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Mancozeb-induced genotoxicity and apoptosis in cultured human lymphocytes

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ABSTRACT

Aims: Mancozeb is a dithiocarbamate fungicide known to be genotoxic and induces tumors in rodents at various sites. There is no report in the literature about its genotoxicity in humans. Here, we investigated the association between mancozeb exposure and induction of genotoxic and proapoptotic changes in cultured human lymphocytes (CHLs).
Main methods: Lymphocytes were isolated from peripheral blood of healthy non-smoking donors. Induction of micronuclei and chromosomal aberrations was recorded both by conventional and flow cytometric methods. Annexin-V FITC was used for the differentiation of apoptotic and necrotic cells by flow cytometry.
Key findings: Mancozeb exposure (0.5, 2 and 5 μg/ml) to CHLs leads to significant induction in the frequency of chromosomal aberrations (CAs) and micronuclei (MN), in a dose-dependent manner. Concomitantly, pro-oxidant potential of mancozeb was also recorded, by increase in the levels of reactive oxygen species (ROS) generation. Our results demonstrated that ROS plays a critical role in the initiation of mancozeb induced apoptosis in CHLs through two ways, primarily through mitochondria-mediated pathway including induction of ROS, decrease in mitochondrial membrane potential ($\Delta\Psi m$), along with cytochrome c release from mitochondria, and activation of the caspase cascade. The other pathway includes increase in ROS, which resulted in activation of NF-κB, expression of FasL and triggered FasL-dependent pathway, which also involves caspase-8. Therefore, exposure to mancozeb can lead to induction of apoptosis in CHLs through both mechanisms.
Significance: The results of study confirm that mancozeb exposure can induce genotoxicity and apoptosis in CHLs, thus pose a potential risk to exposed human population.

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Introduction

Pesticides are used for improving the quality and quantity of flora and fauna without causing much damage to non-target species. Humans are often exposed to pesticides through persistent bio-accumulative residues in the environment apart from occupationally exposed workers, especially farmers and those who are involved in their manufacturing process. Often, their prolonged exposure increases the risk of adverse health effects, including genotoxicity and cancer (Zahm and Blair, 1993). Mancozeb ($C_4H_6MnN_2S_4$)a(Zn)y is a broad spectrum fungicide of ethylene bis-dithiocarbamate (EBDC) family. Mancozeb is a combination of Zinc and maneb which has apparently been shown to cause chronic skin diseases in occupationally exposed workers (Reigart and Roberts, 1999). However, mancozeb has been reported as a multipotent carcinogenic agent in rodents capable of producing tumors of various organs (Belpoggi et al., 2002; Mehrotra et al., 1987). The toxicity of mancozeb and other metal–EBDC pesticides has been attributed to mainly ethylene thiourea (ETU), the major metabolite of mancozeb. ETU is known to have carcinogenic, teratogenic and goitrogenic effects in rodents (WHO, 1988).

Genotoxic effects of mancozeb are well reported in rodents, but in humans no clear information is available about its genotoxicity and its underlying mechanism of action in human. Although, Georgian et al. (1983) and Perocco and Santucci (1989) have shown its chromosomal aberration inducing potential in human cells, but these studies are not enough to classify manozeb as a genotoxic agent. Mancozeb exposure can induce genotoxicity and apoptosis in mouse peripheral blood mononuclear cells (PBMC) through reactive oxygen species (ROS) generation (Calviello et al., 2006). ROS plays an important role in the activation of NF-kB, known to enhance apoptosis via FasL-dependent pathway (Shi et al., 2009). Steenland et al. (1997) reported that mancozeb exposure may increase the risk of thyroid cancer in humans. However, Nordby et al. (2005) claim that mancozeb exposure does not cause thyroid cancer in humans.

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Considering the widespread use of mancozeb, and lack of conclusive information about its genotoxic and carcinogenic risk, the present study was planned and executed using CHLs. The aim of the present study is to explore the genotoxic and apoptosis inducing potential of mancozeb along with underneath molecular events using CHLs.

Materials and methods

Chemicals

Mancozeb, Histopaque-1077, RPMI-1640, 2′,7′-dichlorodihydrofluorescein diacetate dye (DCFH-DA), Rhodamine 123, Propidium iodide (PI), 3[4-dimethylthiazol-2-yl]-2-5-diphenyl tetrazolium bromide (MTT), and Giemsa, were purchased from Sigma Chemical Company (St Louis, CA, USA). Annexin V/PI kit was procured from Becton Dickinson (San Jose, CA, USA) and 4′-6-Diamidino-2-phenylinodole (DAPI) was obtained from Sigma (St. Louis, USA). All antibodies were procured from Cell Signaling Technology (Beverly, USA) and PVDF membrane was purchased from Millipore. The rest of the chemicals were of analytical grade of purity and were procured locally.

Lymphocyte culture

Lymphocytes were isolated from peripheral blood of healthy non-smoking donors. The isolation of lymphocytes was performed as described by Georgieva and Stefanov (1987), with minor modifications. Briefly, 2 ml of RPMI-1640 was added to the 2 ml of blood, layered over Histopaque (3:1 ml), and centrifuged at 300 *g* for 20 min. The media/Histopaque interphase containing lymphocytes was taken out and added to 5 ml RPMI-1640 medium. The suspension was then centrifuged at 300 *g* for 10 min to get the pellet, which was resuspended in RPMI-1640 supplemented with 20% FBS, PHA (0.1 ml) and streptomycin (100 μg/ml) and incubated at 37 °C for a period of 72 h depending upon the experimental conditions at a concentration of 1×10^6 cells/ml.

Mancozeb exposure to CHLs

Mancozeb solutions were prepared immediately before use in DMSO and appropriately diluted in RPMI-1640 complete medium. The final DMSO concentration never exceeded 0.5% (v/v). Cells used as controls were incubated with the vehicle (DMSO) only. CHLs were exposed to mancozeb at appropriate doses.

Chromosome aberration assay

Chromosome aberrations were scored as described by Preston et al. (1987). 1×10^6 of cells were poured to 3.5 ml of RPMI-1640 supplemented with 20% FBS. PHA (0.1 ml) and streptomycin (100 μg/ml) incubated at 37 °C for a period of 72 h. After 48 h of culture initiation, lymphocytes were exposed with mancozeb (0.5, 2.0 and 5 μg/ml) for 24 h. Benzopyrene B(a)P, a carcinogenic polycyclic aromatic hydrocarbon known to induce mutations at dose of 5 μg/ml, was used as a positive control. The cells were treated with colchicines (8 μg/ml) for 2 h before harvesting the cells. The cultures were processed and slides were prepared. All the slides, including mancozeb treated, negative and positive controls were independently coded prior to microscopic analysis. The analysis of chromosome aberrations was performed on 100 metaphase cells per treatment in triplicate. Structural chromosome and chromatid type aberrations were categorized as breaks, rings, fragments and chromatid exchange. In addition, the percent aberration was calculated using the formula: Percent aberration = (Total number of aberrations/Total number of metaphase cells) × 100.

Micronucleus induction assay

The micronuclei induction assay was performed as described by Fenech and Morley (1985). After 48 h of culture initiation, lymphocytes were exposed with mancozeb (0.5, 2.0 and 5 μg/ml) for 24 h. A minimum of 1000 binucleate cells with well-preserved cytoplasm were scored randomly from each sample at a magnification 100×. Micronucleated binucleate cells were identified according to the criteria of Fenech (2003).

Flow cytometric analysis of micronuclei

The procedure was used to prepare the cell suspension for analysis by the flow cytometer as described earlier by Nusse et al. (1994). After 48 h, cultured lymphocytes were exposed with different concentrations of mancozeb (0.5, 2.0 and 5 μg/ml) for 24 h. Further, cell suspension was centrifuged for 5 min at 300 *g* and supernatant was removed, the cell pellet was suspended in solution I (10 mM NaCl, 3.4 mM sodium citrate, 25 μg/ml PI, 0.01 mg RNase from bovine pancreas, and 0.3 μl/ml triton-X). After 1 h at room temperature, an equal volume of solution II was added (78.1 mM citric acid, 40 μg/ml PI, and 0.25 M sucrose). After 15 min, the suspension was filtered through a 53-mm nylon mesh and stored on ice until analyzed on Flow cytometer (Becton-Dickinson LSR II, San Jose, CA, USA) using 'Cell Quest' 3.3 analysis software.

Cell cycle analysis

The cells were cultured at a density of 1×10^6 cells and then exposed with mancozeb (0.5, 2 and 5 μg/ml) for 24 h. Cells were washed twice with cold PBS and centrifuged. The cell pellet was resuspended in 50 μl cold PBS and fixed in 2 ml of 70% ice-cold ethanol. Cells were centrifuged and treated with 0.1% Triton X-100 for 5 min. After incubation, cells were centrifuged and resuspended in 1 ml of PBS. Ribonuclease (100 μg/ml) was then added and the cells were incubated at 37 °C for 30 min. After further centrifugation, cells were resuspended in 1 ml of PBS containing 50 μg/ml PI and incubated for 30 min at 4 °C. The samples were analyzed (excluding debris) by flow-cytometer.

Apoptosis induction assay

Apoptosis was measured using flow cytometry to quantify the levels of detectable phosphatidylserine on the outer membranes of apoptotic cells (Evens et al., 2004). Annexin-V FITC detection Kit was used for the differentiation of apoptotic and necrotic cells. Briefly, 5×10^5 cells were cultured and exposed with different concentrations of mancozeb (0.5, 2 and 5 μg/ml) for 24 h. Annexin-V/PI fluorescence was analyzed for each sample; fluorescence of 10,000 cells was gated and counted using Cell Quest 3.3 software.

TUNEL assay

We used Apo-BrdU terminal deoxynucleotide transferase dUTP nick-end labeling (TUNEL) kit (Molecular Probes, Eugene) as per manufacturer's instruction to measure the extent of apoptosis in macozeb exposed CHLs as DNA breaks expose a large number of 3-hydroxyl ends. Addition of BrdUTP to the TdT reaction serves to label these nicks. Once incorporated into the DNA, BrdU can be detected by FITC (fluorescein isothiocyanate) conjugated anti-BrdU antibody and PI to simultaneously provide apoptosis and cell cycle quantification using flow cytometry. The cell suspensions were washed with phosphate buffer saline (PBS) and the cells (1×10^6) were fixed over night in 70% ethanol. Then cells were washed and labeled with BrdUTP over night, washed again with PBS and incubated with anti-BrdU-FITC antibody followed by counterstaining with PI. Cells were acquired and analyzed on flow cytometer using Cell Quest software 3.3.

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