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Original Article

Zn^{2+}-dependent increase in cells with phosphatidylserine-exposed membranes after treatment with submicromolar concentrations of 2-*n*-octyl-4-isothiazolin-3-one in rat thymocytes

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ABSTRACT — Some household products have high levels of the antimicrobial 2-*n*-octyl-4-isothiazolin-3-one (OIT). Although the diverse effects of OIT are of concern, information regarding its cellular actions is limited. In a previous study, we found that OIT increased intracellular Zn^{2+} levels in rat thymocytes. However, because Ca^{2+} is considered the essential cation that causes cell injury and death, we examined whether Ca^{2+} and Zn^{2+} were involved in OIT-induced cytotoxicity and proposed the mechanisms underlying these results. The effects of OIT on the membrane and cellular parameters of rat thymocytes were examined with a flow cytometer and appropriate fluorescent probes. OIT (0.3-3 μM) increased intracellular Zn^{2+} levels but not intracellular Ca^{2+} levels. Therefore, the involvement of Zn^{2+} was studied further. The simultaneous application of 0.3 μM OIT and 3 μM $ZnCl_2$ significantly increased cells with phosphatidylserine-exposed membranes without changing the dead cells. In contrast, applications of 0.3 μM OIT or 3 μM $ZnCl_2$ alone had no effects. The combination of OIT (0.1-1 μM) and $ZnCl_2$ (1-3 μM) significantly decreased the cellular non-protein thiol contents. These changes that were induced by their combination were completely suppressed by adding an intracellular Zn^{2+} chelator. These results suggested that submicromolar concentrations of OIT induced Zn^{2+}-dependent cytotoxicity in the presence of micromolar concentrations of external Zn^{2+}. Because the threshold of OIT levels that affected cellular parameters in the presence of micromolar concentrations of Zn^{2+} are much lower than the OIT contents in some household products, the adverse effects of OIT are of great concern.

Key words: 2-*n*-octyl-4-isothiazolin-3-one, Antimicrobial, Cytotoxicity, Zinc, Membrane permeability

INTRODUCTION

Although 2-*n*-octyl-4-isothiazolin-3-one (OIT) is used as an antimicrobial in household products, no studies have reported its potentially severe adverse actions except for those involved in occupational and non-occupational contact dermatitis (Aalto-Korte *et al*., 2007; Fukunaga *et al*., 2010). High concentrations of OIT have been detected in some household products (Nakashima *et al*., 2000; Aalto-Korte *et al*., 2007; Kawakami *et al*., 2014). In our previous study (Fukunaga *et al*., 2015), we found that OIT increased membrane Zn^{2+} permeability through the activation of the temperature-sensitive Zn^{2+} pathway, which resulted in increased intracellular Zn^{2+} levels in rat thymic lymphocytes. However, the Zn^{2+}-dependent insults that were induced by OIT were not elucidated. Excessive increases in intracellular Zn^{2+} levels that are induced by OIT may be cytotoxic because Zn^{2+} exhibits a variety of physiological and pathological actions (Sensi *et al*., 2011; Shuttleworth and Weiss, 2011). Indeed, zinc is related to aging and degenerative diseases (Frazzini *et al*., 2006; McCord and Aizenman, 2014). Neverthe-

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less, previous studies have indicated that Ca^{2+} is the most essential cation in cell injury and death (Zhivotovsky and Orrenius, 2011). Currently, there is no evidence that OIT increases intracellular Ca^{2+} levels in mammalian cells. Some cytotoxic chemicals increase the intracellular concentrations of both Ca^{2+} and Zn^{2+} (Chikahisa and Oyama, 1992; Oyama *et al*., 1995; Kawanai *et al*., 2009; Oyama *et al*., 2009), and the external application of Zn^{2+} can augment the cytotoxicity of some compounds (Oyama *et al*., 2007; Matsui *et al*., 2010; Oyama *et al*., 2010). In addition, an excessive increase in the intracellular Zn^{2+} levels has been proposed to cause mitochondrial oxidative stress (Sensi *et al*., 2011; Shuttleworth and Weiss, 2011). Therefore, Zn^{2+} might also be an essential cationic element in chemical-induced cytotoxicity. In the current study, we tested the effects of OIT on intracellular Ca^{2+} levels and examined whether Ca^{2+} and Zn^{2+} were involved in OIT-induced cytotoxicity. Based on these results, we propose a mechanism for the OIT-induced cytotoxicity.

In this study, we used flow cytometric techniques to examine the cellular effects of OIT on rat thymocytes, which are used as an experimental model for several reasons. Because thymocytes can be freshly dissociated without enzymatic treatment, the cell membranes remain intact. Additionally, thymocytes tend to cause apoptosis, which has allowed the study of cell death (Kroemer *et al*., 1998; McLeod and He, 2010; Hernandez *et al*., 2010). Furthermore, several hormones, biological compounds, and chemicals have been shown to induce apoptosis and necrosis in thymocytes (Kroemer *et al*., 1998; Quaglino and Ronchetti, 2001). Thus, thymocytes are susceptible to several types of compounds. In the present study, we examined the hypothesis that OIT induces Zn^{2+}-dependent cytotoxicity in lymphocytes in order to have further insight into OIT-induced cytotoxicity and the toxicological roles of Zn^{2+} (Sensi *et al*., 2011; Haase and Rink, 2014).

MATERIALS AND METHODS

Chemicals

OIT was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Propidium iodide and FluoZin-3 acetoxymethyl (AM) were obtained from Life Technologies (Grand Island, NY, USA). The chelators of external and intracellular Zn^{2+}, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA) and *N*,*N*,*N*′,*N*′-*tetrakis*(2-pyridylmethyl)ethylenediamine (TPEN), and Fluo-3 AM were obtained from Dojin Chemical Laboratory (Kumamoto, Japan). Other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animal and cell preparation

This study was approved by the Committee for Animal Experiments at Tokushima University (No. 14124). The cellular suspension of rat thymocytes was prepared as previously reported (Chikahisa *et al*., 1996; Matsui *et al*., 2008). In brief, thymus glands that were dissected from ether-anesthetized rats were sliced and triturated in Tyrode's solution to dissociate the thymocytes. The cell suspension was passed through a 58-μm diameter mesh and incubated at 36-37°C for 60 min before the experiment. Importantly, the zinc concentration in the Tyrode's solution was 32.4 ± 4.0 nM (Sakanashi *et al*., 2009). This significant increase in zinc concentration was due to the reagents that were used to prepare the solution and that contained trace levels of zinc. Furthermore, the zinc concentration in the solution that was obtained after removing the cells from the cell suspension by filtration (pore diameter: 0.22 μm) was 216.9 ± 14.4 nM (Sakanashi *et al*., 2009). Thus, it was likely that the cell suspension contained trace levels of zinc that resulted from the cell preparation. Zn^{2+} chelators, including DTPA and TPEN, were used to produce a Zn^{2+}-free condition.

Cellular and membrane fluorescence measurements

The membrane and intracellular parameters were measured with a flow cytometer that was equipped with an argon laser (CytoACE-150; JASCO, Tokyo, Japan) and fluorescent probes as previously described (Chikahisa *et al*., 1996; Matsui *et al*., 2008). The excitation wavelength for all of the probes was 488 nm, and the fluorescence emission wavelength was detected at 600 ± 20 nm for propidium iodide and 530 ± 20 nm for the other probes. The fluorescence was analyzed with the JASCO software package (Version 3.06; JASCO International Co., Ltd., Tokyo, Japan). Fluorescence was not detected from the reagents used in the study under the experimental conditions.

To assess cell death, propidium iodide (5 μM) was added to the cell suspensions. The fluorescence was measured at least 2 min after the application of propidium iodide. Fluo-3 AM was used to monitor the changes in the intracellular Ca^{2+} concentrations. The cells were incubated with 500 nM Fluo-3 AM for 60 min before the measurement. Fluo-3 fluorescence was measured in living cells. The cells were incubated with 500 nM FluoZin-3 AM, which is an indicator of intracellular Zn^{2+} levels, for at least 60 min before the fluorescence measurements. In addition, fluorescence was measured in living cells that were not stained with propidium iodide (Matsui *et al*., 2008). The exposure of phosphatidylserine on the outer

surface of cell membranes, which is a phenomenon that occurs during the early stages of apoptosis (Koopman *et al*., 1994), was detected with annexin V-fluorescein isothiocyanate (FITC). The cells were incubated with annexin V-FITC (10 μL/mL) and propidium iodide for 30 min before the measurement. Changes in the cellular contents of non-protein thiols, which were presumably glutathione, were monitored with 5-chloromethylfluorescein diacetate (5-CMF-DA) (Chikahisa *et al*., 1996). The cells were incubated with 1 μM of 5-CMF-DA for 30 min before the fluorescence measurements. The correlation coefficient between the mean intensity of 5-CMF fluorescence and the biochemically estimated contents of cellular glutathione were 0.965 in rat thymocytes (Chikahisa *et al*., 1996).

Presentation and statistical analysis

The data (columns and bars in figures) were expressed as the mean and standard deviation of 4 samples. Each experiment was repeated two or three times. The statistical analyses were performed with analyses of variance with a *post-hoc* Tukey's multivariate analysis. *P* values less than 0.05 were considered significant.

RESULTS

OIT-induced changes in intracellular Ca^{2+} levels

As shown in Fig. 1A, incubation of the cells with 3 μM OIT for 60 min shifted the Fluo-3 fluorescence histogram to a higher intensity, suggesting that OIT increased the intracellular Ca^{2+} levels. The changes in Fluo-3 fluorescence that were induced by OIT reached a steady state within 45-60 min. Ten μM OIT did not increase the intensity of Fluo-3 fluorescence more. OIT (0.3-3 μM) increased the mean intensity of Fluo-3 fluorescence in a concentration-dependent manner, as shown in Fig. 1B. The threshold concentration of OIT for enhancing Fluo-3 fluorescence was 0.3 μM. The augmentation of Fluo-3 fluorescence by 1 μM OIT was significantly diminished in the presence of 10 μM TPEN, which is a chelator of intracellular Zn^{2+} (Fig. 1C). These results suggested that the increase in Fluo-3 fluorescence intensity that was induced by OIT was dependent on intracellular Zn^{2+}.

OIT-induced changes in intracellular Zn^{2+} levels

We previously reported that OIT increased intracellular Zn^{2+} levels through the activation of a temperature-sensitive Zn^{2+} pathway (Zn^{2+} influx) in rat thymocytes (Fukunaga *et al*., 2015). Three μM OIT significantly increased the intensity of FluoZin-3 fluorescence (Fig. 1D). The OIT-induced augmentation of FluoZin-3 fluorescence was completely abolished by 10 μM TPEN, even in the presence of Ca^{2+} (Fig. 1D). Thus, it is likely that OIT increased the intracellular levels of Zn^{2+} rather than those of Ca^{2+}.

Annexin V-FITC and propidium iodide assessments of the OIT-induced cellular changes

Incubation with 0.3-3 μM of OIT for 180 min did not increase the cells exhibiting propidium fluorescence in rat thymocytes (not shown). The percentage of cells with propidium fluorescence under the control condition was 3.8 ± 0.4%. The percentages were 4.1 ± 0.4%, 3.9 ± 0.4%, and 3.5 ± 0.3% for 0.3 μM, 1 μM, and 3 μM, respectively, of OIT. These results suggested that OIT (0.3-3 μM) did not decrease cell viability in rat thymocytes.

Our previous study (Fukunaga *et al*., 2015) suggested that the simultaneous application of OIT and $ZnCl_2$ induces oxidative stress. Thus, we examined the cellular changes with annexin V-FITC and propidium iodide after simultaneous treatment with OIT and $ZnCl_2$ for 60 min. As shown in Fig. 2A, treatment with 0.3 μM OIT or 3 μM $ZnCl_2$ did not affect the cells. In contrast, the simultaneous application of 0.3 μM OIT and 3 μM $ZnCl_2$ significantly increased the number of annexin V-positive living cells without changing the number of cells displaying propidium fluorescence (dead cells) (Fig. 2A). These results, which are summarized in Fig. 2B, suggested that the simultaneous application of OIT and $ZnCl_2$ increased the number of living cells with phosphatidylserine exposed on their outer membrane surfaces, which is a marker for the early stages of apoptosis (Koopman *et al*., 1994) without increasing the number of dead cells. In the presence of 10 μM DTPA, which is a chelator of external Zn^{2+}, the simultaneous application did not change the cells, which indicated that the above findings were a Zn^{2+}-dependent phenomenon.

Cell shrinkage is another marker for the early stages of apoptosis (Maeno *et al*., 2000). The cell volume, which was tentatively estimated with the intensity of forward scatter (a parameter of cell size), of the living cells was significantly reduced when the cells were simultaneously incubated with 0.3 μM OIT and 3 μM $ZnCl_2$. The volumes relative to the control (1.00 ± 0.04) were 1.01 ± 0.03, 0.96 ± 0.03, and 0.91 ± 0.02 in the cells treated with 0.3 μM OIT, 3 μM $ZnCl_2$, and their combination, respectively.

OIT-induced changes in the cellular contents of non-protein thiol in the presence of external Zn^{2+}

The results of Fig. 2B indicated that 0.3 μM OIT pro-

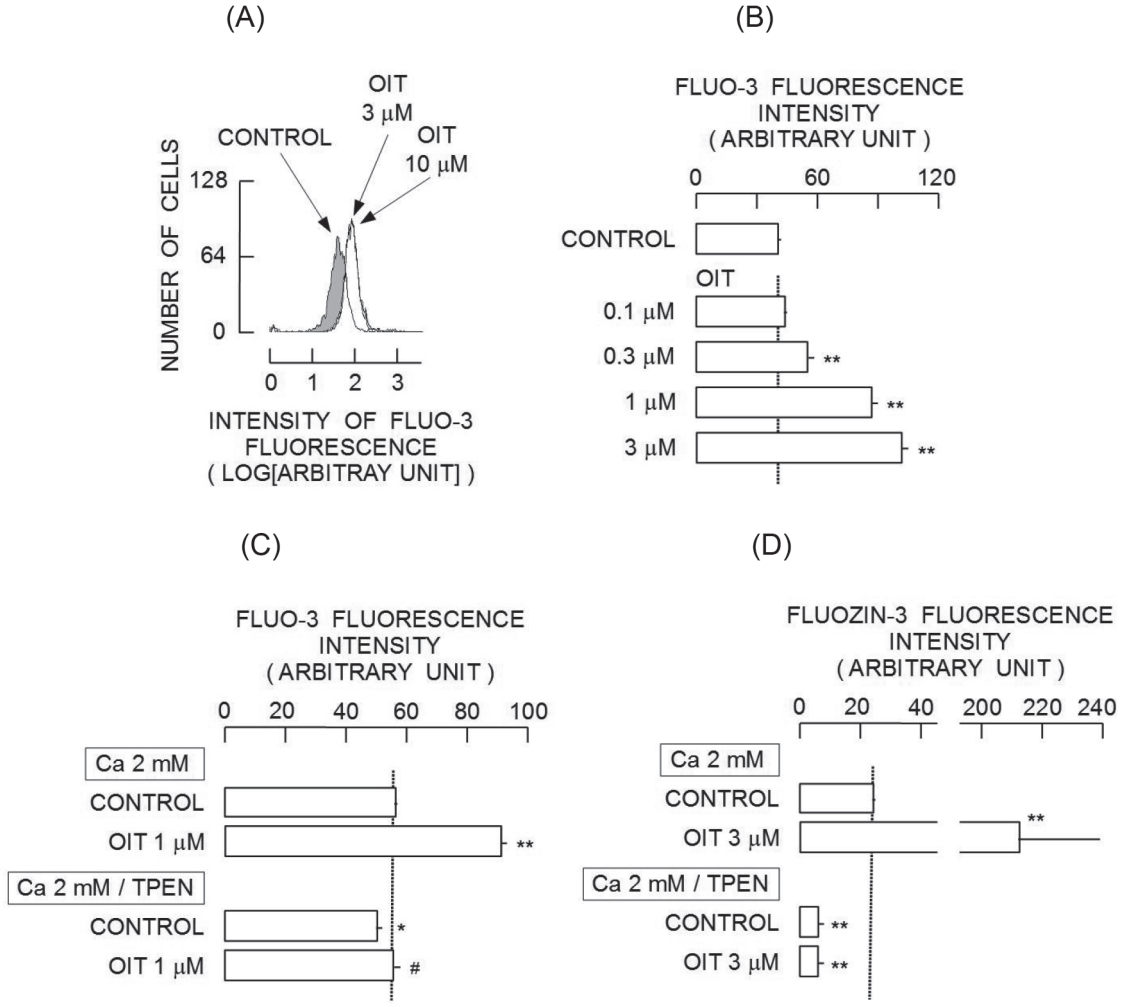

Fig. 1. The 2-*n*-octyl-4-isothiazolin-3-one (OIT)-induced change in Fluo-3 and FluoZin-3 fluorescence in rat thymocytes. (A) OIT-induced shift of Fluo-3 fluorescence in the histogram. Effects were examined 60 min after the application of OIT. Each histogram was constructed from 2,000 cells. The histogram that was obtained from the cells that were treated with 3 μM of OIT is overlapped by the histogram that was obtained from the cells that were treated with 10 μM of OIT. (B) Concentration-dependent increase in the mean intensity of Fluo-3 fluorescence by 0.1-3 μM of OIT. The asterisks (**) indicate a significant difference ($P < 0.01$) between the control group (CONTROL) and the groups of cells treated with OIT. (C) The effects of OIT on Fluo-3 fluorescence in the presence of *N*,*N*,*N*′,*N*′-*tetrakis*(2-pyridylmethyl)ethylenediamine (TPEN). The asterisks (* and **) indicate a significant difference ($P < 0.05$ and $P < 0.01$, respectively) between the control group (CONTROL) in the absence of TPEN and the other groups. The # symbol indicates a significant change between the control group and the OIT-treated group in the presence of TPEN. (D) OIT-induced changes in FluoZin-3 fluorescence in rat thymocytes. The asterisks (**) indicate a significant difference ($P < 0.01$) between the control group (CONTROL) in the absence of TPEN and the other groups of cells.

moted the transition from normal living cells to cells with phosphatidylserine-exposed membranes in the presence of 3 μM of $ZnCl_2$. Thus, we examined the effects of OIT (0.1-1 μM), $ZnCl_2$ (0.3-3 μM), and their combination on the cellular contents of non-protein thiol with 5-CMF fluorescence (Chikahisa *et al*., 1996). The incubation of the cells with 1 μM OIT for 60 min significantly increased the intensity of 5-CMF fluorescence, while this effect was not observed with submicromolar concentrations (0.1-0.3 μM) of OIT (Fig. 3A). Although 3 μM $ZnCl_2$ increased the 5-CMF fluorescence intensity, OIT (0.1-1 μM) significantly decreased the intensity of 5-CMF fluorescence in the presence of 3 μM $ZnCl_2$ (Fig. 3A). Incubation with 0.3-3 μM $ZnCl_2$ for 60 min increased

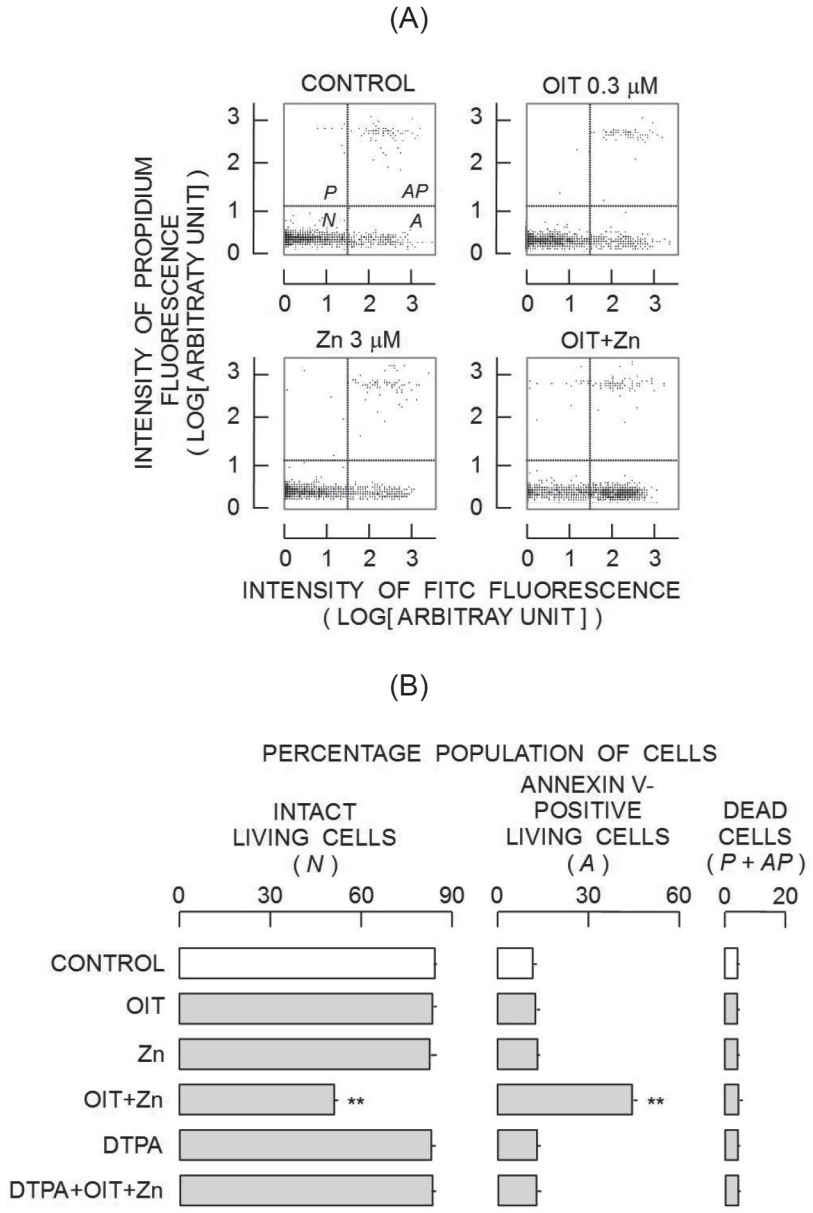

Fig. 2. Changes induced by OIT, $ZnCl_2$, and their combination in cells that were labeled by annexin V-fluorescein isothiocyanate (FITC) and propidium iodide. (A) Changes in the fluorescence cytogram (propidium fluorescence *versus* FITC fluorescence) by 0.3 μM of OIT, 3 μM of $ZnCl_2$ (Zn 3 μM), and their combination (OIT + Zn). The effects were examined 60 min after their application. Each cytogram consisted of 2,000 cells. The N, A, P, and AP areas show the intact living cells, annexin V-positive living cells, dead cells, and annexin V-positive dead cells, respectively. (B) Changes in the cellular percentage after treatment with OIT, $ZnCl_2$, and their combination. The dead cells consisted of the P and AP areas. The asterisks (**) indicate a significant difference ($P < 0.01$) between the control group (CONTROL) and the test groups.

the intensity of 5-CMF fluorescence in a concentration-dependent manner (Fig. 3B). The increase that was induced by 3 μM $ZnCl_2$ was statistically significant. Incubation with 0.3 μM OIT and 0.3 μM $ZnCl_2$ significantly increased the intensity of 5-CMF fluorescence, whereas 0.3 μM OIT greatly reduced the cellular thiol contents in combination with 1-3 μM $ZnCl_2$ (Fig. 3B). The combination of 3 μM $ZnCl_2$ and 0.3 μM OIT did not change

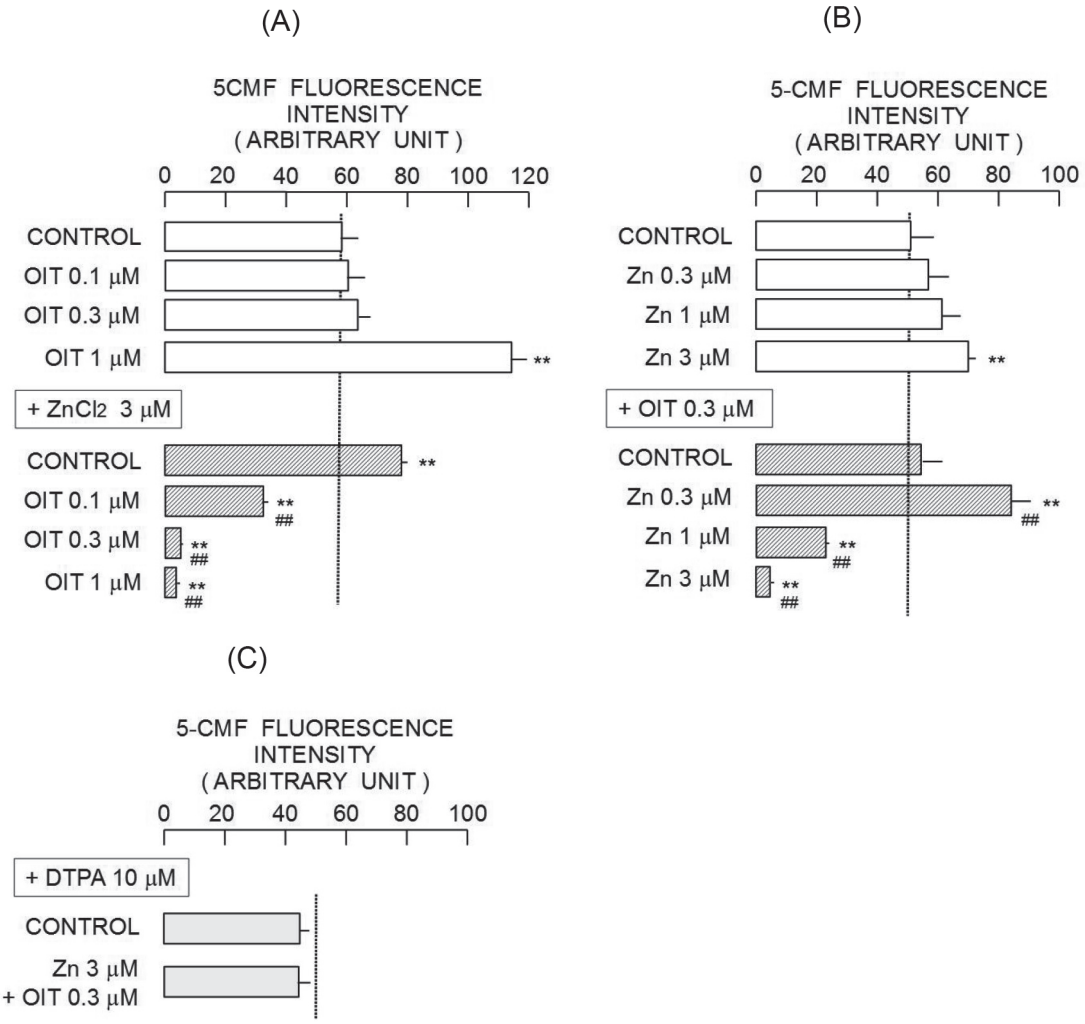

Fig. 3. Changes in 5-chloromethylfluorescein (5-CMF) fluorescence that were induced by treatment with OIT, $ZnCl_2$, and their combination in rat thymocytes. (A) OIT-induced changes in 5-CMF fluorescence in the absence (open columns) and presence (filled columns) of additional $ZnCl_2$ (3 μM). The asterisks (**) indicate a significant difference ($P < 0.01$) between the control group (CONTROL in absence of additional $ZnCl_2$) and all of the other groups. The ## symbols indicate a significant change ($P < 0.01$) between the control group (CONTROL) and the other groups in the presence of additional $ZnCl_2$. (B) The $ZnCl_2$-induced change in 5-CMF fluorescence in the absence (open columns) and presence (filled columns) of 0.3 μM of OIT. The asterisks (**) indicate a significant difference ($P < 0.01$) between the control (CONTROL in absence of OIT) and all of the other groups. The ## symbols indicate a significant difference ($P < 0.01$) between the control group (CONTROL) and the other groups in the presence of 0.3 μM of OIT. (C) The effects of the simultaneous application of 3 μM of $ZnCl_2$ (Zn 3 μM) and 0.3 μM of OIT on 5-CMF fluorescence in the presence of 10 μM of diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA).

the 5-CMF fluorescence in the presence of 10 μM DTPA (Fig. 3C), which indicated that the OIT action was Zn^{2+}-dependent.

DISCUSSION

Zn^{2+}-dependent cytotoxicity of OIT

Ca^{2+} was not thought to be essential in OIT cytotoxicity because 0.1-1 μM of OIT did not increase intracellular Ca^{2+} levels (Fig. 1C). In contrast, OIT activates a temperature-sensitive Zn^{2+} pathway (Fukunaga *et al*., 2015). The increase in membrane Zn^{2+} permeability, which increased the intracellular Zn^{2+} levels, may be a major component in OIT-induced cytotoxicity because Zn^{2+} has physiological and pathological roles in cellular functions (Sensi *et al*., 2011; Shuttleworth and Weiss, 2011). The

OIT-induced increase in the intracellular Zn^{2+} concentrations is dependent on the transmembrane Zn^{2+} gradient (Fukunaga *et al*., 2015). Thus, the simultaneous application of OIT and $ZnCl_2$ further increased the intracellular Zn^{2+} levels, which resulted in a disturbance in intracellular Zn^{2+} homeostasis. In thymocytes, an excessive increase in intracellular Zn^{2+} concentration promotes apoptosis (Mann and Fraker, 2005). Indeed, the combination of OIT and $ZnCl_2$ increased the number of cells with phosphatidylserine-exposed membranes, which is a marker for the early stages of apoptosis, without affecting cell lethality, whereas their application by themselves did not change the cell population (Figs. 2A and 2B). This combination action was completely diminished by removing external Zn^{2+} with DTPA (Fig. 2B). The Zn^{2+}-dependent phenomenon of OIT and $ZnCl_2$ was consistent with the hypothesis that the mobilization of intracellular Zn^{2+} elicits apoptosis (Mann and Fraker, 2005; Frazzini *et al*., 2006).

Micromolar concentrations of $ZnCl_2$ alone increase the cellular levels of non-protein thiol by moderately increasing intracellular Zn^{2+} levels (Kinazaki *et al*., 2011; Seo *et al*., 2012). Moderate increase in intracellular Zn^{2+} level is thought to be cytoprotective. However, the excessive increase in the intracellular Zn^{2+} levels by $ZnCl_2$ occurs under H_2O_2-induced oxidative stress, which increases the cell lethality (Matsui *et al*., 2010). Thus, the intracellular Zn^{2+} levels seem to determine whether Zn^{2+} is cytoprotective or cytotoxic. As shown in Fig. 3A, the simultaneous application of 3 μM $ZnCl_2$ and 0.1-1 μM OIT significantly reduced the cellular levels of non-protein thiol. The combination of 0.3 μM OIT and micromolar concentrations (1-3 μM) of $ZnCl_2$ also decreased the cellular levels of thiol (Fig. 3B). Thus, these results suggested that the combination of micromolar concentrations of $ZnCl_2$ and 0.1-1 μM OIT might induce oxidative stress (Fig. 3C).

Toxicological implications

Information regarding the adverse effects of the OIT that is released from polyvinyl alcohol towels is lacking, except for that related to contact dermatitis (Aalto-Korte *et al*., 2007; Fukunaga *et al*., 2010). The combination of OIT and $ZnCl_2$ increases intracellular Zn^{2+} levels (Fukunaga *et al*., 2015). Zinc is used as a nutritional supplement in adolescents, pregnant women, and infants (Chaffee and King, 2012; Kawade, 2012). The plasma zinc concentration is 10-20 μM and varies depending on nutritional conditions (Yokoi *et al*., 2003; Kuvibidila *et al*., 2006; Potocnik *et al*., 2006). Because Zn^{2+} binds to the sulfhydryl groups of proteins and non-proteins, plasma Zn^{2+} concentrations are thought to be much lower than the plasma zinc concentrations that were described in the above studies. In this study, the combination of 0.1 μM OIT and micromolar concentrations (1-3 μM) of Zn^{2+} significantly reduced the cellular levels of non-protein thiol (Figs. 3A and 3B). Thus, the OIT-induced adverse effects may be of concern because the threshold concentration (0.1 μM = 21 μg/L) of OIT that is required to induce Zn^{2+}-dependent cytotoxicity is lower than the OIT levels in polyvinyl alcohol towels (9.9-281 mg/kg-wt or 28-778 mg/kg-dry) and the minimum inhibitory concentration of OIT for *Aspergillus niger* (0.05 ± 0.01 mg/L) (Williams, 2007; Kawakami *et al*., 2014).

This study revealed that, in the presence of external Zn^{2+}, OIT greatly increased intracellular Zn^{2+} levels, which induced an early stage of apoptosis and which possibly occurred through the oxidative stress that was induced by Zn^{2+}. These findings suggested that OIT elicited the Zn^{2+}-dependent cytotoxic actions on lymphocytes.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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