JOURNAL OF ENVIRONMENTAL SCIENCE AND HEALTH, PART B
https://doi.org/10.1080/03601234.2021.1972721

Evaluation of the cytotoxic and genotoxic potential of the captan-based fungicides, chlorothalonil-based fungicides and methyl thiophanate-based fungicides in human fibroblasts BJ

Andy J. Moo-Muñoz[a], Erika P. Azorín-Vega[b], Ninfa Ramírez-Durán[a], and Pablo Antonio Moreno-Pérez[a]

[a]Laboratory of Medical and Environmental Microbiology University, Autonomous of the State of Mexico, Paseo Tollocan, State of Mexico; [b]National Radiopharmaceutical Research and Development Laboratory, National Institute for Nuclear Research, La Marquesa-Ocoyoacac, State of Mexico

ABSTRACT

The objectives of this study were to examine cytotoxic and genotoxic damage in human BJ fibroblasts caused by three pesticides used worldwide by trypan blue dye exclusion assays and to measure the relative level of phosphorylated histone H2A.X by flow cytometry at different concentrations. Captan-based fungicide and methyl thiophanate-based fungicide (100 and 1000 μM) showed immediate cytotoxic effects; furthermore, after 24 h, captan-based fungicide, chlorothalonil-based fungicide and methyl thiophanate-based fungicide caused cytotoxic effects in the concentration ranges of 40–100 μM, 30–100 μM and 150–1000 μM, respectively. All fungicides generated DNA damage in the treated cells by activating ATM and H2A.X sensor proteins. The three fungicides tested generated DNA double-stranded breaks and showed cytotoxicity at concentrations 33, 34, and 5 times lower (captan, chlorothalonil and thiophanate-methyl respectively) than those used in the field, as recommended by the manufacturers.

ARTICLE HISTORY
Received 30 October 2020
Accepted 17 August 2021

KEYWORDS
Genotoxicity; cytotoxicity; fungicide; viability; phosphorylation

Introduction

Pesticides are important for food production as they maintain or increase agricultural production yields to feed the rising world population.[1] Among these pesticides are fungicides, such as captan-based fungicides [N-(trichloromethylthio)-cyclohex-4-ene-1,2-dicarboximide], chlorothalonil-based fungicides (2,4,5,6-tetrachloroisophthalonitrile), and methyl thiophanate-based fungicides [1, 2-bis-(3-methoxy carboxy-2-thioureidobenzene)], which are widely used in the pre- and postharvest of crops to control of phytopathogenic fungi, greenhouses, foliar treatment, soils, seeds, cereals, fruits, vegetables, and ornamentals.[2–4] These fungicides are used in Germany, Australia, Austria, Belgium, Spain, the United States, France, Holland, Mexico and Portugal, among others countries.[5]

Recently, it was reported that 530,000 tons of fungicides and bactericides were used for agricultural applications worldwide,[6] but there is insufficient information on their toxicity since manufacturers do not provide it. The World Health Organization estimated 3 million cases of acute poisoning, including suicides, and more than 2 million deaths caused by pesticides.[7] These figures have led to the study of the effects of pesticides in biological models such as their effects on the development of zebrafish (*Danio rerio*),[8] physiological metabolism and metabolic disturbances in Sprague Dawley rats,[9,10] and genotoxicity in blood cells and hepatoma (HepG2) cells and toxicity in *Daphnia magna*.[11,12]

Cell toxicity assays are widely used to detect a variety of agents that show direct cytotoxic effects.[13] The main kinase activated in response to DNA double-strand breaks is ataxia telangiectasia mutated protein kinase (ATM). The ATM protein exists as a dimer, but it is rapidly dissociated and phosphorylated at serine 1981 in response to damage from ionizing radiation. Once activated, ATM phosphorylates several factors, including histone H2A.X. Phosphorylation of the variant histone H2A.X at serine 139 (phospho-H2A.X) by ATM is an important indicator of DNA damage. As the level of DNA damage increases, the degree of phosphorylation of histone H2A.X increases and accumulates at sites where a DNA double-strand break has occurred, which is often used as an indicator of the level of DNA damage present in the cell.[14]

This work evaluated cytotoxic and genotoxic damage in human BJ fibroblasts exposed to three pesticides used worldwide by trypan blue dye exclusion assays and measured the relative level of phosphorylated histone H2A.X by flow cytometry.

Materials and methods

Chemical fungicides evaluated

CAPTAN ULTRA® 50 WP (captan-based fungicide) as a wettable powder (with 500 g a.i./kg) was purchased from

CONTACT Pablo Antonio Moreno-Pérez saieto@hotmail.com

Arysta LifeScence México, SA de CV Saltillo, Coahuila de Zaragoza, México. Coraza® 720 S (chlorothalonil-based fungicide) in aqueous suspension (with 720 g of a.i./L) was purchased from Promotora Técnica Industrial, S.A. de C.V., Morelos, Mexico. Cercobin®-M (methyl thiophanate-based fungicide) as a wettable powder (with 700 g of a.i./kg) was purchase from BASF Mexicana, S.A. de C.V., D.F. Mexico.

Fungicide concentrations used

Preliminary tests were conducted to determine the lethal concentration (LC_{50}) of each fungicide to be evaluated in human fibroblasts. The concentrations varied between 0.5 and 1000 µM based on their active ingredient. The final concentrations of the cytotoxicity tests were 5, 10, 20, 30, 40.50, 60, 80 and 100 μM for captan-based fungicide and chlorothalonil-based fungicides 100, 150, 200, 250, 300, 400, 500, 800 and 1000 μM for methyl thiophanate-based fungicide. All concentrations were diluted in phosphate buffer saline (PBS).

Cell culture

Human skin fibroblasts (BJ) ATCC™ CRL-2522 were kindly donated by The National Institute for Nuclear Research, Mexico. Cells were cultured in RPMI-1640 medium (Gibco Invitrogen Co, USA) supplemented with 10% fetal bovine serum (FBS, Gibco Invitrogen, USA), 3.7 g/L sodium bicarbonate, 2 mM glutamine (Sigma Aldrich, USA), penicillin (100 U/mL) and streptomycin (100 mg/mL) (Gibco Invitrogen) at 37 °C in an incubator containing 5% CO_2 with 85% humidity. After reaching a confluence of approximately 80%, cells were harvested using trypsin (0.25% in PBS; GIBCO Invitrogen).[15]

Cell viability assay

The viability of BJ human fibroblasts was examined with the trypan blue dye-exclusion test. Approximately 3.5×10^5 cells were resuspended in simple RPMI medium (free of FBS) in 1.5 mL microtubes (EF0030108116, Eppendorf™, Germany), and fungicides were added to a final volume of 500 µL. Subsequently, cells were incubated for 1 and 24 h at 37 °C in an incubator containing 5% CO_2 with 85% humidity. A 10 μl aliquot of the cell suspension was combined with 10 µl of trypan blue, followed by incubation for 5 min at room temperature. Live and dead cells were counted using a hemocytometer and an inverted microscope (AE2000, Motic™, Hong Kong).[7] Cells suspended in RPMI medium in the absence of fungicides served as the negative control. Values from three independent experiments were averaged, and results were expressed as percentages.

Measurement of phosphorylated H2A.X

DNA damage was assessed by flow cytometry. Approximately 3.5×10^5 human BJ fibroblasts resuspended in SBF-free RPMI medium were treated for 24 h with fungicides at different concentrations as follows: captan-based fungicide (46 and 100 µM), chlorothalonil-based fungicide (56 and 100 µM) and methyl thiophanate-based fungicide (300 and 570 µM) with a final volume of 500 µL at 37 °C in an incubator containing 5% CO_2 with 85% humidity. A cell suspension, which was exposed to 10 Gy using the Gamma-Cell 220 system (60Co) (Atomic Energy, Canada), served as the positive control. Cells resuspended in FBS in the absence of fungicides served as the negative control. Subsequently, cells were washed with PBS and centrifuged at 3000 rpm for 5 min. The measurement of the active form of ATM and H2A.X was performed using the Muse™ Cell Analyzer and the Muse™ Multi-Color DNA Damage Kit (MCH200107, Luminex, USA), which contained an antibody that specifically recognized ATM phosphorylated at serine 1981 (anti-(Ser1981)-ATM) and a conjugated antibody that specifically detected phosphorylated histone H2A.X (H2A.X-PECy5, MerckMillipore®, USA).[16] Results were calculated using Muse™ Multi-Color DNA Damage software.

Statistical analysis

Statistical analysis was performed using SPSS 25.0 software (IBM, USA). Results were expressed as mean ± standard

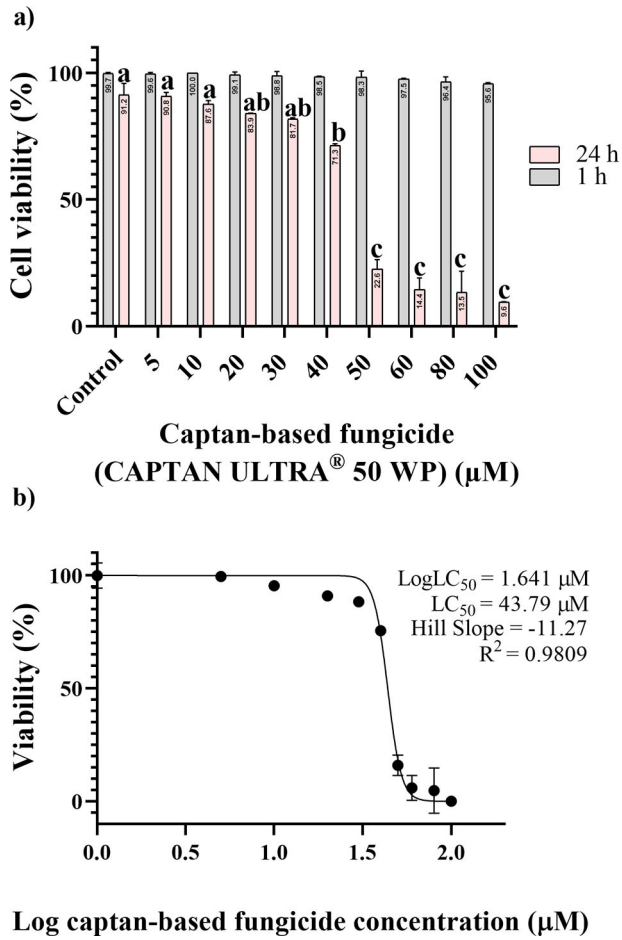

Figure 1. Cytotoxic effects of captan-based fungicide in BJ human fibroblasts (ATCC® CRL-2522™). a) Percentage (%) of viable cells after exposure for 1 and 24 h at different concentrations compared with the control (without treatment). Different letters (a, b, c) represent a significant difference between treatments based on one-way ANOVA and Tukey's post hoc test ($p < 0.05$). b) Dose-response curve. The percentage of cell viability was associated with an increase in the logarithmic concentration of the fungicide after 24 h of exposure.

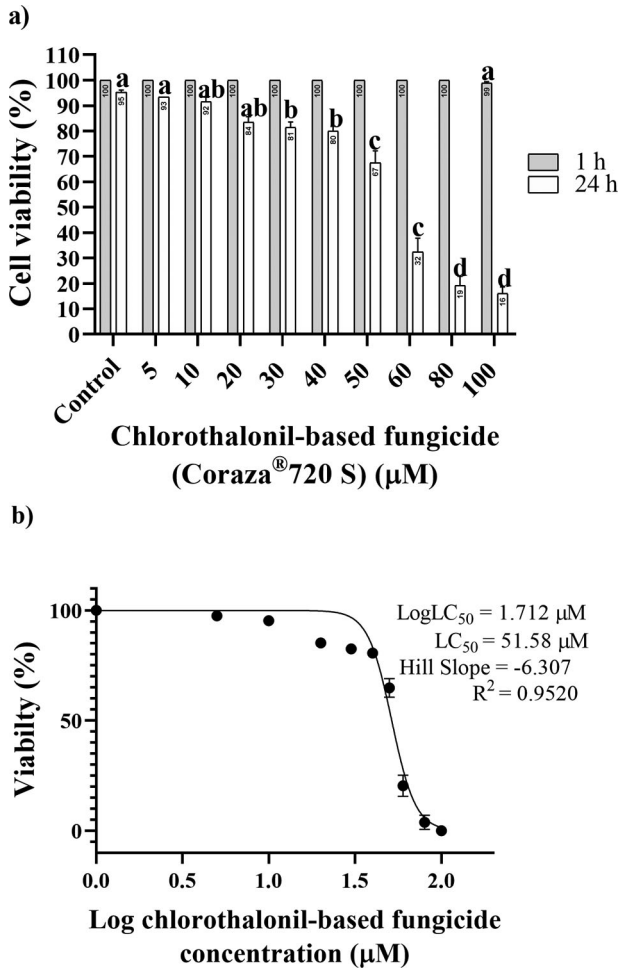

Figure 2. Cytotoxic effect of chlorothalonil-based fungicide in BJ human fibroblasts (ATCC® CRL-2522™). (a) Percentage (%) of viable cells after exposure for 1 and 24 h at different concentrations compared with the control (without treatment). Different letters (a, b, c, d) represent a significant difference between treatments based on one-way ANOVA and Tukey's post hoc test ($p < 0.05$). (b) Dose-response curve. The percentage of cell viability was associated with an increase in the logarithmic concentration of the fungicide after 24 h of exposure.

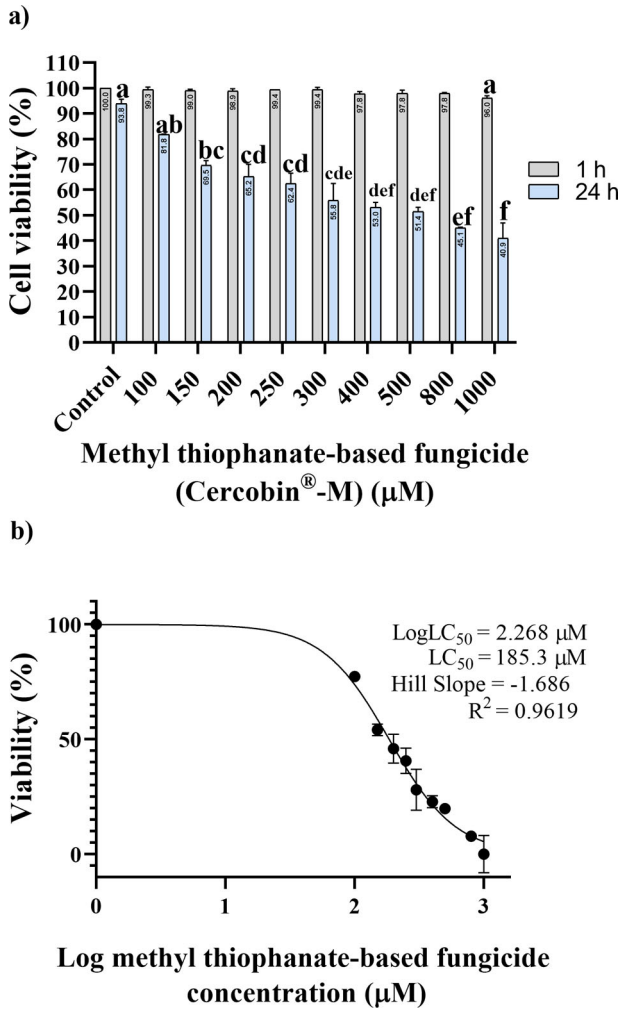

Figure 3. Cytotoxic effect of methyl thiophanate-based fungicide in BJ human fibroblasts (ATCC® CRL-2522™). a) Percentage (%) of viable cells after exposure for 1 and 24 h at different concentrations compared with the control (without treatment). Different letters (a, ab, bc …) represent a significant difference between treatments based on one-way ANOVA and Tukey's post hoc test ($p < 0.05$). (b) Dose-response curve. The percentage of cell viability was associated with an increase in the logarithmic concentration of the fungicide after 24 h of exposure.

error of the mean (SEM) from at least three independent experiments. Statistical significance was assessed by one-way ANOVA, followed by Tukey's post-hoc test in cases of significance (set at $p < 0.05$). Normalized data fit a sigmoidal dose-response curve, and LC_{50} values were calculated by nonlinear regression using Prism 8 software (GraphPad, USA).

Results

Cytotoxic action of fungicides

After 1 h of exposure, captan-based fungicide did not show a statistically significant difference ($p < 0.05$) in the viability of BJ human fibroblasts compared with the control (Fig. 1a). However, after 24 h of exposure to treatment, a significant reduction ($p < 0.05$) in cell viability (40–100 µM) of less than 50% was observedd. The LC_{50} was 43.79 μM (Fig. 1b).

In the case of the chlorothalonil-based fungicide (100 µM), this sample showed a statistically significant difference ($p < 0.05$) in the cell viability of BJ human fibroblasts after 1 h of exposure compared with the control. After 24 h of exposure, a significant reduction ($p < 0.05$) in cell viability was observed with lower concentrations (30–100 µM) (Fig. 2a). The dose-response curve showed decreased cell viability associated with the increased fungicide concentration. The IC_{50} for chlorothalonil-based fungicide was 51.58 μM (Fig. 2b).

Methyl thiophanate-based fungicide showed a statistically significant difference from the control ($p < 0.05$) in the cellular viability of BJ human fibroblasts after 1 h of exposure (1000 μM) (Fig. 3a). After 24 h of exposure, a significant reduction ($p < 0.05$) in cell viability was observed, indicating that methyl thiophanate-based fungicide possesses cytotoxic activity at all studied doses (150–1000 μM). The LC_{50} was 185.3 µM (Fig. 3b).

Genotoxicity by flow cytometry

Dispersion plots of the phosphorylation of ATM kinases and H2A.X by fungicides allowed the quantification of the

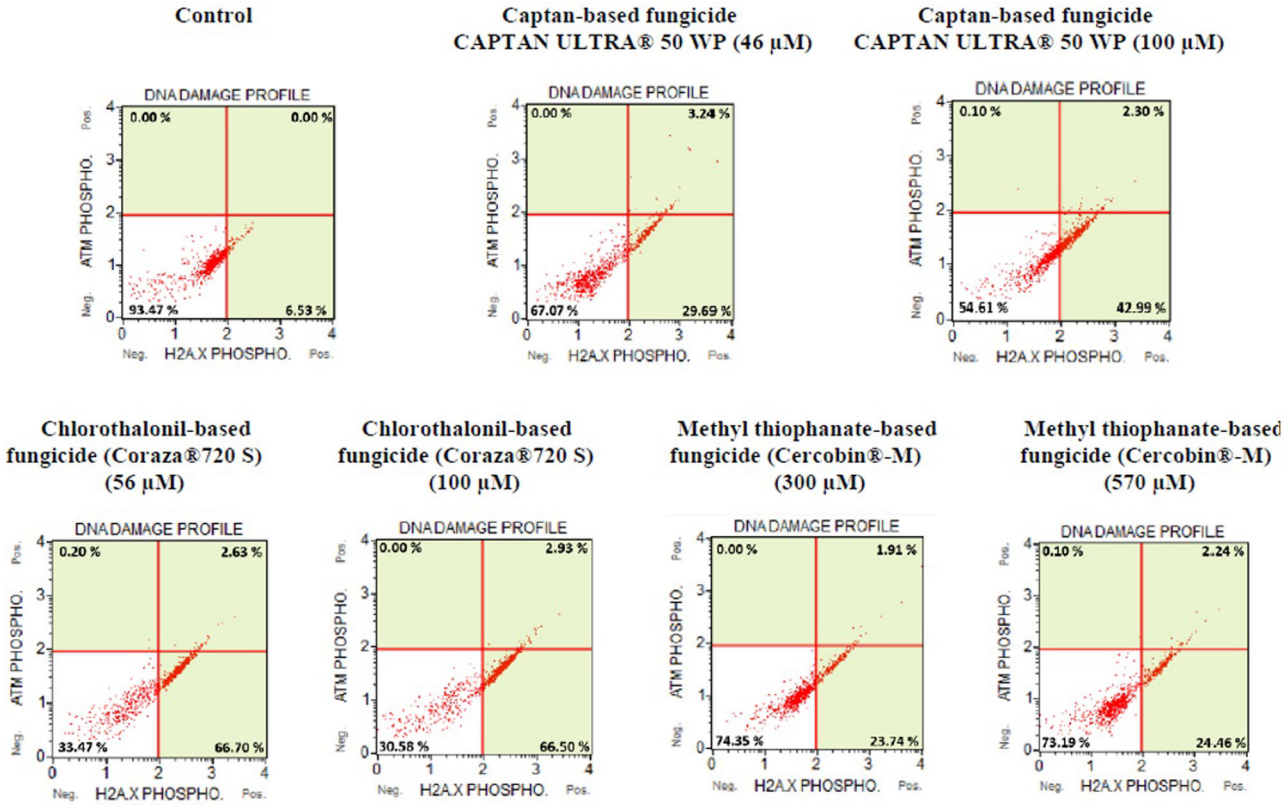

Figure 4. Phosphorylation of H2A.X quantified by cellular analysis of cultures exposed to three fungicides. Dispersion plots show the phosphorylation of DNA damage sensors (ATM and H2A.X) in BJ human fibroblasts exposed to captan-based fungicide (46 and 100 µM), chlorothalonil-based fungicide (56 and 100 µM) and methyl thiophanate-based fungicide (300 and 570 µM) for 24 h. The redistribution of events toward the upper left of the quadrants suggests an increase in ATM activation, while H2A.X activation is reflected in the redistribution of events toward the upper part of the dot diagram indicating the severity of the damage. The quantification of the results is shown in the plots.

genotoxic damage after 24 h at different fungicide concentrations. DNA damage caused by these fungicides is notorious, as the level phospho-H2A.X increased after treatment and its relationship with the applied dose (Fig. 4). The phospho-H2A.X levels of captan-based fungicides were 29.6% for the 46 µM concentration and 42.9% for 100 µM. Conversely, the levels for the chlorothalonil-based fungicides were 63.7 and 66.5% for 56 µM and 100 µM concentrations, respectively; and the latter concentration associated with the highest DNA damage (2.93%). Finally, for the methyl thiophanate-based fungicide, the phospo-H2A.X activation percentages were 23.7 and 24.4% for treatments with concentrations of 300 and 570 µM, respectively; and with a percentage of DNA double-strand breaks of 2.24%.

Data obtained from the percentage of DNA double-strand breaks after 24 h of treatment with captan-based fungicide, chlorothalonil-based fungicide and methyl thiophanate-based fungicide showed statistically significant differences ($p < 0.05$), with methyl thiophanate-based fungicide generating the most damage (>12% at a concentration of 570 µM) (Fig. 5).

Discussion

Captan-based fungicide belongs to the chemical group of phthalimides. It is a multisite fungicide, which indicates that these molecules affect a series of different fungal structures and metabolic pathways.[17] Captan-based fungicide Captan has adverse effects on *Danio rerio* embryos by decreasing the heart rate, inhibiting growth, and causing teratogenic damage.[2] It also has genotoxic effects in mammalian cells,[18] causing biological alterations such as hematotoxicity, hepatotoxicity, and immunological alterations in rats.[19] The treatments of *Saccharomyces cerevisiae* with captan-based fungicide (20 µM) for 6 h reduced their viability by 90% as there was membrane damage and necrosis due to captan's reaction with thiols.[20] At 30 μM, captan caused apoptotic and necrotic cell death, increased intracellular levels of Ca^{2+} and Zn^{2+}, and decreased the concentration of thiolic compounds in cells by increasing cytotoxicity caused by hydrogen peroxide (H_2O_2) increases in rat thymic lymphocytes.[21] It also induced cytotoxicity and peroxidation in the hepatocytes of Wistar rats treated with 25–1000 µM.[22] In contrast, captan at 100 μM not affect the viability of JEG-3 human placental choriocarcinoma cells.[23] The present study found that concentrations of captan-based fungicide, starting at 40 μM generate cytotoxic effects in BJ human fibroblasts, which cause a decrease in cell viability by up to 90% at a concentration of 100 μM. It is worth mentioning that the tested concentration (100 μM, equivalent to 0.06 g/L) of captan-based fungicide was 33 times lower than the lowest dose recommended for use by the manufacturer (2 g/L).

Chlorothalonil is a systemic fungicide belonging to the chemical group of chloronitriles with multisite contact

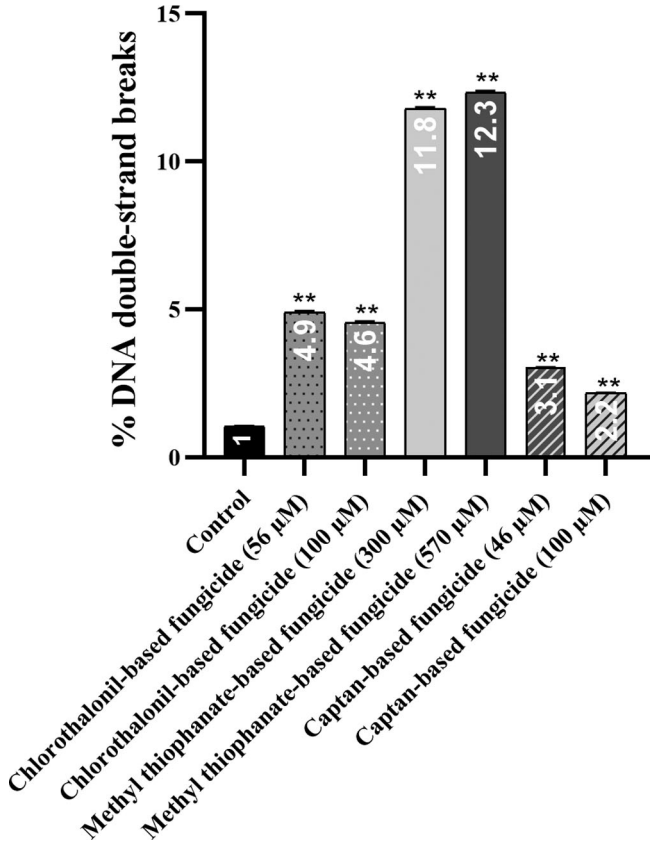

Figure 5. Effect of fungicides on the level of double-strand breaks in DNA in BJ human fibroblasts (ATCC® CRL-2522™) after 24 h of exposure. Cell damage DNA was analyzed by flow cytometry using the Muse® Multi-Color DNA Damage Kit. Values are adjusted based on cell number/µL. Data are presented as the mean ± SEM. Two Asterisk (**) show a significant difference between groups ($P < 005$) versus control.

activity.[17] It can reach different tissues and organs, which may involve the generation of oxidative stress, thereby causing cytotoxicity.[24] The chlorothalonil-based fungicides has been reported to cause advers effects such as dermatitis, eye irritation, gastrointestinal problems, hepatotoxicity, abnormal development and reproduction, and possibly cancer.[3,25–27] This fungicide induces apoptotic cell death[28] and cytotoxicity in *Perna perna* hemocytes after 96 h of exposure by decreasing mitochondrial activity and altering lysosomal integrity.[29] In *Ciona intestinalis* larvae, at concentrations above 100 nM of chlorothalonil can stop their embryonic stage.[30] In rat thymocytes, a concentration of 1 μM of chlorothalonil increases the oxidative stress of cells in the presence of H_2O_2, thereby exerting significant cytotoxicity.[31] This compound generates organic peroxides and metabolites that produce free radicals by reacting with membrane lipids causing cell death and decreasing viability at concentrations of 250–1000 μM.[22] Therefore, the generation of these compounds may indicate the cause of cell death and the decreased viability when using this fungicide at the concentrations applied to BJ fibroblasts in this study. It is noteworthy, the highest dose of chlorothalonil-based fungicide evaluated in this study (100 µM, equivalent to 37 µL/L) was 34 times lower than the lowest dose recommended for use in the field (1.25 mL/L).

The active ingredient thiophanate methyl belongs to the chemical group of thiophanates that interfere with DNA synthesis, mitosis of fungal cells, and polymerization of microtubular proteins.[17] Studies reported that methyl thiophanate-based fungicides induced oxidative stress and genotoxic changes and immunomodulatory effects[32]; histopathological damage in rat blood, liver, and kidneys;[33] production of reactive oxygen species,[34] and DNA damage.[35] Methyl thiophanate affects hematological parameters and increases the production of reactive oxygen species, es well as the oxidation of protein products and malondialdehyde levels in the erythrocytes of rats treated with 300, 500, and 700 mg/kg body weight after 24 h of treatment.[34] Doses of 300 and 500 mg/kg body weight of methyl thiophanate-based fungicide caused hematotoxicity, nephrotoxicity, and hepatotoxicity in rats; this may have been due to increases in H_2O_2 and malondialdehyde levels as well as oxidative proteins in the liver and kidneys.[33] Therefore, this fungicide may have oxidizing effects that reduced cell viability compared with to the other fungicides tested in this study (captan-based fungicide). The highest concentration of methyl thiophanate-based fungicide (1,000 µM) reduced viability up to 20%; this concentration (equivalent to 0.4 g/L) was 5 times lower than the lowest dose (2 g/L) that growers use in the field.

In the cellular response to double-strand breaks in DNA, histone H2A.X is rapidly phosphorylated at serine 139 by ATM kinase near double-strand breaks in DNA.[36] Histone H2A.X is phosphorylated after the activation of ATM, and this phosphorylated isoform (phospho-H2A.X) is a sensor of DNA damage. The foci of phospho-H2A.X in the nuclei are signs of damaged DNA that are subject to repair.[37] Occupational exposure can be significant, the genotoxic effect of fungicides show dose-dependent DNA damage and can have a significant genotoxic effect in in vitro human cell models.[11]

Captan-based fungicide inhibits synthetic DNA processes and induces DNA-protein crosslinking and modifications in DNA bases, which indicates that is has great mutagenic potential.[38] Exposure to 20 µM of captan-based fungicide after 2 h resulted in a dose-dependent increase in the number of phospho-H2A.X-positive cells and suggests that DNA damage is related to replication stress resulting in the formation of double-strand breaks in DNA.[18] In the present investigation, an increase in phospho-H2A.X was observed after 24 h of exposure to 46 µM captan-based fungicide. This confirms that this fungicide can generate double-strand breaks in DNA and is a potential genotoxic agent at the evaluated doses.

A comet assay showed that chlorothalonil-based fungicides, induced DNA damage at doses of 2–40 µM, causing breaks in DNA chains.[39] Simultaneously, chlorothalonil disrupted the histone methylation and DNA methylation associating with the modulation of estrogenic signaling pathways that are capable of disrupting spermatogenesis in rats.[28] In this study, chlorothalonil-based fungicide induced the phosphorylation of histone H2A.X, an indicator of DNA damage after the double strand disruption, caused by

treatment (46 and 100 µM) of this fungicide for 24 h in BJ fibroblasts.

Specific interactions of methyl thiophanate with DNA have been reported as an initiating event of DNA damage caused by this fungicide that triggered further DNA damage, which, when not processed by the cellular DNA repair machinery, has genotoxic and carcinogenic implications.[35] Long-term methyl thiophanate-based fungicide exposure induces genomic damage that is positively correlated with exposure time.[40] The intraperitoneal administration of methyl thiophanate-based fungicide (300, 500, and 700 mg/kg body weight) produced mutagenic effects in leukocytes of rats after 24, 48 and 72 h.[34] Methyl thiophanate-based fungicide also acts as an oxidative stressor, producing reactive oxygen species that lead to mitochondrial dysfunction, oxidative DNA damage, and mutagenesis.[41] Therefore, chronic exposure to this fungicide can trigger genotoxic events in the DNA of exposed organisms. The results showed that methyl thiophanate-based fungicide promotes the phosphorylation of H2A.X in BJ fibroblasts, indicating the presence of double-strand breaks in DNA. ATM activation and increased expression of phospho-H2A.X indicated cellular DNA damage that was promoted by the fungicides in human BJ fibroblasts.

Conclusions

Captan-based fungicide, chlorothalonil-based fungicide and methyl thiophanate-based fungicide showed cytotoxicity and genotoxicity in human BJ fibroblasts by reducing their viability and activating H2A.X-mediated DNA double-strand damage. It also caused cytotoxicity at lower concentrations (33, 34, and 5 times lower, respectively) than those recommended for use by manufacturers. The data obtained in this study endorsed the importance of controlling the use of fungicides, reducing their application, and regulating their environmental exposure as they represented a risk to human health.

Acknowledgments

The authors acknowledge Consejo Nacional de Ciencia y Tecnología (CONACYT) under project number 2018-000068-02NACF-23822 for the scholarship awarded to the main author. This work was conducted as part of the activities of the Laboratorio Nacional de Investigación y Desarrollo de Radiofármacos, Conacyt.

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