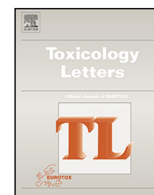
Toxicology Letters 249 (2016) 1–4

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet



Mancozeb, a fungicide routinely used in agriculture, worsens nonalcoholic fatty liver disease in the human HepG2 cell model

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HIGHLIGHTS

- In the present study, we found that the hepatic toxicity of Mancozeb, exacerbated fatty acid-induced steatosis.
- We found a strong reduction of cell viability, to below 50%, for mancozeb concentrations ranging 1–100 ppm. Indeed, 48 h of exposure to 100 ppm caused total cell death, as assessed by an MTT test.
- We found that the fungicide increased the amount of intracellular lipid droplets, with respect to fatty acid treatment alone, from 0.1 ppm to 100 ppm.
- The fungicide has an ability to modify the function of different enzymes, such LDH and cytochrome c, leading to cell death.

ARTICLE INFO

Article history:
Received 4 February 2016
Received in revised form 2 March 2016
Accepted 21 March 2016
Available online 22 March 2016

Keywords:
Mancozeb
Fungicide hepatoxicity
NAFLD
HepG2

ABSTRACT

Mancozeb, a manganese/zinc ethylene-bis-dithiocarbamate, is a fungicide routinely used in pest control programs. However, it has been found to have deleterious effects on human health and on the environment. Indeed, its massive use has raised the issue of possible health risks for agrarian communities; the molecule can also reach human cells via the food chain and alter metabolism, endocrine activity and cell survival. In particular, mancozeb induces many toxic effects on hepatic cell metabolism. For this reason, we investigated its effect in an *in vitro* model of hepatic damage, namely fatty acid-induced nonalcoholic fatty liver disease in the HepG2 cell line. We found that the hepatic toxicity of the fungicide exacerbated fatty acid-induced steatosis, as manifested by an increase in intracellular lipid droplet accumulation. Furthermore, mancozeb altered cell metabolism and induced cell death through upregulation of lactate dehydrogenase and cytochrome c, respectively, in dose-dependent manners. Therefore, mancozeb may play an important role in the pathogenesis and progression of chronic disease in humans and represents a danger for human health in high doses
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1. Introduction

Despite their popularity and extensive use, fungicides are now raising serious concerns about the health risks to farmers when applying them or working in treated fields, and to the general population, who can be exposed to residues present in food and drinking water (Damalas and Eleftherohorinos, 2011). Mancozeb is a non-systemic agricultural fungicide that controls many fungal diseases in a wide range of field crops, fruits, nuts, vegetables, and ornamentals, so is widely used in agriculture (Lamberti et al., 2014; Maroni et al., 2000). It is a manganese/zinc ethylene-bis-dithiocarbamate fungicide (Fig. 1) that reacts with, and inactivates, the sulfhydryl groups of amino acids and enzymes in fungal cells. This results in disruptions in lipid metabolism, respiration, and production of adenosine triphosphate (Afsar and Demirata, 1987). In larvae, mancozeb extends development time, alters fecundity, and increases time of pupation and pupae mortality (Adamski

Abbreviations: NAFLD, nonalcoholic fatty liver disease; HepG2, hepatocarcinoma cell line; LDH, intracellular lactate dehydrogenase; MTT, tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FA, fatty acids (oleic 18.

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http://dx.doi.org/10.1016/j.toxlet.2016.03.004
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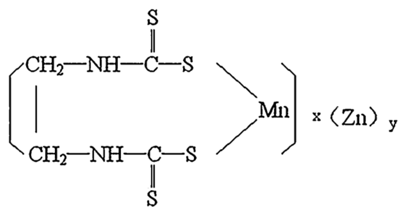

Fig. 1. Chemical structure of mancozeb.

et al., 2011): indeed, larval ultrastructure undergoes a series of malformations, such as invagination of the nuclear envelope, swelling of nuclei and endoplasmic reticulum, decreases in cytoplasmic glycogen content, and increased translucency of the cytoplasm.

Although mancozeb has been reported to have low acute toxicity with an oral LD50 value of 8 g/kg/day in Wistar rats (Edwards et al., 1997), studies have shown that it induces apoptosis in embryos, has immunomodulatory effects, induces tumors of the skin and toxic epidermal necrolysis, affects oocyte meiotic spindle morphology, and impairs fertilization rate, even when used at very low concentrations (Dennis et al., 2010; Ghisari et al., 2015; Goldoni et al., 2014; Goldner et al., 2010).

Moreover, mancozeb is metabolized into ethylenethiourea, which is known to inhibit thyroid peroxidase in rats and primates, to induce malformations of the neural tube in rats and frogs, and to cause thyroid cancers in rats and mice (Axelstad et al., 2011). A relationship has been reported between intake of mancozeb-treated vegetables and deleterious effects on the liver of rats (Adjrah et al., 2013), and thus may contribute to the development of liver disease. Also, when administered daily to mice for 30 days, it significantly decreased liver weight and the quantity of protein (Ksheerasagar and Kaliwal, 2003).

These findings suggesting a toxicity to liver prompted us to evaluate the effect of mancozeb on a hepatocarcinoma (HepG2)-based model of nonalcoholic fatty liver disease (NAFLD).

2. Materials and methods

2.1. Cell culture and treatments

The hepatoma HepG2 cell line was obtained from American Type Culture Collection (ATCC HB 8065). All cell culture materials were purchased from GIBCO/Invitrogen (Milan Italy), unless otherwise stated. HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/ml streptomycin, and 100 μg/ml antimycotic. The cells were grown on tissue culture plates (BD Bioscience-Falcon, San Jose, USA) in an incubator with a humidified atmosphere (95% air/5% CO_2 v/v) at 37 °C for 48 h until 80% confluence, then washed, and exposed for 24–48 hrs to mancozeb at different concentrations (0.1–500 ppm in serum-free DMEM). Before treatment, mancozeb was solubilized in 100% DMSO, then diluted in serum-free medium to reach 0.5% DMSO. In vitro steatosis was induced by incubating the hepatocytes with 6 mM of a 1:1 v/v mixture of oleic (18:1) and linoleic acid (18:2) (Sigma Aldrich, Milan, Italy).

2.2. MTT assay

HepG2 cells (1.0 × 105), cultured in 24-well plates in DMEM, were exposed to mancozeb at 0.1, 1, 10, 100, or 500 ppm for 24 h or at 1, 10, or 100 ppm for 48 h. Cell viability was assessed by measuring the reduction of the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-5-(3 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT; Sigma Aldrich). Mitochondrial dehydrogenates of living cells reduce the tetrazolium ring, yielding a blue formazan product that can be measured spectrophotometrically. The optical densities obtained are directly proportional to the number of living cells. The cytotoxic effect of a sample is evaluated by the percentage of living cells present in the sample, in relation to the cells treated only with the solutions. After treatment, the medium was replaced by a solution of 1 mg MTT/ml in DMEM medium without phenol red. After 3 h of incubation, the liquid was aspirated and the insoluble formazan produced dissolved in 0.1 M HCl in isopropanol. The optical densities of the obtained solutions were measured at 570 nm using a Beckman DU 640 spectrometer (Beckman, Milan, Italy).

2.3. Oil red O staining

For the evaluation of steatosis, 1.0 × 105HepG2 cells were seeded on 24-well plates and treated for 24 h with fatty acids. After treatment, the cells were exposed for 24 h to mancozeb. The cells were then rinsed with cold phosphate buffered saline (PBS) and fixed in 4% v/v paraformaldehyde for 30 min, and stained with Oil Red O (0.5% v/v). Images of cells were captured using an optic microscope, and stained lipid droplets were extracted with isopropanol (60% v/v) for quantification by measuring absorbance at 510 nm.

2.4. Western blotting

Proteins were extracted using RIPA lysis buffer (R0278, Sigma Aldrich), and concentrations determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Milan Italy). Whole cell extracts and equal amounts of protein (30 μg) were fractionated by SDS-PAGE and transferred to a nitrocellulose membrane using a specific apparatus according to the manufacturer's protocols (Bio-Rad Laboratories, Milan Italy). After incubation with 5% w/v non-fat milk in Tris-buffered saline (10 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 60 min, the membrane was washed once with tween-20 in Tris-buffered saline (TBST) and incubated with antibodies (purchased from Santa Cruz Biotechnology, CA, USA) against lactate dehydrogenase (LDH: H-160, rabbit polyclonal IgG) (1:1000), cytochrome c (H-1049, rabbit polyclonal IgG) (1:500), or actin (sc-1616, goat polyclonal IgG I–19) (1:1000) at room temperature for 2 h. Membranes were washed three times for 10 min and incubated with a 1:10000 dilution of horseradish peroxidase-conjugated anti-rabbit, anti-rabbit and anti-goat antibodies respectively at room temperature for 1 h. Blots were washed with TBST three times and developed with the ECL system (Amersham Biosciences, UK) according to the manufacturer's protocol.

All experiments were done in triplicate.

3. Results

3.1. HepG2 cell viability

Cell viability was evaluated with an MTT test after 24 h and 48 h of exposure to mancozeb at different concentrations (Fig. 2). The fungicide was highly cytotoxic at all concentrations. Indeed, a strong reduction in cell viability (to below 50%) was observed at 24 h, with a 48-hour exposure to 100 ppm causing total cell death.

3.2. Intracellular lipid accumulation

To evaluate in the effect of mancozeb on liver steatosis, we assessed intracellular lipid accumulation with Oil Red O staining in HepG2 cells exposed to fatty acids (Fig. 3). We found that the

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