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# Ziram, a dithiocarbamate fungicide, exhibits pseudo-cytoprotective actions against oxidative stress in rat thymocytes: Possible environmental risks

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ABSTRACT

Ziram, a dithiocarbamate fungicide, protects various vegetables and fruits against infections by fungus. Recently, there have been increasing anxieties about the risks in the use of dithiocarbamate fungicides. Our previous studies showed that $Zn^{2+}$ was a determinant of Ziram cytotoxicity. In addition, $Zn^{2+}$ is linked to $H_2O_2$ cytotoxicity. Therefore, in this study, we aimed to test the hypothesis that Ziram could augment the cytotoxicity of $H_2O_2$ by examining the changes induced by Ziram in some cellular parameters in rat thymic lymphocytes subjected to $H_2O_2$-induced oxidative stress using flow-cytometric methods with fluorescent dyes. Ziram significantly attenuated $H_2O_2$-induced cell death at sublethal concentrations. However, in the cells under oxidative stress elicited by $H_2O_2$, Ziram promoted the changing over from intact cells to living cells with exposed phosphatidylserine (PS) on plasma membranes, whereas it inhibited the transition from PS-exposed living cells to dead cells. Ziram significantly augmented $H_2O_2$ actions, including reduction of cellular glutathione levels and elevation of intracellular $Zn^{2+}$ concentrations. Conversely, it attenuated $H_2O_2$-induced depolarization of mitochondrial membrane potential. Ziram at sublethal concentrations seems to exhibit promotive and suppressive actions on the process of cell death caused by $H_2O_2$. Ziram increased the number of living cells with exposed PS, a phenomenon characteristic of early stages of apoptosis. Thus, it is concluded that Ziram exhibits pseudo-cytoprotective actions against $H_2O_2$-induced oxidative stress. Ziram at sublethal concentrations exerts promotive and suppressive actions on the process of cell death caused by oxidative stress.

## 1. Introduction

Ziram, a dithiocarbamate fungicide, is widely used in agriculture to protect various fruits and vegetables against fungal infections (Berrada et al., 2010; Tsakiris et al., 2011; López-Fernández et al., 2012). Recently, there have been increasing anxieties about the environmental and health risks in Ziram use (Tsakiris et al., 2011; Lozowicka et al., 2015). In our previous studies (Kanemoto-Kataoka et al., 2015, 2017), we investigated the adverse actions of Ziram in rat thymocytes. Ziram markedly increased the intracellular $Zn^{2+}$ concentrations ($[Zn^{2+}]i$) and decreased the cellular glutathione content ([GSH]i). In addition, the agent increased $Zn^{2+}$-dependently the cells that were positive to annexin V. $Zn^{2+}$ chelators greatly attenuated the increase in cell lethality by Ziram. Furthermore, a synergistic increase in cell lethality was observed after simultaneous application of micromolar $ZnCl_2$ and Ziram at a sublethal concentration. Therefore, it was suggested that $Zn^{2+}$ was a determinant of Ziram cytotoxicity (Kanemoto-Kataoka et al., 2017).

Ziram was reported to inhibit both superoxide dismutase and catalase activities (Sbrana et al., 1995). Zinc pyrithione, a zinc ionophore, and $ZnCl_2$ increased cell vulnerability to $H_2O_2$-induced oxidative stress by increasing $[Zn^{2+}]i$ levels (Matsui et al., 2010; Oyama et al., 2012). Therefore, Ziram might be suggested to increase cell vulnerability to $H_2O_2$-induced oxidative stress via an increase in $[Zn^{2+}]i$ levels and/or induction of oxidative stress. In this study, we tested this hypothesis by cytometrically investigating the adverse actions of sublethal concentrations of Ziram in rat thymic lymphocytes. However, Ziram, at sublethal (nanomolar) concentrations, exhibited a cytoprotective action against $H_2O_2$-induced oxidative stress. Therefore, we explored the possible mechanisms by which Ziram might decrease $H_2O_2$-induced cell death. The present study might provide some insights into the risks in

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**Table 1**
Fluorescent probes used in this study.

| Fluorescent probes / manufacture |
|---|
| Propidium iodide (PI) / Invitrogen (Eugene, OR, USA) |
| Annexin V-FITC / Invitrogen |
| FluoZin-3-AM / Invitrogen |
| Fluo-3-AM / Dojin Chemical Laboratory (Kumamoto, Japan) |
| 5-Chloromethylfluorescein diacetate (5-CMF-DA) / Invitrogen |
| JC-1 / Invitrogen |

the use of Ziram.

## 2. Materials and methods

### 2.1. Reagents

Ziram (purity, 99.9%) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Fluorescent dyes used in this study are listed in Table 1. Other chemicals were purchased from Wako Pure Chemicals, unless otherwise stated.

### 2.2. Animals and cell preparation

The Committee for Animal Experiments at the University of Tokushima approved the present study (No. 05279). The cell suspension was prepared as previously described (Chikahisa et al., 1996; Sakanashi et al., 2009; Matsui et al., 2010). Briefly, thymus glands were dissected from anesthetized rats. The slices of thymus glands were ground in chilled Tyrode's solution. The cell suspension was incubated at 36–37 °C for 1 h before the experiment. The suspension contained 216.9 ± 14.4 nM zinc derived from the cell preparation. Ziram (0.003–1 mM in 2 μL of dimethyl sulfoxide) were added to the cell suspensions (2 mL) to achieve final concentrations of 0.003–1 μM, and incubated at 36–37 °C for 1–3 h, depending on experimental purposes. A sample (100 μL) was analyzed using a flow cytometry to evaluate the Ziram-induced changes in membrane and cellular parameters.

Oxidative stress was induced by 100 μM $H_2O_2$. The treatment with 100 μM $H_2O_2$ for 3 h induced cell death in about 20% of cell population under present experimental conditions. The population of dead cells caused by $H_2O_2$ was increased by adding $ZnCl_2$ and decreased by adding a chelator of intracellular $Zn^{2+}$ (Matsui et al., 2010).

### 2.3. Fluorescence measurements

Membrane and cellular changes were evaluated using a flow cytometer (CytoACE-150; JASCO, Tokyo, Japan) and respective fluorescent dyes. Wavelength for dye excitation was 488 nM. Dye emissions were detected at a wavelength of 530 ± 20 nm for FITC, 5-CMF, Fluo-3, FluoZin-3, and JC-1 (green) fluorescence and 600 ± 20 nm for PI and JC-1 (red) fluorescence. Data analyses were performed with JASCO software (Version 3.06; JASCO).

Cells with PI fluorescence were dead. 5-CMF, Fluo-3, and FluoZin-3 fluorescence were recorded from cells without PI fluorescence (living cells). Phosphatidylserine (PS) exposed on membrane surface, an event at initial phase of apoptosis, was revealed with annexin V-FITC (Koopman et al., 1994). The change in cellular glutathione content ([GSH]i) was estimated with 5-CMF-DA. The coefficient of correlation between 5-CMF fluorescence and [GSH]i was 0.965 (Chikahisa et al., 1996). Changes in mitochondrial membrane potentials were estimated with 1 μM JC-1 (Smiley et al., 1991). The cells were treated with 1 μM JC-1 for 1 h before the fluorescence measurement. Fluo-3-AM and FluoZin-3-AM at 1 μM were used to estimate the changes in intracellular $Ca^{2+}$ and $Zn^{2+}$ levels ([$Ca^{2+}$]i and [$Zn^{2+}$]i), respectively.

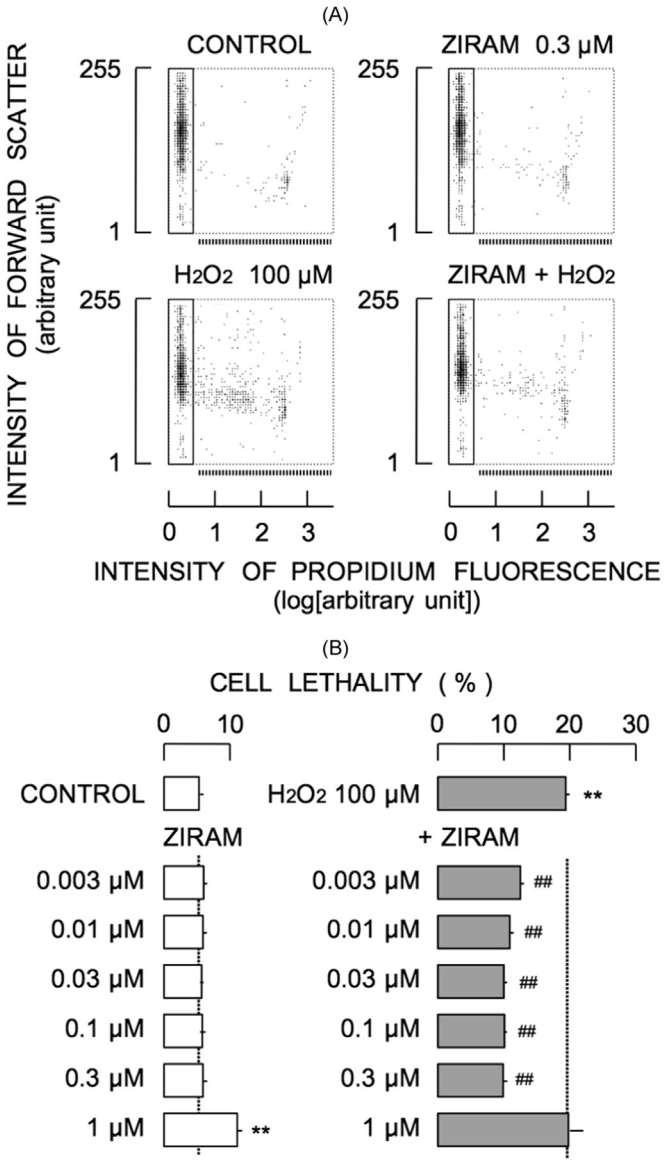


**Fig. 1.** Changes in the population of cells exhibiting PI fluorescence by Ziram, $H_2O_2$, and their combination. Effects were examined 3 h after the respective application(s). (A) Changes in cytogram (forward scatter versus PI fluorescence) by Ziram, $H_2O_2$, and their combination. Each cytogram included 2500 cells. Dotted line under cytogram indicates the area of cells exhibiting PI fluorescence. (B) Cell lethality (percentage of cells exhibiting PI fluorescence) after treatment with Ziram or Ziram + $H_2O_2$. Asterisk (**) indicates significant difference ($P < 0.01$) between the control group (control) and drug-treated cells. Symbol (##) indicates significant difference ($P < 0.01$) between the $H_2O_2$-treated cells and cells co-treated with $H_2O_2$ and Ziram. Data represent the mean cell lethality and standard deviation of four samples. Dotted bars are placed for comparison with the respective control.

### 2.4. Statistical analysis and data representation

Statistical differences were confirmed using analysis of variance followed by Tukey's *post-hoc* test. *P*-values $< 0.05$ were considered statistically significant. Data represent the means ± standard deviation of four samples. Each experiment was carried out in triplicate, unless otherwise specified.

## 3. Results

### 3.1. Attenuation of $H_2O_2$-induced increase in cell lethality by Ziram

Treatment of the cells with 100 μM $H_2O_2$ for 3 h increased the