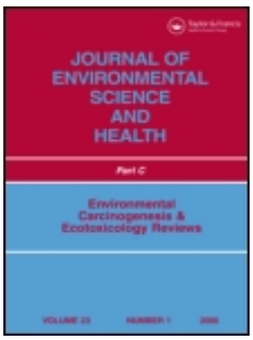

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Genotoxicity and immunotoxic effects of 1,2-dichloroethane in Wistar rats

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ABSTRACT

Dichloroethane is widely used as a solvent, degreasing agent and in a variety of commercial products, and is known for being a ubiquitous contaminant in the environment. Important sources principally include the emissions from industrial processes, improper consumption, storage, and disposal methods. In view of the fact that the mechanism of its genotoxicity has not been satisfactorily elucidated, the acute in vivo toxicological impact is assessed in *Rattus norvegicus*. A systematic investigation has been made involving the use of conventional methods along with molecular and flow cytometric approaches. The micronucleus and chromosomal aberration frequencies were significantly elevated in bone marrow cells exposed to three concentrations at multiple treatment durations indicating positive time- and dose-response relationships. The mitotic index significantly decreased in similar concentrations in contrast to normal control. Separate studies were performed on blood cells for comet assay. It revealed dichloroethane-induced DNA damage in all exposures readily explainable in a dose- and time-dependent manner. Recent molecular techniques were further employed using leukocytes for the cell apoptosis/cycle and mitochondrial membrane potential employing propidium iodide staining and rhodamine-123, respectively. The effect on mitochondrial membrane permeability, cell cycle phases, and the DNA damage was analyzed through flow cytometry. These indicators revealed dichloroethane treatment decreased the mitochondrial membrane potential, affected the cell cycle, and confirmed the DNA damage, leading to apoptosis of the cells of the immune system responsible for immunotoxic effects of dichloroethane on rat leukocytes.

KEYWORDS

Apoptosis; comet assay; flow cytometry; genotoxicity; mitochondrial membrane potential

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Introduction

The genotoxicity of pesticides in nontarget organisms and their influence on worldwide ecosystems is of great concern.[1] Due to the heavy use of pesticides and the consequent potential environmental impact, it is imperative to assess the genotoxic potential of manufactured chemicals such as organochlorines. This concern is shared by many researchers and agencies.[2–4] One such compound of the organochlorine group is 1,2-dichloroethane (EDC), which has a major use as an intermediate in the manufacture of vinyl chloride. It has also been utilized in the production of tri- and tetrachloroethylenes, ethylamines, and trichloroacetic acid, as well as a solvent, a metal degreaser, and as a finish remover. Other applications include scavenging lead in gasoline, cleaning equipment and textiles, extraction of oil from seeds, processing animal fats, manufacturing pharmaceuticals, and as a pesticide.[5,6] According to International Agency for Research on Cancer (IARC) classification, EDC is considered to be possibly carcinogenic to humans (Group 2B). However, American Conference of Governmental Industrial Hygienists has classified EDC carcinogenicity as A4, meaning not classifiable as human carcinogen due to limited evidence derived so far. Overwhelming observations on EDC focused on hepatic DNA, inducing strand breaks.[7,8] An early study however reported a low level of DNA alkylation in other organs as well.[9] DNA adduct formation following exposure to EDC has been reported using different exposure routes.[10]

Some of the mutagenic studies did appear in *Drosophila melanogaster* using various conventional endpoints, sex-linked recessive lethal, and chromosome nondisjunction. In many instances, these early studies demonstrated mutagenicity, but only tested with a single dose as opposed to the recommended multiple doses by current Organisation for Economic Co-operation and Development (OECD) guidelines[11]; therefore, these results cannot be considered positive. Taking account of the broad use of organochlorines in general and EDC in particular, qualify for close scrutiny.

It is informative to consider studies with other important organochlorine compounds tested for in vivo genotoxicity.[12–14] The most reliable genotoxicity evaluation in mammals is a combination of chromosomal aberration (CA) and micronuclei (MN) testing.[15] Therefore a micronucleus test (MNT), in conjunction with CA, was preferred in the present study, among other sophisticated parameters, which can give clues for both clastogenic damage and damage to the mitotic apparatus with aneugenic consequences. The mitotic index (MI) assay, also undertaken in this investigation, is considered useful to characterize proliferating cells and to identify compounds that inhibit or induce mitotic progression; thus it is an effective measure of cell proliferation kinetics in mammalian systems.[16,17]

The genotoxicity of any compound cannot be judged accurately by a single parameter and the use of multiple genetic assays is a standard protocol; therefore, of late, some protocols directly endorsing DNA damage following chemical insult have been favored. The comet assay is one such test; most suitable, fast, and practiced globally for assessing DNA damage with usefulness in biomonitoring.[18,19] In

vitro studies on human lymphocytes with a greater focus on organochlorine pesticides bear testimony to this.[20]

Flow cytometry is a powerful tool to reveal cell distribution in the three major phases of cell cycle, G1, S, and G2/M, based on the analysis of cellular DNA content detected by DNA intercalator; propidium iodide. It also allows quantification of the percentage of apoptotic cells in the Sub G1 phase.

Among all the organelles involved in apoptosis, the role of mitochondria have been deciphered the most in recent years.[21] Mitochondrial membrane potential (Ψm) integrity plays an important role not only in the induction of apoptosis but also in the localization of various proteins into the mitochondria for cell proliferation and survival. The mode of action of organochlorines is linked to the activation of mitochondrial intrinsic apoptotic pathway and to the perturbations in the cell cycle progression.[22] The study of eukaryotic mitochondria could reveal the energetic mode of action for EDC during acute poisoning of metabolizing tissues.[23] In this study we attempt to provide a mechanistic basis for EDC-induced apoptosis observed in rat leukocytes by correlating alterations we detected in Ψm and cell cycle progression.

Molecular events leading to cyto- and genotoxicity caused by acute exposure to EDC have not been systematically investigated, so the study was designed to provide a dose response relationship of mammalian mutagenicity and acute toxicity of EDC by intraperitoneal route of administration, since there is not even a single study available for evaluating the genotoxicity of EDC on these lines. Therefore, the present study was conducted to determine if EDC can cause (i) cytogenetic and genotoxic alterations by CA, MNT, and MI assays; (ii) DNA strand breaks by comet assay; (iii) cellular stress, measured in terms of changes in Ψm; and (iv) cell cycle alterations using flow cytometry; in white blood cells (WBCs) of exposed rats.

Materials and methods

Study design and experimental animals

The acute dose was calculated in accordance with OECD and further considered revised draft TG September 2013 OECD guideline for the testing of chemicals.[24] The dose and mode of administration was selected because this route of administration would maximize the chemical exposure to bone marrow.[25]

The male Wistar rats were procured from Indian Institute of Integrative Medicine, Jammu, quarantined and acclimatized, and then divided by stratified randomization into 5 groups, each comprising five male animals, housed in stainless steel wire cages. Two groups served as controls and three received treatments with specific concentrations of EDC for a specified time with an injection volume of 100 μl. The regular feed was of commercial standard food and water *ad libitum*. All the rats were 8–10 weeks old with an average body weight of 100 $\pm$ 2 gms and were

kept in controlled conditions (12 h dark and light period, temperature; 22 ± 2°C and humidity; 60%–70%). The sacrifice of rats was in compliance with the recommended regulations formulated by the Ethical Committees of the Aligarh Muslim University, Aligarh and Indian Institute of Integrative Medicine, Jammu.

Reagents

1,2-Dichloroethane (EDC, CAS#107-06-2, 99.6%) was purchased from Sigma-Aldrich Chemicals, Bangalore, India. Rhodamine 123 (Rh-123), propidium iodide (PI), sodium bicarbonate, phosphate buffered saline (PBS), tris buffered saline and electrophoresis reagents were all purchased from Sigma Aldrich Co, USA. Becton Dickinson FACS lysing solution was procured from San Jose, CA, USA. DNase-free RNase was purchased from USB Corporation, USA. Tris buffer, bromophenol blue, potassium chloride, methanol, colchicine, fetal bovine serum, Giemsa, and May-Grunwald stain were procured from Himedia Pvt Ltd, India. Glacial acetic acid, cyclophosphamide, and trichloroacetic acid were obtained from Fisher Scientific Ltd and Merck Specialties Pvt. Ltd, India. Remaining chemicals used were also of molecular grade.

Treatment

The stock solution of EDC was prepared by dissolving in distilled water. Sublethal concentrations were calculated on the basis of median lethal dose (LD_{50}), which for EDC is 807 mg/kg b.wt.[26] The sublethal concentrations ranged from low (10% of $LD_{50;}$ 80.7 mg/kg b.wt.), medium (20% of $LD_{50;}$ 161.4 mg/kg b.wt.) to high (30% of $LD_{50;}$ 242.1 mg/kg b.wt.). All the concentrations administered intra-peritoneally having five animals per treatment. Bone marrow flushed and other cell types were screened after completion of specified durations. Concurrently positive control (cyclophosphamide; 40 mg/kg b.wt.) and normal control (water) run simultaneously for comparative analysis of the data.

Bone marrow cell experiments

Micronucleus test

The micronucleus test was carried out according to the protocol of Schmid[27] on the rats treated with EDC. The flushing of bone marrow cells (BMCs) from both the femurs was collected as a fine suspension into a tube containing 1ml fetal bovine serum (FBS). The centrifugation was carried out for 10 min at 1000 rpm. The pellet was resuspended in FBS. The suspension so prepared was smeared onto the pre-cleaned slides and air-dried. The slides were fixed in 100% methanol for 5 min following the differential staining carried in May-Grunwald and Giemsa. The clearing of slides for both (CA and MNT) was done in xylene and permanently mounted in DPX.

Independently coded slides were tested for analyzable MN and the polychromatic erythrocyte (PCE) to normochromatic erythrocyte (NCE) cell ratio was recorded. The MN frequency expressed as mean percent micronulceated cells were scored by analyzing the number of micronucleated PCEs (MNPCEs) in 2000 PCEs per animal. The PCE:NCE ratio was taken to estimate the cytotoxic effect in 1000 erythrocytes per animal.[28] The final observation and representative photography was carried out at *100X* (Olympus U-PMTV microscope mounted with optical zoom camera), under oil immersion.

Chromosomal aberration assay

For the assessment of chromosomal damage, two hours prior to sacrificing of rats, colchicine (0.004 mg/g b.wt.) was injected intra-peritoneally. The slide preparation and the staining followed the procedural details of Preston and colleagues.[29] The femurs were removed and bone marrow was flushed using a syringe containing 0.56% KCl solution, which served as hypotonic solution, pre-incubated for 30 min at 37°C. Next, the suspension was centrifuged at 1500 rpm and the supernatant was discarded. The pellet was fixed in glacial acetic acid: methanol (1:3 v/v). The suspension having the cells was then prepared for microscopic examination by placing 3–4 drops on precleaned, chilled, ethanol-dipped slides, flame, and air-dried, which were stained with 5% Giemsa (25–30 min). Properly spread metaphases were randomly analyzed for chromosomal aberrations and photographed at *100X* in oil immersion (Olympus U-PMTV microscope mounted with optical zoom camera).

The metaphase cells from 1000 cells per concentration per animal both in exposed and control replicates, expressed in percentage were considered separately for calculating MI.

Alkaline single cell gel electrophoresis assay

The DNA damage in our study was assessed by alkaline comet assay as described earlier by Singh and colleagues[30] with some modifications. Before moving on to the experiment, retro-orbital bleeding was employed for blood collection.[31] On the slides precoated with normal melting agarose (NMA), a coat of 75 μl of 0.5% low melting point agarose (LMPA) mixed with 10 μl of whole blood was added. The coverslip was placed on the slide, kept on a slide tray resting on icepacks until the agarose layer hardens (5–10 min). The coverslip was removed and the slide was slowly lowered into a cold, freshly prepared lysing solution (pH = 10). A 1% triton X-100 solution was added afresh to solubilize cellular proteins, leaving the DNA as nucleoids in cold conditions for a minimum of 2 hours. Electrophoresis was performed under pH > 13 alkaline conditions at 300 milliamperes and 24 volts ($\sim$0.74 V/cm) for 40 min. The slides were coated with neutralization buffer (pH = 7.5) dropwise and kept for 5 min, followed by staining the slides with 80 μl of 1X ethidium bromide and dipped in chilled distilled water to remove excess stain. The slides were examined under an Olympus fluorescence microscope (IX51) equipped

with an excitation filter (510 nm) and a barrier filter (590 nm) at 40X magnification using computerized image analysis system (Komet 5.5).

Flow cytometric methods

Isolation of WBCs

The blood collected from retro-orbital plexus of sensitized rat in 0.1% EDTA was used in this study also. After the incubation of blood for 30 min at room temperature in the dark, 500 μl of it was taken in a centrifuge tube and 1 ml FACS lysing solution was added to it and further incubated for 10 min. The cells were centrifuged (2000 rpm) for 10 min at 25°C, the supernatant was discarded and the pellet washed in 1 ml FACS lysing solution and centrifuged again for 5 min (2000 rpm). This step was repeated twice. The pellet was suspended in 500 μl of PBS having only WBCs in it.

Cell cycle phase distribution

The effect of the test agent on different phases of the cell cycle in leukocytes was explored by flow cytometry. Completion of treatment durations was followed by harvesting of leukocytes by centrifugation at 1000 rpm (5 min). The harvested cells were washed twice with PBS, fixed in 70% cold ethanol for 48 h. After fixation, the cells were washed again with PBS, and subjected to RNase digestion (400 μg/ml) at 37°C for 45 min. Finally, the cells were incubated with propidium iodide (10 μg/ml) and analyzed immediately with a FACS ARIA II flow cytometer (Becton Dickinson, USA). The fluorescence intensity of sub-G_1 cell fraction represents the apoptotic cell population. A total of 10,000 events were acquired for cell cycle analysis.[32]

Measurement of mitochondrial membrane potential (Ψm) for cellular energy status

The method of Majeed and colleagues[33] was followed for flow cytometric measurements of change in Ψm as a result of mitochondrial perturbation using the fluorescent dye Rh-123. For this, the leukocytes were incubated with 5 μM Rh-123 for 60 min before the termination of the experiment. Cells were washed with PBS at 1500 rpm for 5 min at room temperature and the pellet was re-suspended in 300 μl PBS. The decrease in intensity of fluorescence due to a decrease in Ψm among 10,000 cellular events was analyzed in the FITC channel using a FACS ARIA II flow cytometer. Leukocytes were identified with the characteristics of forward scatter and side scatter. A similar gate was used to reduce debris and other contaminants for all groups.

Statistical analysis

The results were expressed as mean values with standard error (mean ± S.E.). All the treatments performed in triplicate for every parameter. Statistical analysis was performed using *Statistical Package for Social Sciences (SPSS) Version 20.0*.

Table 1. Micronuclei induction in polychromatic erythrocytes observed in the bone marrow cells of *Rattus norvegicus* treated *in vivo* with different doses of dichloroethane at various durations.

Group & dose	Time (h)	Total PCEs scored	Total number of MNPCEs	Mean frequency of MN per 1000 PCEs ± S.E.	P/N ratio
Normal control (NC)	24	2010	2	0.67 ± 0.33	0.796 ± 0.04
	48	2031	1	0.37 ± 0.31	0.784 ± 0.03
	72	2024	1	0.37 ± 0.30	0.789 ± 0.04
Positive control (PC) (40 mg/kg b. wt.)	24	2031	24	8.01 ± 1.73**	0.507 ± 0.01*
	48	2039	16	5.33 ± 1.45*	0.514 ± 0.00*
	72	2024	14	4.66 ± 2.33*	0.509 ± 0.01*
EDC I (80.7 mg/kg b.wt.)	24	2064	12	4.01 ± 1.15*	0.701 ± 0.03*
	48	2039	8	2.67 ± 1.20**	0.754 ± 0.02**
	72	2139	7	2.33 ± 1.31*	0.739 ± 0.02**
EDCII (161.4 mg/kg b.wt.)	24	2133	15	5.00 ± 1.73**	0.691 ± 0.02**
	48	2191	13	4.33 ± 1.76**	0.699 ± 0.03
	72	2164	10	3.34 ± 1.85*	0.684 ± 0.01**
EDCIII (242.1 mg/kg b.wt.)	24	2039	17	5.67 ± 1.45**	0.614 ± 0.02**
	48	2043	14	4.66 ± 1.85**	0.622 ± 0.01**
	72	2012	11	3.67 ± 1.76**	0.637 ± 0.04*

Normal control (water); positive control (cyclophosphamide); EDC I (80.7 mg/kg b.wt); EDC II (161.4 mg/kg b.wt); EDC III (242.1 mg/kg b.wt);PCEs (Polychromatic erythrocytes); MNPCEs (micronucleated polychromatic erythrocytes); *statistically significant values at.
*p<0.05, **p<0.01, ***p<0.001*(One way ANOVA, post hoc Tukey)*.

Statistical significance of the difference was defined as $p < 0.05$ using one-way analysis of variance (One way ANOVA); *post hoc Tukey*.

Results

Micronucleus test

The results of the MN assay for dichloroethane (Table 1) showed significant time- and dose-dependent increase in micronucleated polychromatic erythrocytes (MNPCEs) in rats treated with EDC. The maximum number of micronuclei induction in PCEs was observed on administration of the highest dose, registering a mean frequency of 5.67 ± 1.45 at 24 h (Table 1). However a significant decrease in mean number of MNPCEs was observed at each concentration and duration ($p < 0.05$). The cytotoxicity indicator P/N ratio reflected dose dependent depression for each concentration.

Chromosomal aberration

These results have been presented in Table 2 with a maximum significant chromosomal aberration of 6.34 ± 1.69 observed at highest concentration in 24 h post-treatment ($p < 0.05$). Comparatively, the injurious effect of CA was found lesser in other two durations of each concentration. The exposed groups differ significantly on comparison with control groups as the one way ANOVA; *post hoc Tukey* is applied. The observed aberrations include breaks, gaps, translocations, stickiness, pulverization, acentric fragments, dicentrics, and polyploidy, which were not unique for any treatment qualitatively; however, a dose- and time-dependent increase in

Table 2. Incidence of *in vivo* chromosomal aberrations and mitotic index recorded in the bone marrow cells of *Rattus norvegicus* exposed to multiple doses of dichloroethane at various durations.

		Structural aberrations							
Group & dose	Time (h)	Break	Gap	Fragment	Ring	Dicentric	Deletion	Mean frequency of aberrations ± S.E.	Mean percent MI ± S.E.
Normal control (NC)	24	0	1	0	1	1	0	0.62 ± 0.01	2.78 ± 0.23
	48	0	1	1	1	1	1	0.94 ± 0.29	2.94 ± 0.20
	72	0	0	0	0	1	1	0.62 ± 0.01	2.82 ± 0.15
Positive control (PC) (40 mg/kg b. wt.)	24	9	10	4	3	3	2	8.31 ± 2.08**	1.41 ± 0.15**
	48	7	5	4	3	3	3	6.80 ± 1.79**	1.46 ± 0.11***
	72	7	6	3	2	2	2	6.45 ± 1.31**	1.43 ± 0.05***
EDCI (80.7 mg/kg b.wt.)	24	5	4	3	3	4	4	5.63 ± 1.56*	2.44 ± 0.15
	48	4	2	3	2	3	2	4.97 ± 1.4*	2.42 ± 0.13
	72	2	2	1	0	2	1	2.07 ± 0.92*	2.23 ± 0.12
EDCII(161.4 mg/kg b.wt.)	24	5	5	4	4	4	2	6.30 ± 1.69**	2.31 ± 0.09
	48	4	3	3	2	3	3	4.89 ± 1.50**	2.08 ± 0.13
	72	2	2	2	1	3	6	3.28 ± 1.11**	2.12 ± 0.11
EDCIII (242.1 mg/kgb.wt.)	24	5	4	4	3	4	4	6.34 ± 1.69***	2.14 ± 0.02*
	48	4	3	3	2	4	4	5.13 ± 1.46**	2.02 ± 0.08*
	72	3	2	3	1	3	5	4.46 ± 1.33**	2.07 ± 0.07

Normal control (water); positive control (cyclophosphamide); EDC I (80.7 mg/kg b.wt); EDC II (161.4 mg/kg b.wt); EDC III (242.1 mg/kg b.wt); *statistically significant values at
* $p < 0.05$, **$p<0.01$, ***$p<0.001$*(One way ANOVA, post hocTukey)*.

Table 3. Incidence of *in vivo* DNA damage in rat blood cells exposed to various doses of dichloroethane at multiple durations.

Group & dose	Time (h)	Mean head DNA ± SEM	Mean tail DNA ± SEM	Mean tail Length ± SEM	Mean OTM ± SEM	Mean tail coefficient ± SEM
Normal control (NC)	24	97.54 ± 0.30	2.45 ± 0.30	1.15 ± 0.34	0.18 ± 0.02	1.81 ± 0.38
	48	93.59 ± 0.43	6.40 ± 0.43	2.84 ± 0.62	0.90 ± 0.15	3.26 ± 0.38
	72	85.27 ± 0.96	14.72 ± 0.96	3.86 ± 0.46	0.86 ± 0.07	3.34 ± 0.50
Positive control (PC) (40 mg/kg b. wt.)	24	65.09 ± 1.70	34.90 ± 1.70	38.14 ± 2.62	10.37 ± 0.72	9.33 ± 0.77
	48	62.73 ± 1.05	37.27 ± 1.05	37.00 ± 0.85	6.92 ± 0.23	8.74 ± 1.11
	72	54.36 ± 2.14	35.63 ± 2.14	55.29 ± 3.35	14.73 ± 0.78	11.25 ± 0.83
EDC I(80.7 mg/kg b.wt.)	24	67.26 ± 1.68**	32.73 ± 1.68*	34.67 ± 1.16*	6.70 ± 0.33**	9.09 ± 1.25*
	48	63.08 ± 3.10***	36.91 ± 3.10**	36.10 ± 4.03**	10.78 ± 1.27***	10.69 ± 1.39**
	72	64.17 ± 1.96***	35.82 ± 1.96**	50.68 ± 3.89**	12.73 ± 1.37***	10.8 ± 80.96**
EDC II (161.4 mg/kg b.wt.)	24	63.02 ± 2.70**	36.97 ± 2.70*	44.40 ± 3.68**	9.92 ± 0.98**	8.82 ± 0.84*
	48	49.26 ± 3.81**	50.73 ± 3.81**	39.65 ± 3.29*	16.20 ± 1.42***	10.40 ± 1.03**
	72	54.40 ± 2.91***	45.59 ± 2.91**	57.22 ± 4.90**	19.40 ± 2.03***	13.97 ± 1.85**
EDC III(242.1 mg/kg b.wt.)	24	54.63 ± 1.89**	45.36 ± 1.89*	63.20 ± 3.15**	19.87 ± 1.14***	13.07 ± 0.83**
	48	50.01 ± 2.85***	49.98 ± 2.85*	48.00 ± 3.74***	16.31 ± 1.27***	13.15 ± 1.35**
	72	57.99 ± 2.57***	42.00 ± 2.57***	65.07 ± 4.60***	19.52 ± 1.48***	13.84 ± 1.05**

Normal control (water); positive control (cyclophosphamide); EDC I (80.7 mg/kg b.wt); EDC II(161.4 mg/kg b.wt); EDCIII(242.1 mg/kg b.wt); Olive tail moment (OTM);*statistically significant values at.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *(One way ANOVA, post hoc Tukey)*.

total aberrations was observed. The study was further extended to observe MI to characterize proliferating cells. A significant dose-dependent reduction in the MI (Table 2), points to inhibitory effect on cell division.

Comet assay

The assay gave an insight of DNA damage in a single cell. The results of DNA damage induced by EDC expressed as mean tail DNA, tail length, and Olive tail moment (OTM) of comet have been summarized in Table 3 with representative comet micrographs in Figure 1. Treatment of rats with different concentrations of EDC at multiple durations resulted in a significant elevation in the migration of DNA ($p < 0.05$) when compared with the normal control. EDC caused a dose- and time-dependent increase in the OTM, indicating an increase in DNA damage as a function of median OTM of the comet at all the post-treatment concentrations and durations. The maximum DNA damage 19.87 ± 1.14 was observed at highest concentration at 24 h (Table 3). The images clearly indicate and the table authenticate that when blood cells were exposed to EDC, the percentage of DNA in the tail of the comet as well as the tail length was substantially increased. With increasing doses, more DNA moved

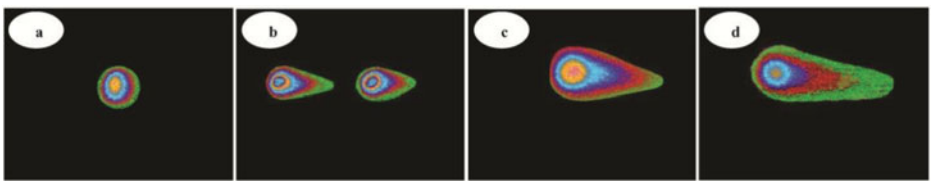

Figure 1. Representative comet images of control (a) and dichloroethane exposed blood cells of *Rattus norvegicus* treated in vivo with different doses of dicholoroethane showing slight (b) medium (c) and highly damaged cells (d) exhibiting increasing DNA fragmentation.

out of the comet head as shown in the representative Figure 1(b-d), alternatively no tail was observed in the control (Figure 1a).

Cell cycle analysis

The effect on cell cycle phase distribution was observed on treatment of different EDC concentrations in *Rattus norvegicus* after 24, 48, and 72 h durations. In 24 h treatment, EDC induced a maximum apoptotic cell population of 38.0 ± 2.71. For normal control the apoptotic percentage was 8.9 ± 0.72, and positive control (cyclophosphamide; 40 mg/kg b. wt.) showed 100% cell population in the apoptotic phase (Table 4). In 48 h treatment of EDC, apoptotic cell population increased comparatively compared to the normal control. In 72 h treatment, the apoptotic cell population increased further with maximum apoptotic cell percentage of 86.3 ± 5.01 at the highest concentration compared to the respective normal control of 10.3 ± 0.64 (Table 4).

It is thus concluded that EDC showed both concentration- and time-dependent increase in the apoptotic cell percentage of WBCs. Thus the compound inflicted significant apoptosis in rat WBCs in time- and dose-dependent manner.

Analysis of mitochondrial membrane potential

After EDC treatment (10%, 20%, and 30% of LD_{50}), *Rattus norvegicus* were examined for the effect on Ψm in WBCs after 24, 48, and 72 h; measured by flow cytometry. EDC showed both concentration- and time-dependent decrease in Ψm in all the WBC types (Table 5). However, maximum significant effect on Ψm was observed in neutrophils ($p < 0.05$). The representative flow cytometric images have been presented in Figure 2. It is also worth mention here that EDC was effective in decreasing

Table 4. Flow cytometric analysis of cell cycle phase distribution in rat WBCs after propidium iodide staining treated with different doses of dichloroethane at various durations.

Group & dose	Time (h)	Sub-G1	G1	S	G2/M
Normal control(NC)	24	8.9 ± 0.72	80.6 ± 3.11	8.6 ± 1.06	1.9 ± 0.15
	48	5.5 ± 0.46	90.1 ± 2.62	2.6 ± 0.36	1.7 ± 0.17
	72	10.3 ± 0.64	82.6 ± 1.53	4.0 ± 0.45	3.1 ± 0.06
Positive control (PC) (40 mg/kg b. wt.)	24	100 ± 0.12	0.0 ± 0.12	0.0 ± 0.06	0.0 ± 0.06
	48	77.9 ± 4.81	22.1 ± 1.25	0.0 ± 0.06	0.0 ± 0.12
	72	91.5 ± 7.52	5.9 ± 1.15	0.1 ± 0.06	2.5 ± 0.06
EDCI (80.7 mg/kg b.wt.)	24	3.5 ± 0.20	92.9 ± 3.94**	0.3 ± 0.06***	3.3 ± 0.51***
	48	15.1 ± 0.79	83.2 ± 3.71	0.8 ± 0.25***	0.9 ± 0.31*
	72	79.3 ± 4.88***	17.8 ± 0.71***	1.2 ± 0.10***	1.7 ± 0.06***
EDCII (161.4 mg/kg b.wt.)	24	33.4 ± 2.21***	60.4 ± 3.91***	3.4 ± 0.26***	2.8 ± 0.60**
	48	43.8 ± 1.90***	53.9 ± 4.20***	0.6 ± 0.21***	1.7 ± 0.25
	72	77.0 ± 6.40***	19.0 ± 0.20***	1.9 ± 0.06***	1.9 ± 0.15**
EDCIII (242.1 mg/kg b.wt.)	24	38.0 ± 2.71***	55.0 ± 1.80***	4.0 ± 0.36***	3.0 ± 0.25**
	48	33.4 ± 1.19***	57.2 ± 3.90***	7.2 ± 0.10***	2.2 ± 0.10
	72	86.3 ± 5.01***	12.1 ± 0.17***	1.1 ± 0.06***	0.5 ± 0.06***

Normal control (water); positive control (cyclophosphamide); EDC I (80.7 mg/kg b.wt); EDC II (161.4 mg/kg b.wt); EDC III (242.1 mg/kg b.wt);Sub-G_1, G_1, S and G_2/M represent different phases of cell cycle along with the mean percentage of cells ± S.E.; *statistically significant values at
*$p < 0.05$; **$p < 0.01$;***$p < 0.001$*(One way ANOVA, post hoc Tukey)*.

Table 5. Induced Ψm decrease in rat WBCs exposed to different concentrations of dichloroethane at various durations in flow cytometric analysis.

Group & dose	Time (h)	Mean Ψm ± S.E.				
		Neutrophils	Monocytes	Lymphocytes	Platelets	Eosinophils
Normal control (NC)	24	0.2 ± 0.06	2.5 ± 0.32	2.0 ± 0.21	1.6 ± 0.31	0.2 ± 0.09
	48	0.5 ± 0.21	12.0 ± 0.47	19.3 ± 0.35	3.4 ± 0.25	0.7 ± 0.15
	72	13.8 ± 0.85	13.4 ± 0.31	17.2 ± 1.01	12.8 ± 0.42	5.2 ± 0.47
Positive control (PC) (40 mg/kg b. wt.)	24	10.9 ± 0.85	14.4 ± 0.95	14.1 ± 0.32	11.3 ± 0.35	16.4 ± 1.12
	48	19.0 ± 0.26	14.7 ± 0.61	26.2 ± 0.36	19.1 ± 0.26	11.1 ± 0.26
	72	54.0 ± 0.40	27.2 ± 0.42	25.3 ± 0.35	93.2 ± 1.72	81.1 ± 0.67
EDCI (80.7 mg/kg b.wt.)	24	3.9 ± 0.31*	10.0 ± 0.50***	10.6 ± 0.21***	2.5 ± 0.40	2.9 ± 0.21*
	48	33.3 ± 0.36***	25.2 ± 0.10***	19.9 ± 1.00	29.6 ± 0.81***	16.3 ± 0.57***
	72	52.6 ± 0.98***	50.8 ± 0.79***	43.2 ± 0.42***	67.4 ± 1.30***	67.7 ± 1.55***
EDCII (161.4 mg/kg b.wt.)	24	25.3 ± 1.57***	16.0 ± 0.85***	10.9 ± 0.44***	18.6.3 ± 1.23***	12.3 ± 1.16***
	48	28.0 ± 0.58***	24.4 ± 0.26***	41.8 ± 0.75***	17.0 ± 0.25***	15.7 ± 0.90***
	72	60.5 ± 0.86***	63.4 ± 0.50***	38.7 ± 1.55***	35.0 ± 1.85***	53.6 ± 1.14***
EDCIII (242.1 mg/kg b.wt.)	24	41.0 ± 2.63***	17.6 ± 1.36***	12.4 ± 1.04***	29.9 ± 3.71***	12.4 ± 0.97***
	48	59.2 ± 0.25***	29.6 ± 1.14***	40.7 ± 0.35***	36.1 ± 0.51***	26.9 ± 0.75***
	72	86.4 ± 0.46***	70.1 ± 0.90***	49.5 ± 1.21***	85.8 ± 0.99***	85.9 ± 1.07***

Normal control (water); positive control (cyclophosphamide); EDC I (80.7 mg/kg b.wt); EDC II (161.4 mg/kg b.wt); EDC III (242.1 mg/kg b.wt);*statistically significant values at
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$*(One way ANOVA, post hoc Tukey)*.

Ψm even in 24 h treatment. Our results suggest that the apoptotic induction is the result of decrease in Ψm in WBCs.

Discussion

The serious lack of studies on genotoxic effects of EDC in mammalian models prompted this investigation on *Rattus norvegicus*. Our results show that the test chemical induced significant cytogenetic damage in BMCs evidenced by the increase in MN and CAs. The increase in MNPCEs, especially 24 h post-treatment thereafter, a gradual decrease at other two durations is noteworthy and quite opposite to an earlier study by Jessen and colleagues.[34] The cytotoxicity indicator P/N ratio reflects a dose-dependent depression in most cases is also clearly evident. The alteration in the ratio may be due to accelerated differentiation from erythroblasts to form erythrocytes, inhibition of erythroblast division or impaired erythroblast division recovery.[35]

During our observations on CAs, a surge in aberrations in the early duration and a decline in later intervals could be due to a number of factors such as elimination

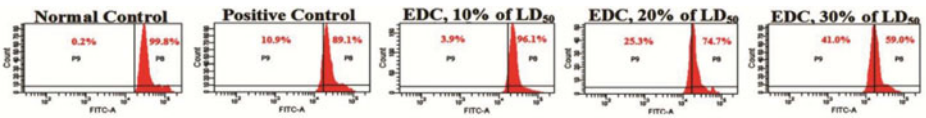

Figure 2. Representative flow cytometric images of the effect of dicholoroethane on mitochondrial membrane potential (Ψm). Induced Ψm decrease in rat WBCs on treatment of different concentrations of EDC at 24 h duration. Figures show the representative FACS images from a single animal exhibiting decline in the Rh-123 fluorescence as a function of EDC concentration in Neutrophils. P_8 and P_9 respectively represent mean percentage of intact and decreased Ψm in Neutrophils. Similar experiments were performed for Monocytes, Lymphocytes, Platelets, and Eosinophils at 48 and 72 h.

of chemical or metabolites from the body, repairing of the damaged genetic material and removal of chromosomes with damaged genetic material.[36] The ability of EDC to inhibit or induce mitotic progression indicates a possible cellular death or delay in cell proliferation kinetics; similar observations were reported by Öcal and associates[37] and Sant'Anna and colleagues.[38]

Similar to related organohalides, EDC is a potent agent of DNA damage. Recently Blasiak and colleagues[39] and Hossain and researchers[40] suggested that such chemicals interact with cellular DNA by producing free radicals through direct or indirect effects and thereby create DNA lesions in the exposed cell types. This may culminate in the occurrence of cell cycle disturbances and aberrant mitoses leading to cell death. Thus, the primary target of chemical damage by EDC is nuclear DNA.[41,42] Previous investigations of the induction of strand breaks by EDC focused on hepatic DNA.[7,8]

It has been reported earlier that EDC can increase the frequency of micronuclei, another marker of DNA damage, in three metabolically competent human cell lines: AHH-1, h2E1, and MCL5.[43] The comet assay indicates that the maximum DNA damage observed 24 h post-treatment of EDC at the highest concentration is in concordance with the previous studies.[44,45] It must be noted that even the lowest concentration of EDC increased DNA damaging effect significantly when compared with untreated control; as has been concluded by Cheng and colleagues[46] in human lymphocytes.

Jagetia and Adiga[47] and Jagetia and associates[48] suggested that accumulation of DNA damage is the hallmark of cell death. The increased Olive tail moment found in the present scenario is a pointer to this effect and may be due to direct induction of DNA strand breaks or modification in DNA that can turn into strand-breaks.[49,50] Also the conjugation reaction of EDC with GSH catalyzed by glutathione S-transferase(s) to yield S-(2-chloroethyl)-glutathione, a sulfur-half-mustard that has been demonstrated to be an alkylating agent. The structure S-[2-(N7-guanyl)ethyl]GSH was proposed as the major DNA adduct following GSH conjugation in multiple test systems exposed to EDC and being potent in inducing mutations including S-(2- chloroethyl)-L-cysteine and N-acetyl-S-(2-chloroethyl)-L-cysteine.[45] Although some mutagenic studies of EDC did appear in insects and plants producing sex linked lethal, chromosome nondisjunction, and strand breaks.[51]

The induction of DNA damage by EDC cannot be ascribed to a single mechanism but may be due to interplay of several different mechanisms. Other than these mechanisms, apoptosis induction for enhanced cytotoxicity and operation of other unknown mechanisms in producing the chemically induced DNA damage by EDC cannot be ruled out. The exact mechanism of DNA damage by EDC is not fully understood.

In our study, exposed cells with DNA damage showed an increase in migration of DNA fragments (comet tail) from the nucleus (comet head), which is considered a feature closely associated with the necrotic/apoptotic death process.[52,53] Apoptotic

form of cell death is characterized by DNA fragmentation. One of the known methods used for confirming DNA fragmentation is cell cycle analysis by flow cytometry. The measure of apoptosis is the fraction of cells in sub-G1 phase, indicating the DNA of these cells has undergone fragmentation and DNA loss. Further, the position of sub-G1 peak can provide us further information about apoptosis and distinguish it from necrosis. Necrosis can peak into sub-G1 area but close to G1 due to the loosely packed DNA while apoptosis peak will be far off from G1 towards y-axis as DNA underwent fragmentation and florescence falls drastically.

Moreover, EDC action of apoptosis-induction and its underlying mechanisms were accordingly studied using flow cytometry. Thus far, to our knowledge, no study has been conducted to provide insight into the cytotoxic effects of EDC on WBC functions and their mechanism of action in vivo; therefore, the potential mechanistic action of EDC should be elucidated for its health risk assessment. Flow cytometric assays have been applied for evaluating cell activities to screen the toxicity of xenobiotics.[54,55] Therefore, the present study was conducted to provide highly reliable results using flow cytometric approach.

Our analyses of cell cycle distribution showed that EDC can induce apoptosis in a time-dependent manner in rat leukocytes. Physiologically, apoptotic cells significantly contribute in immune suppression[56]; therefore, the action of EDC-induced apoptosis might reduce the number of functional leukocytes with concurrent reduction in lymphocyte number and as a result lower levels of immune function as observed by Munson and colleagues[57] in CD-1 mice, wherein EDC elicited an immunotoxic response with a significant dose-related reduction in IgM response to sheep erythrocytes and an accompanying 30% decrease in total leukocyte number. The various other molecular mechanisms of the EDC effect need to be further investigated.

The mitochondrial pathway, characterized by the increased mitochondrial membrane permeability and the subsequent release of cell death mediators from the mitochondria is one of the important apoptotic pathways for the cells of the immune system.[58,59] In the present research, Ψm decreased in EDC exposed cells of the immune system at all the post-treatment durations in a concentration-dependent manner, these results are in coherence with the hypothesis that Ψm decrease is a cellular event in the mitochondrial driven apoptosis.[60,61]

The significant decrease in Ψm may release the apoptogenic signals that activate caspases, which mediate the activation of apoptotic signal transmission.[62,63] Our observations confirmed that EDC induces apoptosis, suggesting that EDC can target the Ψm-related apoptotic pathway. Further, our results indicate that the decrease in Ψm in rat WBCs correlated with EDC concentration and exposure time, suggesting that EDC may act as an uncoupler of oxidative phosphorylation in mitochondria, which diminishes membrane potential in a concentration-dependent manner. This provokes the idea that at higher concentrations EDC is more toxic to oxidative phosphorylation in mitochondria similar to the effects described by Cetkauskaite and associates[23] for the dianaline herbicide pendaimethalin.

Flow cytometric methods have been widely used for clinical research; however, its applicability has been limited to a few immunotoxicological evaluations. It has been demonstrated in the present work and in other studies[64,65] that flow cytometric methods are promising alternatives to standard genotoxicity testing approaches using cell monolayers and in vivo screening assays.

Conclusions

Thus, based on information available, we propose that EDC produces clastogenicity in rat BMCs in a dose- and time-dependent manner under the experimental conditions used for this study. The results of MNT, CA, and MI assays elucidate a plausible mechanism of induction of cytogenetic damage. These molecular analyses led us to conclude that EDC has a potential for inducing cellular and genetic toxicity, manifested as DNA damage in the peripheral blood, disturbances in cell cycle, Ψm dysfunction, activation of apoptosis, and appearance of $SubG_1$ apoptotic peak in cell cycle in the cells of the immune system in treated Wistar rats.

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