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Toxicogenetic evaluation of dichlorophene in peripheral blood and in the cells of the immune system using molecular and flow cytometric approaches

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HIGHLIGHTS

- This is the first report on genotoxicity of Dichlorophene (DCP) using highly sensitive molecular and flow cytomertic approaches.
- Rats treated with DCP induced DNA damage in peripheral blood.
- DCP induces apoptosis and alters mitochondrial membrane potential in leukocytes.

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ABSTRACT

Dichlorophene; a halogenated phenolic compound with wide applications as a fungicide, bactericide and antiprotozoan. Dichlorophene spray also has therapeutic use in the disease digital dermatitis. In guinea pigs, a few studies obtained mixed results in dicholorophene sensitization tests. In consideration of the fact, that the mechanism of its genotoxicity has not been adequately elucidated lead to present study assessing the acute *in vivo* toxicological impact in *Rattus norvegicus*. A systematic research has been made encompassing the use of molecular and flow cytometric approaches. The study was designed on blood cells for comet assay which revealed dichlorophene induced DNA damage in all exposures understandable in time dependent manner. The feasibility of this assay was also established as an effective, fast and accurate method with a great potential in biomonitoring. Contemporary molecular techniques were further engaged using leukocytes for the cell apoptosis/cycle and mitochondrial membrane potential employing propidium iodide staining and rhodamine 123 respectively. The effect on cell cycle phases and mitochondrial membrane permeability was analyzed through flow cytometry. These indicators exposed that dichlorophene decreased the mitochondrial membrane potential, altered the cell cycle and confirmed the DNA damage leading to apoptosis of the cells of the immune system accountable for immunotoxic effects of dichlorophene on rat leukocytes.

1. Introduction

The threat of organochlorine pesticides and also with other chemicals is so serious that even commonly used chemicals have been reported to be mutagenic to human population (Saghir et al., 2001). The ever increasing risk of hazardous chemicals to human exposure is imminent. In recent times, our attention has been drawn to human-induced environmental changes that pose potential threat to life (Topashka-Ancheva and Gerasimova, 2012; Van der Sluijs et al., 2015). Because of the heavy use of pesticides now a days, it is of vital importance to assess the genotoxic potential of chemicals including organochlorines. This concern is shared by many researchers and agencies (IARC, 1979; ATSDR, 2001; Berkowitz, 2014). One such compound of the organochlorine group is dichlorophene (DCP); Chemically, represented as 2,2′-methylenebis(4-chlorophenol), a halogenated phenolic compound

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with numerous applications. It is used as a fungicide, bactericide and antiprotozoan (Gemmel and Johnston, 1981; Kintz et al., 1997). The dichlorophene spray have found therapeutic use in the disease digital dermatitis (Ghashghaei, 2007). Also the reports in guinea pig studies were mixed which were obtained in dicholorophene sensitization tests (Yamarik, 2004). Some derivatives have been used as antibacterial and antifungal agents which were found to be potent inhibitors of glucose-6-phosphate dehydrogenase in yeast (Wang and Buhler, 1981; Kintz et al., 1997). A recent study on DCP in our laboratory focused on *in vivo* mutagenic potential of dichlorophene in *Rattus norvegicus* by using chromosomal aberration (CA), micronucleus (MN) induction and mitotic index (MI) in bone marrow cells (BMCs) (Lone et al., 2013), but research on DCP is seriously lacking. Taking account of the broad use of organochlorines in general and DCP in particular, qualify for close scrutiny.

The very basic principle that genetic alterations are found in all cancers is partly connected to the application of genotoxicity to predict potential carcinogenicity. Genotoxicity explains the ability of chemicals to alter the genetic material leading to its transmission during cell division (Eastmond et al., 2009; Kang et al. 2013). Further a compound cannot be judged accurately by a single parameter and the multiple genetic assays is a standard protocol; therefore, of late, some protocols directly endorse DNA damage following chemical insult. The comet assay is one such test; most suitable, fast and practiced globally for assessing DNA damage with usefulness in biomonitoring (Muid et al., 2012; Wada et al., 2015). It will be interesting to site important compounds belonging to organochlorines tested for *in vivo* genotoxicity (Blakey et al., 2008; ICH, 2008; Eastmond et al., 2009). *In vitro* studies by Jamil et al. (2005) on human lymphocytes with emphasis on organochlorine pesticides bear testimony to this.

The flow cytometric assays are highly sensitive and add on as power 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 of cell cycle and further deciphers the role of mitochondrial membrane potential (Ψm) integrity in the induction of apoptosis. Not a single study could be found with respect to dichlorophene on these lines. However, with other compounds some references could be found.

In recent years the role of mitochondria among all the organelles involved in apoptosis, have been deciphered the most (Chandel, 2014). 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 (Saquib et al., 2012). The universal model system such as eukaryotic mitochondria could reveal the energetic mode of action during acute poisoning of metabolizing tissue (Cetkauskaite et al., 2006). We attempt to co relate this study with apoptosis on mechanistic basis through Ψm and cell cycle analysis.

The molecular events leading to cyto- and genotoxicity caused by acute exposure to DCP have not been thoroughly investigated, so the study was designed to provide a dose response relationship of mammalian mutagenicity and acute toxicity of DCP by intraperitoneal route of administration, since there is not even a single study available for evaluating the genotoxicity of DCP on these lines. Therefore, in our study DCP induced DNA damage and apoptosis were assessed in Wistar rats by use of sensitive molecular assays and techniques. The present study was conducted to determine; if DCP can cause: i) DNA strand breaks by comet assay; ii) cellular stress, measured in terms of changes in Ψm; and iii) cell cycle alterations using flow cytometry; as indicators of apoptosis in white blood cells (WBCs) of exposed rats.

2. Materials and methods

2.1. Study design

The assays were performed at the Molecular Gene-Tox Laboratory (Aligarh Muslim University, Aligarh) and Cancer Pharmacology Laboratory (Indian Institute of Integrative Medicine, Jammu). The acute dose was calculated and conducted in accordance with OECD (1997) and further considered revised draft TG September 2013 OECD guideline for the testing of chemicals. The dose and mode of administration was selected as this route of administration would maximize the chemical exposure to the cells (Patlolla and Tchounwon, 2005).

2.2. Animals

The animals were procured from Indian Institute of Integrative Medicine, Jammu; quarantined and acclimatized and then divided by stratified randomization into 5 groups, each comprising five animals, housed in stainless steel wire cages. Two groups served as controls and three groups received treatments with specific concentrations of DCP for a specified time. The regular feed was of commercial standard food and water *ad libitum*. All the rats were of 8–10 weeks old with an average body weight of 100 ± 1 gms and 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. It must be noted that at the end of a specific interval, 5 rats per duration were sacrificed by cervical dislocation and immediately followed by the procedures required to obtain the necessary tissues for various molecular and flow cytometric methods.

2.3. Reagents

Dichlorophene (DCP, CAS#97-23-4, 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. Cyclophosphamide and bromophenol blue were procured from Himedia Pvt Ltd, India. Remaining chemicals used were also of molecular grade.

2.4. Treatment

The stock solution of DCP was prepared by dissolving in distilled water. Sub-lethal concentrations were determined by calculating median lethal dose (LD_{50}) by probit analysis (Farah et al., 2004). The LD_{50} of DCP was calculated as 669 mg/kg b.wt. The sub-lethal concentrations ranged from low (10% of $LD_{50;}$ 66.9 mg/kg b.wt.), medium (20% of $LD_{50;}$ 133.8 mg/kg b.wt.) to high (30% of $LD_{50;}$ 200.7 mg/kg b.wt.). All the concentrations administered intraperitoneally having 5 animals per treatment. Bone marrow flushed and other cell types were screened after completion of specified durations. Concurrently positive control (cyclophosphamide; 0.02%) and normal control (water) run simultaneously for comparative analysis of the data.