-dichlorofluorescin (DCF) in butachlor treated PBMN cells. Qualitative analysis revealed a concentration dependent increase in the fluorescence intensity of DCF in butachlor treated cells as compared to the untreated control cells (Fig. 6A). At higher concentrations of 500 μM butachlor, the fluorescence intensity of DCF was substantially reduced due to decrease in the number of cells because of cell death. The quantitative analysis of ROS mediated changes in DCF fluorescence by flow cytometry demonstrated about 2.1 and 2.3-fold increase in ROS generation at butachlor doses of 25 and 50 μM, respectively (Fig. 7). Interestingly, a significant shift in FL1 DCF spectra was observed with the marked decrease in MnXI values at higher concentrations of 100, 250, and 500 μM butachlor, due to significant reduction in cell population.

Furthermore, the changes in mitochondrial activity, using a cationic fluorescent probe Rh123, have been noticed through fluorescence microscopic analysis of butachlor treated PBMN cells. The probe accumulates electrophoretically in the strong negatively charged matrix of mitochondria in control cells, whereas it undergoes a butachlor concentration dependent reduction in the mitochondrial fluorescent intensity, as observed in Fig. 6B. However, the quantitative analysis by flow cytometry showed the initial increase in the fluorescence intensity (MnXI) values as 21.3% and 59.1% in 50 and 100 μM butachlor treated PBMN cells as compared to 17.4% in control. Also, an altered peak pattern and a significant shift in Log FL1 Rh123 peak at higher concentrations of 100, 250 and 500 μM butachlor were observed as compared to the solvent control (Fig. 8).

3.6. Butachlor induced cell death (late apoptosis/necrosis) in human PBMN cells.

Flow cytometry data on cell cycle analysis of PI-stained untreated and butachlor treated cells exhibited an increase in sub-G1 peak with concomitant reduction in G1 phase (Supplementary Fig. 1). A significant increase in the proportions of dead cells in sub-G1 phase were recorded as 2.1 ± 0.49%, 10.4 ± 2.4%, 15.3 ± 0.7%, 16.0 ± 1.6%, 16.9 ± 1.58% and 24.2 ± 3.9%, in cells exposed to 25, 50, 100, 250 and 500 μM of butachlor, respectively. The proportion of dead cells in the solvent control population was 1.4 ± 0.2%. Significant G2/M arrest (7.2 ± 0.08, 8.0 ± 0.6 and 8.5 ± 0.3) was observed in cells exposed to 50, 100 and 250 μM butachlor, compared to 3.7 ± 0.18% in control cells (Fig. 9). Butachlor induced cell death was further validated by use of the annexin V-PE and 7-AAD apoptotic assay, which exclusively generate fluorescence signals upon binding of annexin V-PE with phosphatidylserine on the plasma membrane of apoptotic/necrotic cells and necrotic cells with 7-AAD. Based on the annexin V-PE and 7-ADD staining, 97.3% of cells in solvent control were alive and 0.01%, 0.09% and 2.57% of cells were classified as being in early, late and necrotic stages. However, exposure to 25, 50, 100, 250 and 500 μM butachlor resulted in necrosis, marked by a shift of 3.28%, 3.34%, 3.88%, 35.4% and 67.9%, dead cells into the upper left quadrant of the annexin V^-/7-AAD^+ plot, which represents necrosis. Almost 0.85%, 1.01% and 0.36% of cells fall in the upper right quadrant of the annexin V^+/7-AAD^+ plot, which represents the formation of late apoptotic cells at an exposure of 100, 250 and 500 μM butachlor (Table 3). Virtually, no cells were observed in the lower right quadrant, specifically representing the apoptosis. The necrotic index at 250 and 500 μM butachlor were determined to be 33.7 and 66.1, respectively (Supplementary Fig. 2).

Table 2
Butachlor induced micronuclei formation in human PBMN cells.

Treatment	Dose	BNMN/1000 cells	NDI
Control	0	9.0 ± 2.01	1.92
DMSO (%)	1.0	10.0 ± 1.88^{ns}	1.92
MMS (μM)	100	22.0 ± 3.02*	1.80
Butachlor	25	19.0 ± 2.93*	1.90
(μM)	50	29.0 ± 2.18*	1.85
	100	34.0 ± 3.44*	1.80

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.
* $p < 0.05$.

4. Discussion

Several pesticides are known to induce genetic alterations leading to DNA damage, and have been categorized as potent genotoxicants (Padmavathi et al., 2000; Zeljezic and Garaj-Vrhovac, 2002; Saquib et al., 2009). The systemic herbicide butachlor investigated in this study, is one such an important environmental pollutant, which poses a potential threat to the agro-ecosystem and human health through food chains (Sapna et al., 1995; Debnath et al., 2002). This substituted chloroacetanilide compound has been reported to cause a dose-dependent increase in the frequency of chromosomal aberrations in cultured human lymphocytes (Sinha et al., 1995), mutagenicity in *Salmonella typhimurium* strain TA 100 (Hsu et al., 2005), induction of micronuclei in the cat fish erythrocytes (Ateeq et al., 2002) and stomach tumors in rats (Thake et al., 1995). However, there has been a paucity of systematic and comprehensive information on the (i) binding affinity of butachlor for DNA molecule (ii) ability of butachlor to produce intracellular reactive oxygen species (ROS) and oxidative stress and (iii) mitochondrial dysfunction and DNA damage in human PBMN cells, ensuing apoptosis and/or necrosis. It was therefore, considered important to investigate the butachlor–DNA interaction in vitro, and its potential to elicit oxidative stress leading to mitochondrial membrane disruption and DNA damage in human PBMN cells.

The fluorescence studies suggested strong binding of butachlor with DNA, resulting in formation of the butachlor–ctDNA binary complex. The quenching of intrinsic fluorescence of butachlor upon addition of ctDNA could be due to masking of butachlor chromophores within the helix due to surface binding at the reactive nucleophilic sites on the heterocyclic nitrogenous bases of ctDNA molecule. The quenching constant (Ksv) determined using the Stern–Volmer algorithm, is indicative of the affinity of this herbicide toward DNA. The linearity of the Stern–Volmer plot suggests the (i) existence of one binding site for the ligand in proximity of fluorophore, or (ii) more than one binding site equally accessible to the ligand (Ghosh et al., 2008). Thermodynamic parameters such as the changes in free energy (ΔG) due to ligand binding also provided insight into the binding mode. The negative ΔG value for the interaction of ctDNA with butachlor indicates the spontaneity of the complexation. The interaction process has been found to be entropy driven and the major contribution of ΔG comes from positive ΔS. The observation that both the ΔH and ΔS for butachlor binding to DNA were positive is consistent with complexation of ctDNA with butachlor involving hydrophobic interaction, which together with hydrogen bonds, van der Waals and electrostatic interactions play a significant role in molecular recognition (Ross and Subramanian, 1981). Since, the complex metabolic activation of butachlor pathway leads to the generation of DNA-reactive metabolites like CDEPA, DEA and 2,6-diethylbenzoquinone imine (DEBQI) in human liver (Coleman et al., 2000), it would be

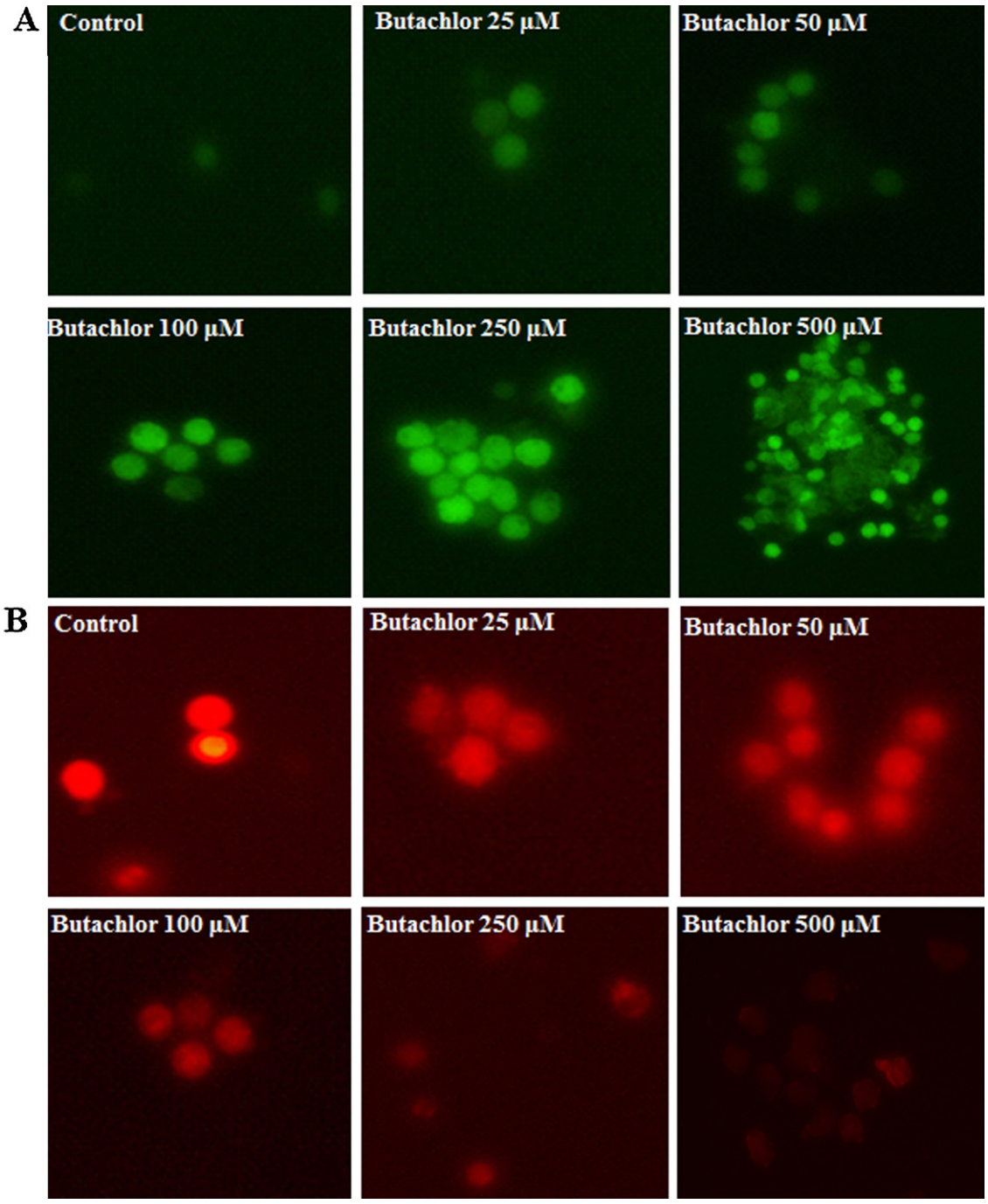

Fig. 6. Fluorescence analysis of the changes in intracellular ROS generation and mitochondrial membrane potential in butachlor treated PBMN cells. Panel 6 (A) shows the butachlor concentration dependent enhancement in green fluorescence of DCF due to ROS generation up to 500 μM. Fig. 6 (B) shows the reduction in the intensity of Rh123 fluorescent probe in treated cells. Fluorescence decrease at higher concentration of 500 μM signifies the cell death due to necrosis.

Table 3
Effect of butachlor on PBMN cell population by flow cytometric analysis.

Butachlor (μM)	Live (%)	Early apoptosis (%)	Late apoptosis (%)	Necrosis (%)
Control	97.3 ± 2.3	0.01 ± 1.4	0.09 ± 2.3	2.57 ± 1.0
25	96.6 ± 3.2	0.01 ± 1.8	0.07 ± 1.6	3.28 ± 1.8
50	97.0 ± 2.6	0.03 ± 1.9	0.09 ± 3.5	3.34 ± 2.3
100	95.7 ± 4.8	0.10 ± 2.3	0.85 ± 1.8	3.88 ± 1.5
250	63.1 ± 5.4*	0.32 ± 2.8	1.01 ± 1.2	35.4 ± 4.6*
500	31.7 ± 6.3*	0.04 ± 3.2	0.36 ± 3.4	67.9 ± 5.3*

Data represent the mean ± S.D of two independent experiments done in triplicate.
* $p < 0.01$ vs control by one way ANOVA (Dunnett's test).

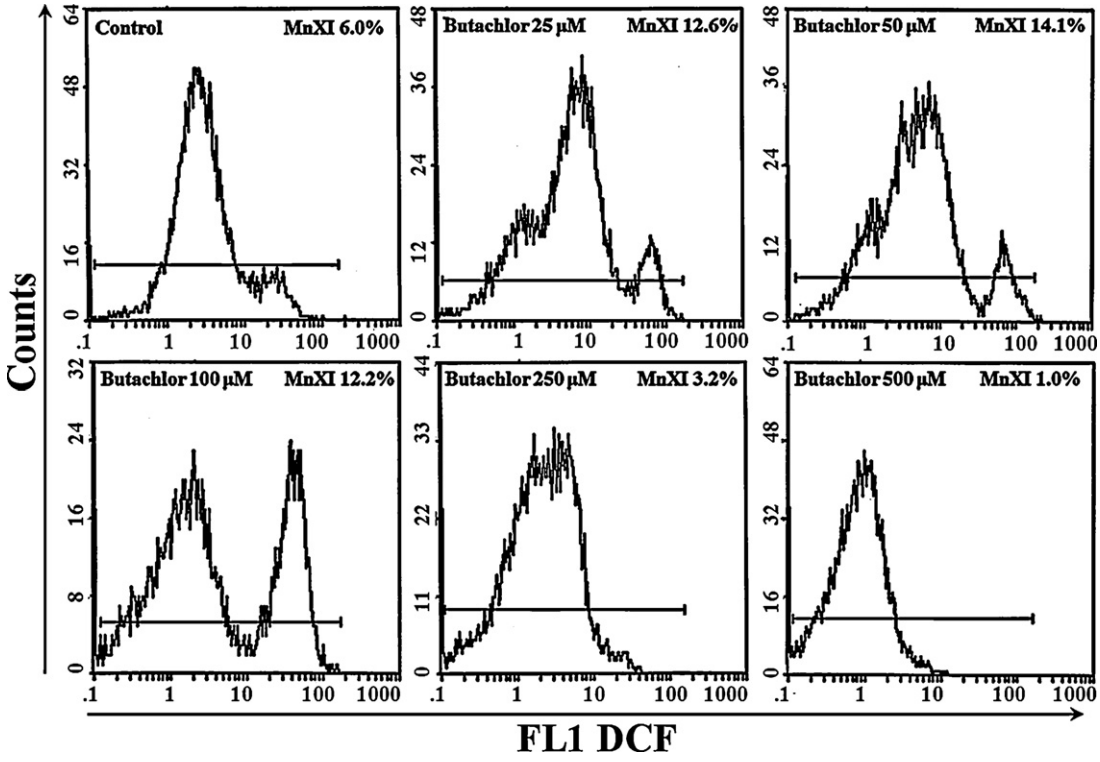

Fig. 7. Flow cytometric analyses of butachlor induced ROS generation. The panel shows the MnXI values in solvent control (0.1% DMSO) and treated PBMN cells in concentration range of 25–500 μM butachlor.

interesting to understand the molecular interaction of these carcinogenic metabolites of butachlor in future studies.

In order to provide a better understanding of the butachlor induced genotoxicity, the cellular damage in terms of DNA strand breaks and chromosomal breakage were assessed in PBMN cells by use of sensitive techniques like comet and CBMN assays, fluorescence spectroscopy and flow cytometry. Human PBMN cells have been extensively used in toxicity studies because of the ease of

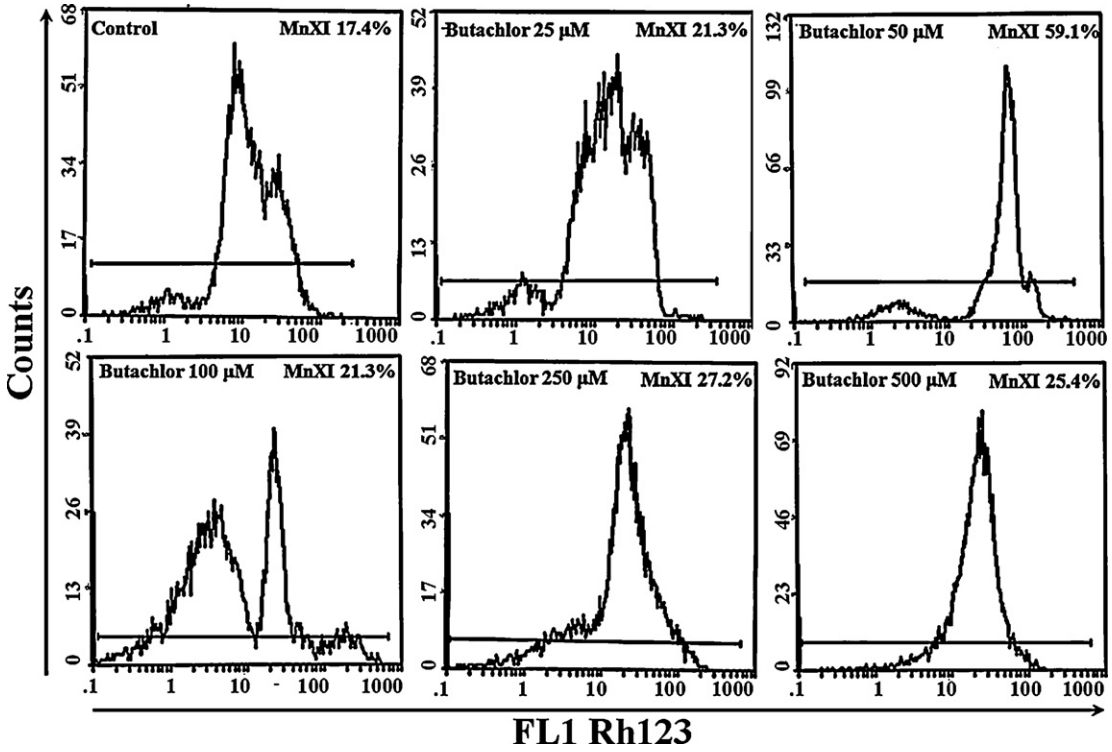

Fig. 8. Flow cytometric analyses of mitochondrial membrane potential ($\Delta\Psi m$) in PBMN cells. The panel shows the MnXI values in solvent control (0.1% DMSO) and treated PBMN cells in concentration range of 25–500 μM butachlor.

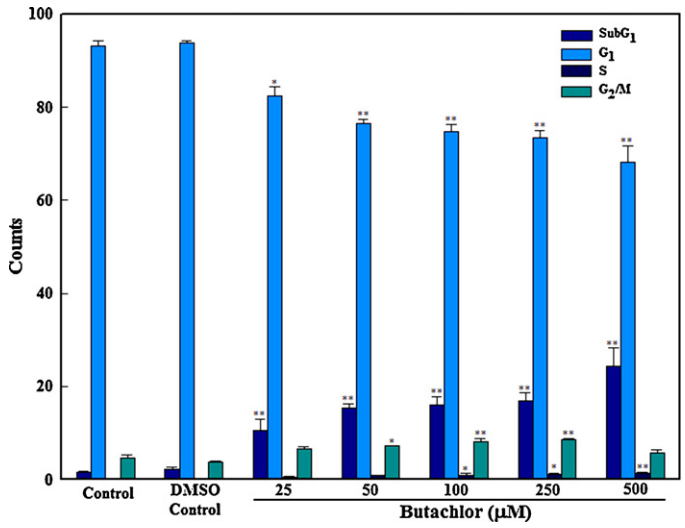

Fig. 9. Effect of butachlor on cell cycle progression by flow cytometry analysis. Each histogram represents mean ± S.D. values of different phases of cell cycle obtained from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to solvent control using one-way ANOVA.

handling of these cells without undue sub-culturing, proficiency in metabolic activity, inexpensive and easy process of isolation for environmental and occupational risk analysis in healthy and exposed human donors with known medical histories, which makes these cells more relevant for risk assessment and genotoxic effects in humans. In spite of the information on the wider applications and associated environmental risk of butachlor, there is still a lack of genetic- and cyto-toxicological data for this herbicide in human cellular system. Our comet assay results in PBMN cells revealed a significant increase in the OTM and comet tail length, as an index of butachlor induced DNA damage in a concentration dependent manner. The damage in treated cells is attributed to the interaction of butachlor and possibly its metabolites with cellular DNA, resulting in the formation of frank strand breaks. In this study, the butachlor doses varying from 25 to 500 μM (equivalent to 7.5–300 ppm) for short-term exposures of 1–3 h, were chosen after thorough optimization of sub-lethal cellular DNA damage under different experimental conditions. Quantifiable DNA damage was obtained within the above selected doses, except the formation of 8-oxodG, where the adduct formation was observed only at higher concentration, perhaps due to lower sensitivity of the antibody based detection through ELISA. The doses reported in literature for in vitro treatment in PBMN cells as 20 ppm (Sapna et al., 1995) and in vivo in different experimental models viz. fish (2.5 ppm), tadpole (1.5 ppm) and green algae (100 ppm) ranging from 1.5 to 100 ppm for an extended exposure time of 48, 72 and 96 h and in zebra fish up to 30 days (Ateeq et al., 2006; Baorong et al., 2010; Chang et al., 2011). Although, the doses chosen in this study draw closer to the doses reported in literature but still the inter-experimental variability in exposure time period makes the data comparison more difficult.

The genotoxicity data on PBMN cells corroborates with the earlier studies, which demonstrated the herbicides butachlor and alachlor induced chromosomal aberrations (CA), sister, chromatid exchanges and DNA adducts formation in human lymphocytes (Ribas et al., 1996; Kevekordes et al., 1996; Ateeq et al., 2005). Comparatively weaker positive response of butachlor in inducing sister chromatid exchange in primary rat tracheal epithelial (RTE) and Chinese hamster ovary (CHO) cells has been reported (Wang et al., 1987; Lin et al., 1987), and these studies have not elucidated the mechanistic aspect of butachlor induced DNA and mitochondrial damage. Ateeq et al. (2006) have suggested that butachlor induces apoptosis, and cytologically demonstrated the nuclear blebbing, mitochondrial deformities, electron-dense cytoplasm, plasma membrane damage and heterochromatinization in walking catfish (*Clarious batrachus*) at 2.5 ppm after 96 h exposure. However, as a typical feature of apoptosis, no characteristic ladder formation due to internucleosomal DNA degradation was observed, which is an indication of necrosis rather than apoptosis. Our fluorescence and flow cytometry studies with ROS and mitochondria specific probes Rh123 and DCFH-DA, explicitly demonstrated the mitochondrial membrane disruption with altered ($\Delta\psi m$) due to increased ROS production leading to cellular necrosis.

Our results of CBMN assay have demonstrated the butachlor induced genetic instability in human PBMN cells. The treatment of cells with butachlor for 24 h with subsequent addition of Cyto B resulted in formation of binucleate cells having variable number of micronuclei as function of butachlor concentration, which is suggestive of the clastogenicity of butachlor. Significant increase in the numbers of MN formation both in the mononucleated and binucleated cells at the butachlor dose range of 25, 50 and 100 μM vs untreated controls, unequivocally demonstrated the DNA damaging potential and anti-karyokinesis activity of these herbicides in human cells. These results support the earlier studies indicating the clastogenic potential of butachlor (Panneerselvam et al., 1999; Mahli and Grover, 1987; Dhingra et al., 1990).

The flow cytometry data suggested the plausible role of intracellular ROS production and induced oxidative stress in butachlor induced DNA and mitochondrial damage. The initial increase in the Rh123 fluorescence intensity (MnXI) values in 50 and 100 μM butachlor treated PMBN cells as compared to control is most likely due to enhanced Rh123 uptake caused by the interaction of butachlor with cell membrane components, which possibly increases the permeation of plasma and/or mitochondrial membranes, as also reported by Ouedraogo et al. (2000) in case of human skin fibroblasts photosensitized by fluoroquinolones. Altered mitochondria can no longer retain Rh123 and leaks out from the mitochondrial membrane to the cytoplasm, and this relocalization from the mitochondrial to cytosol is an index of mitochondrial impairment with the disruption of mitochondrial membrane (Ouedraogo et al., 2000). Moreover, subsequent reduction in MnXI values at 100, 250 and 500 μM butachlor, marked with altered peak pattern and significant shift in Log FL1 peak, as compared to the solvent control, suggests cell necrosis at greater butachlor concentrations. Indeed, the chemical structure of butachlor indicates its potential for the generation of reactive intermediates such as quinoneimine intermediate, formaldehyde and other aldehydes. These electrophiles have been suggested to react preferentially with soft nucleophiles such as glutathione (GSH) and other SH-containing moieties, causing depletion of GSH, and thus rendering the cells susceptible to toxic action of these reactive intermediates (Ashby et al., 1996). Thus, with the compromised antioxidizing ability of butachlor exposed cells, and incessant intracellular ROS generation may lead to oxidative stress. Our results of intracellular ROS measurement based on peroxide-dependent oxidation of DCFH-DA to fluorescent 2′,7-dichlorofluorescin (DCF) exhibited a similar FL1 spectral patterns as obtained with the Rh123 probe. The initial increase in ROS production at 25 and 50 μM butachlor is responsible for the mitochondrial and DNA damage. Subsequent dose dependent increase in MnXI values at greater concentrations is attributed to cellular necrosis possibly because of disruption of plasma membrane. This supports our Rh 123 fluorescence based results on butachlor induced mitochondrial dysfunction and also our earlier observations with phorate treated human amniotic epithelial (WISH) cells, where DCF fluorescence intensity was substantially decreased at 500 or 1000 μM phorate due to significant cell death (necrosis) (Saquib et al., 2012).

Furthermore, the appearance of sub-G1 peak in cell cycle analysis at increasing concentrations of butachlor suggested the possible involvement of late apoptotic/necrotic pathway, which might be triggered by alteration in mitochondrial and lysosome functions (Nicoletti et al., 1991; Ravi et al., 2010). A recent study suggested that mitochondrial events of necrosis involves opening of a pore in the inner mitochondrial membrane, referred as mitochondrial permeability transition pore (MPTP), and loss of $\Delta\Psi m$ (Kitsis and Molkentin, 2010). Opening of the MPTP results in mitochondrial swelling and rupture of outer mitochondrial membrane during necrosis. Damage to lysosomal membranes is known to release lysosome protease into intracellular spaces, which affects the neighbor cells, and triggers cell death due to necrosis (Leist and Jaattela, 2001). Our flow cytometric data with the annexin V-PE and 7-ADD explicitly demonstrated the butachlor induced cell death attributed to late apoptosis and necrosis. Virtually, no annexin V^+-PE cells in lower right quadrant at any butachlor concentrations, signifies the integrity of plasma membrane and no externalization of phosphatidylserine (PS) due to loss of membrane assembly, which is indicative of early apoptosis (Fadok et al., 1992; Martin et al., 1995; Diaz and Schroit, 1996). However, appearance of significant annexin V^+-PE and 7-ADD^+ cells population in the upper left quadrant suggests the complete loss of cell membrane integrity, eventually causing cell death due to necrosis in butachlor treated human cells. It is concluded that the comprehensive investigations are warranted to assess the human health risk assessment in butachlor exposed populations in the occupational settings, and possibly determine the correlations of butachlor exposure with genetic abnormalities including cancer. This study demonstrated that the butachlor has a capacity to bind DNA with high affinity. It can also induces intracellular ROS, which eventually triggers the development of DNA strands breaks, chromosomal and mitochondrial membrane damage leading to cell cycle alterations and necrosis, which is indicative of butachlor genotoxicity and clastogenicity in exposed human PBMN cells.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox.2012.07.014.

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