

CYTOTOXIC EFFECTS OF TRIAZOLE FUNGUCIDES

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SUMMARY

Fungicides are at high risk exposure compounds both pre and post harvest time due to their usage during storage. Fungicides have various benefits on agricultural struggle. Apart from that, studies showed that fungicides also have mutagenic, carcinogenic, chronic toxic effects on nervous system, immune system, hormonal and reproductive system. Triazole group fungicides, prochloraz, tebuconazole, triadimenole and triadimefone, are commonly used in the world. In the study, it is aimed to emphasize about their importance on human and environmental health. And, we evaluated their potential cytotoxic effect on HeLa cells by using MTT and LDH tests. Before the tests application, the parameters like cell counts, incubation times, pesticide concentrations and test circumstances were examined for test optimization. As a result, for MTT cytotoxicity test IC_{50} values of prochloraz, tebuconazole, triadimefone and triadimenole were recorded in order as 0.4, >1, 0.24 and 0.37 mg/ml. As well for LDH test these values were identified in order as 0.065, 0.985, 0.052 and 0.042 mg/ml. MTT test, used to identify hazards on mitochondrial activity and respiratory chain, showed that cytotoxic effect of prochloraz, triadimefone and triadimenole was dose dependently increased. While tebuconazole showed cytotoxic effect on concentration range studied, it was seen that IC_{50} values for LDH test which is used to identify membrane damage were lower than IC_{50} values compared with MTT test. The obtained results would draw attention to expand researches on toxic effects of triazole fungicides which are identified as cytotoxic for human and environment health. Also, it is believed to take necessary precautions for usage of these compounds.

Key words: Triazole fungicides, MTT test, LDH test, Cytotoxicity

INTRODUCTION

Fungicides are widely used to kill or inhibit fungi or fungal spores that threaten to ruin greenhouse crops through incorporation into the soil and foliar application. On the contrary single fungicide applications, multiple applications over a growing season often result in the fungicide's permanency in agricultural soil. This permanency can perennially affect soil microbial communities and consequently damage soil fertility or health. Therefore, there is an increasing concern on environmental health and eventually on human health (1). Epidemiological researches have stated a causal connection between human exposure to fungicides and mutagenic, carcinogenic, endocrine disrupting, chronic toxic effects on nervous system, immune system (2-4).

Over the last three decades, a broad spectrum of triazoles has been advanced for the treatment of fungal diseases in humans, animals and crops (5). The most used triazole fungicides are prochloraz, tebuconazole, triadimefon and triadimenol. They are used to control field crops, fruit and vegetables, leaves and other plant diseases in Europe, Australia, Asia, South America and Turkey (6, 7). The fungicides disrupt fungal membrane structure by inhibiting ergosterol biosynthesis. Tebuconazole, triadimefon and triadimenol penetrate the plant and disperse in the leaf tissue continuously and uniformly. The fungicides have protecting, curative and eradicating activity in some cases (8-10).

Studies showed that these four commonly used triazole fungicides have mutagenic, clastogenic, aneugenic, neurotoxic, cytotoxic and endocrine disrupting effects (2, 11). Besides, they have possible developmental dopaminergic neurotoxicity and spatial memory effects by inhibiting dopamine intake, regulating retinoic acid metabolism (3, 12, 13).

Cytotoxicity assays are widely used in vitro toxicological studies. The LDH and MTT tests are the most common employed for the detection of cytotoxicity or cell viability following exposure to toxic substances. As cell lines, it is generally preferred diploid human fibroblast lines (e.g. WI-38) and tumour cell lines (e.g. HeLa) in toxicity studies (14). MTT test is depend on the capacity of mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide) into an insoluble coloured formazan product which is measured spectrophotometrically. Reduction of

MTT can only occur in metabolically active cells. Therefore, the level of activity is a measure of the viability of the cells. As to LDH assay; lactate dehydrogenase (LDH) enzyme is a stable cytoplasmic enzyme. When cell lyses and membrane damage occurs, cell culture release into supernatant quickly. LDH reduces nicotinamide adenine dinucleotide (NADH) to NAD^+ by the oxidation of pyruvate to lactate. Decrease in absorbance of NADH consumption is measured by spectrophotometer.

In the study, we evaluated cytotoxic effect potentials of triazole fungicides (prochloraz, tebuconazole, triadimefon and triadimenol) by using MTT and LDH tests on HeLa cells. We aimed to determine cytotoxic activities of the mostly used fungicides and to raise awareness about the use of the fungicides depending on the amount of consumption.

MATERIAL AND METHODS

Materials; Prochloraz (Pestanal, 45631), tebuconazole (Pestanal, 32013), triadimefon (Pestanal, 45693) and triadimenol (Pestanal, 46138) were purchased from Sigma (Germany). Dimethylsulfoxid, trypsin and triton X-100 (Biomatik, Canada), disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium chloride, absolute alcohol and ethylenediamine tetra acetic acid (Sigma, Germany), sodium hydroxide (Merck, Germany), trypan blue (Fluka, Switzerland) were obtained from the different companies. Fetal bovine serum (FBS), Dulbecco's modified eagle medium (DMEM), penicillin-streptomycin and sodium dodesyl sulphate (SDS) and phosphate buffer saline (PBS) were purchased from Multicell-Wisent Inc. (Quebec, Canada). The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent was purchased from Sigma (St. Louis, USA). Cytotoxicity detection with lactate dehydrogenase activity kit was obtained from Roche Company (Indianapolis, USA). In the study, microplate reader spectrophotometer (Biotek-EPOCH, Germany), CO_2 incubator (Heracell-Thermo-Scientific, USA), microscope (CKX4-Olympus, Japan), laminar cabinet (Tezsan, Turkey) were used.

Preparation of the fungicide solutions; Prochloraz, tebuconazole, triadimefon and triadimenol were dissolved in 5 ml DMSO to prepare 1 mg/ml of stock solutions. Higher concentrations more than 1 mg/ml could not be prepared due to the solubility problems. The working solutions were

diluted with distilled water to 10 µg/ml concentration and then filtered via 0.5 µm filter.

Cell line, culture condition and treatment: The cervical cancer cell line (HeLa) was provided by Department of Genetics, Istanbul University, Turkey. The cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% streptomycin and penicillin at 37°C in a 5% CO₂ and 95% O₂ in a humidified cell incubator. Growth medium was changed every 2 days. Cells grown to 75–85% confluence were washed with PBS, trypsinized with 3 ml of 0.25% (v) trypsin - 0.03 % (v) EDTA and diluted with fresh medium. The passage number range for both cell lines was maintained between 20 and 25. The optimum cell concentration as determined by the growth profile of the cell line was 10⁵ cells/ml.

Cells were exposed to various concentrations of the fungicides. In the study, the exposure concentrations for all fungicides were determined as 0.0078-1 µg/ml. The control and treated cells were incubated for 24 h in 37°C, 5% CO₂ incubator. By using the trypan blue exclusion test to determine cell viability, we found that there was no difference in cell count for 1% DMSO treated cells and untreated (control) cells.

Cytotoxicity by MTT test; 20 µl MTT solution was added to each well of 96 well microplate including the cell line that was incubated at 37 °C, 5% CO₂ with test substances. After shaking for 5 min, the cells were incubated at 37 °C for one hour. The supernatant of the wells was thrown gently. 100 µl DMSO was added to each well. Later, it was shaken for 5 min at 150 rpm. The intensity of colour was measured at 590 nm (against the reference wavelength of 670 nm) via microplate reader spectrophotometer.

Cytotoxicity by LDH test; Cell lines were incubated with test substances at 37 °C, %5 CO₂ for 24 hours. After incubation, the cell viability was evaluated using LDH test according to cytotoxicity detection kit. Except the blank and water wells, mediums on other wells were thrown. Wells were rinsed with 50 µl PBS. 5 µl lysis solution was added to three wells including triton X-100 and it was shaken for 15 min at 150 rpm. 100 µl reaction mixtures were added to all wells except water wells. It was incubated for 30 min at 15-25 C⁰ in the dark. The assay was conducted immediately by mixing the media with the assay reagent prepared by mixing two separate solutions (diaphorase / NAD⁺ mixture and iodotetrazolium chloride / sodium lactate mixture). After 30 minutes incubation in dark, 50 µl stop solution

was added to the wells. Measurements were done at 490 nm (against the reference wavelength of 600 nm) via microplate reader spectrophotometer. As positive control (100% cell lysis), 10% triton X-100 was used.

Evaluation of the results; The number of dead cells (Inhibition concentration, IC) in % was calculated by comparing absorbance values of test compounds and solvent/positive controls group. By subtracting the absorbance of control and blank solvent absorbance of each sample corrected absorbance values were obtained. Based on average absorbance values calculation was done for each microplate repeats. From each compound six concentrations were tested in triplicates and each test was repeated twice. IC calculated according to the below formula as the percentages of solvent/positive controls;

$$\% \text{inhibition} = 100 - (\text{corrected mean OD}_{\text{substance}} \times 100 / \text{corrected mean OD}_{\text{solvent/positive control}})$$

While the viability % was calculated by solvent control for MTT test, the taken respond by using triton X-100 was considered as 100% cytotoxic in LDH test. The significance of the difference between the groups in accordance with the data obtained from experiments was evaluated with one way ANOVA test by using Minitab-13 statistic program. Significance level was accepted as $p < 0.05$.

RESULTS AND DISCUSSION

In the study, the features of the selected fungicides are shown in Table 1. The information was taken from the database such as Pesticide Properties and Veterinary Substances Database (Inchem, 1979; PPDB, 2012; VSDB, 2012).

Table 1: Features of the studied triazole fungicides

Substance	Chemical Name (IUPAC)	ADI (mg/kg)	NOAEL (mg/kg)	WHO classification
Prochloraz	<i>N</i> -propyl- <i>N</i> -[2-(2,4,6-trichlorophenoxy)ethyl] imidazole-1-carboxamide	0.01	0.90	II, moderately hazardous
Tebuconazole	(<i>RS</i>)-1- <i>p</i> -chlorophenyl)-4,4-dimethyl-3-(1 <i>H</i> -1,2,4-triazol-1-ylmethyl) pentan-3-ol (1 <i>RS</i> , 2 <i>RS</i> ; 1 <i>RS</i> , 2 <i>SR</i>)-1-(4-chlorophenoxy)-3,3-dimethyl-1-(1 <i>H</i> -1,2,4-triazol-1-yl) butan-2-ol	0.03	> 10.8	II, moderately hazardous
Triadimenol	(<i>RS</i>)-1-(4-Chloro-phenoxy)-3,3-dimethyl-1-[1,2,4]-triazol-1-yl- butan-2-one	0.05	> 8	II, moderately hazardous
Triadimefon		0.03	2.5	III, slightly hazardous

HeLa cells were exposed to four fungicides (prochloraz, tebuconazole, triadimefon, triadimenol) for 24 h and cytotoxicity was determined with LDH and MTT tests. Before determining the cytotoxic activities of the compounds, different exposure times and cell densities were tried by using MTT test. Cells were seeded in the different density (10^4 - 5×10^4 - 10^5 - 5×10^5 per 100 μ l per well) in wells. According to the test, significant difference was not observed among the results obtained from different cell densities ($p > 0.05$).

When evaluated the obtained results after exposure to four fungicides at different exposure times (24 and 48 hrs), it was shown that the repeatability decreased at the incubation time with 48 hrs (Table 2).

Table 2: The effects of exposure times on the cytotoxic activities of four fungicides.

Test substance		Absorbance	
		24 hour	48 hour
	Prochloraz	0.628 \pm 0.012	0.658 \pm 0.022
	Tebuconazole	0.408 \pm 0.020	0.480 \pm 0.032
	Triadimefon	0.700 \pm 0.018	0.715 \pm 0.028
	Triadimenol	0.610 \pm 0.009	0.622 \pm 0.019

The effects of substances on mitochondrial activity and respiratory chain were observed to be more frequent than damage on membrane. The cytotox-

icity results presented in Table 3 and 4 were expressed as the concentration inhibiting 50% of the cell growth (IC_{50}).

Table 3: Mortality % and IC_{50} values obtained by the MTT assay for test substances

Test substance Cons. ($\mu\text{g/ml}$)	Cell Death %							IC_{50} ($\mu\text{g/ml}$)
	0.016	0.032	0.063	0.13	0.25	0.50	1	
Prochloraz	0.91	7.70	11.25	23.81	35.36	60.18	88.65	0.40
Tebuconazole	-	-	-	-	10.33	33.74	42.25	>1
Triadimefon	-	1.62	13.88	27.46	48.02	66.67	88.45	0.24
Triadimenol	-	3.55	3.85	25.74	37.99	73.96	83.49	0.37

Table 4: Mortality % and IC_{50} values obtained by the LDH assay for test substances

Test substance Cons. ($\mu\text{g/ml}$)	Cell Death %							IC_{50} ($\mu\text{g/ml}$)	
	0.008	0.016	0.032	0.063	0.13	0.25	0.50		1
Prochloraz	31.00	34.87	42.77	45.15	53.10	67.51	74.02	77.38	0.065
Tebuconazole	28.10	29.70	27.89	29.39	36.57	40.96	47.16	52.07	0.985
Triadimefon	32.28	36.67	40.50	53.87	70.35	76.71	81.10	85.54	0.052
Triadimenol	30.68	39.67	45.40	50.67	65.24	72.21	82.28	92.77	0.042

Considering the results of the two tests, a statistically significant difference was observed in terms of effects on cells. For MTT test, IC_{50} values of prochloraz, tebuconazole, triadimefone and triadimenole were determined 0.4, >1, 0.24 and 0.37 $\mu\text{g/ml}$, respectively. For LDH test, their values were identified 0.065, 0.985, 0.052 and 0.042 $\mu\text{g/ml}$, respectively. It was seen that IC_{50} values for LDH test which is used to identify membrane damage are lower than IC_{50} values for MTT test.

For these triazole fungicides, the cytotoxicity assay has not been evaluated through LDH and MTT assay on the cell line before. For mammals, the acute oral LD_{50} values are 1023, 1700, 300 and 721 mg/kg for prochloraz, tebuconazole, triadimefon, triadimenol, respectively (15-17). So, the results of the present study show that the IC_{50} values obtained by using MTT and LDH assay from this study are directly proportional to the LD_{50} values for these fungicides.

In conclusion, we aimed to emphasize their importance on human and environmental health. When their potential cytotoxic effects were evaluated by using MTT and LDH tests on HeLa cells, tebuconazole showed no cytotoxic effect while prochloraz showed the highest cytotoxicity effect. Nevertheless, for each tested substances cytotoxic activity was detected in a concentration-dependent increase and calculated IC_{50} values were higher than reported ADI values by FAO and WHO. It is believed that these obtained results, will draw attention to expand researches on toxic effects of triazole fungicides which are identified as cytotoxic for human and environment health. Furthermore, it is believed to take necessary precautions for usage of these compounds.

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REFERENCES

1. Fang, H., Tang, F.F., Zhou, W., Cao, Z.Y., Wang, D.D., Liu, K.L., Wu, X.W., Yu, Y.L. (2012). *J Environ Sci Health* 47:104-110.
2. Birkhoj, M., Nellemann, C., Jarfelt, K., Jacobsen, H., Andersen, H.R., Dalgaard, M., Vinggaard, A.M. (2004). *Toxicol Appl Pharmacol* 201(1):10-20.
3. Xi, J., Yang, Z., Zeng, C., Hu, X., Wang, J. (2012). *Behav Pharmacol* 23:727-734.
4. Filipov, N.M., Lawrence, D.A. (2001). *Toxicol Sci* 62 (2):185-186.
5. Dmytriyeva, O., Klementiev, B., Berezin, V., Bock, E. (2013). *Exp Toxicol Pathol* 65(5):591-593.
6. FAO (Food and Agricultural Organizations of United Nations). (2009). *Prochloraz (407/TC) US: FAO specifications*.
7. Ugurlu, S. (2009). Pesticide Risk Assesment and management in Turkey. Ministry of Agriculture and Rural Affairs, Ankara Plant Protection Central Research Institute Ankara.
8. FAO (Food and Agricultural Organizations of United Nations). (2000). *Tebuconazole (494/TC) US: FAO specifications*.
9. FAO (Food and Agricultural Organizations of United Nations). (2011). *Triadimefon (398/TC) US: FAO specifications*.

10. FAO (Food and Agricultural Organizations of United Nations). (2011). *Triadimenol* (352/TC) US: FAO specifications.
11. Demir, H. (2005). Methidathion ve triadimenol pestisitlerinin insan lenfosit kültürlerindeki genotoksik etkileri. The master thesis, unpublished, Gazi University, Institute of Science, Ankara.
12. Walker, Q.D., Lewis, M.H., Crofton, K.M., Mailman, R.B. (1990). *Toxicol Appl Pharmacol* 102(3):474-85.
13. Reeves, R., Thiruchelvam, M., Baggs, R.B., Cory-Slechta, D.A. (2003). *Neurotoxicol* 24:839-850.
14. Ekwall, B., Silano, V., Paganuzzi-Stammati, A., Zucco F. (1998). Toxicity tests with mammalian cell cultures. P. Bourdeou, (Ed.), *Toxicity tests with Mammalian Cell Cultures* (75-97). California: John Wiley & Sons.
15. INCHEM. International Programme on Chemical Safety (IPCS). *Pesticide Residues in Food*. (1979). <http://www.inchem.org/>.
16. PPDB (Pesticide Properties Database). (2012). <http://www.pesticide-info.org/>.
17. VSDB (Veterinary Substances Database). 2012. <http://sitem.herts.ac.uk/aeru/vsdb/index.htm>.

