Natural Science Research Univ. Tokushima (Peer-Reviewed Paper)
Vol. 24, No.3 (2010) p.13-20

$ZnCl_2$ and vitamin C, known as antioxidants, differently potentiate the cytotoxicity of H_2O_2 in rat thymocytes: Cytometric analysis using forward and side scatters

Takuya Kawanai*, Soya Harada*, Chiharu Kubota*, Erika Hashimoto, Yasuo Oyama

Laboratory of Cellular Signaling, Faculty of Integrated Arts and Sciences, The University of Tokushima, Tokushima 770-8502, Japan

Corresponding author: Yasuo OYAMA (E-mail: oyama@ias.tokushima-u.ac.jp)

* *Member of '2009 Journal Club for Undergraduate Studies'*

Abstract

The 'antioxidant hypothesis' proposes that antioxidant nutrients afford protection against chronic diseases by decreasing oxidative damages. The ability of zinc to retard oxidative processes has been recognized for many years. However, the application of $ZnCl_2$ potentiates the cytotoxicity of H_2O_2. Thus, some antioxidants may be cytotoxic under certain oxidative conditions. Therefore, in this study, the effect of vitamin C, one of antioxidant nutrients, on the cells treated with H_2O_2 has been examined to see if vitamin C potentiates the cytotoxicity of H_2O_2. Experiments were carried out with flow cytometer and rat thymocytes. Vitamin C also potentiated the cytotoxicity of H_2O_2. The increase in cell lethality induced by the combination of H_2O_2 and $ZnCl_2$ was associated with the increase in population of shrunken cells with increased intensity of side scatter. However, it was not the case for the combination of H_2O_2 and vitamin C. The profile of cytotoxicity induced by H_2O_2 and vitamin C was different from that by H_2O_2 and $ZnCl_2$. It may be suggested that the effects of zinc and vitamin C varies from cytoprotective to cytotoxic, being dependent on the type of oxidative stress.

Keywords: *Zinc; Vitamin C; Hydrogen peroxide; Cytotoxicity.*

1. Introduction

Zinc and vitamin C supplements are available for common people to promote and/or maintain good health conditions (Prasad, 2008; Mandl et al., 2009; Maggini et al., 2010). However, $ZnCl_2$ potentiates the cytotoxicity of H_2O_2 in rat thymocytes (Matsui et al., 2009) by inducing excessive increase in intracellular Zn^{2+} concentration (Matsui et al., 2010). Thus, although the ability of zinc to retard oxidative processes has been recognized for many years, zinc is cytotoxic under certain oxidative stress. Vitamin C is also known as an anti-oxidant and it protects the body against oxidative stress (Padayatty et al., 2003). However, hydroxyl radical, a potent oxidative radical, is generated by vitamin C and H_2O_2, being independent of mediation by transition metals (Smirnoff and Cumbes, 1989; Nappi and Vass, 2000). Therefore, the combination of vitamin C and H_2O_2 may induce cytotoxic action. In this study, the effects of $ZnCl_2$ and vitamin C on the cytotoxicity of H_2O_2 were studied to see if vitamin C potentiates the cytotoxicity of H_2O_2 and to compare the actions of $ZnCl_2$ and vitamin C on H_2O_2 cytotoxicity.

The 'antioxidant hypothesis' proposes that antioxidant nutrients afford protection against chronic diseases by decreasing oxidative damages. However, randomized primary and secondary intervention trials failed to show any consistent benefit from the use of antioxidant supplements on cardiovascular diseases or cancer risk, with some trials even suggesting possible harm in certain subgroups (Stanner et al., 2004). Since both zinc and vitamin C are recognized to be against oxidative stress, this study may give an insight to elucidate the cytotoxic action of 'antioxidants' under certain oxidative condition.

2. Materials and methods

2.1. Chemicals

H_2O_2 was purchased from Sumitomo Chemical Co. (Osaka, Japan). Propidium iodide was obtained from Molecular Probes Inc. (Eugene, Oregon, USA).

Other chemicals (NaCl, $CaCl_2$, $MgCl_2$, KCl, glucose, HEPES, NaOH, and $ZnCl_2$) were supplied from Woko Pure Chemicals (Osaka, Japan).

2.2. Animals and cell preparation

This study was approved by the Committee for Animal Experiments in the University of Tokushima (No. 05279). The procedure to prepare cell suspension was similar to that previously reported (Chikahisa and Oyama, 1992; Chikahisa et al., 1996). In brief, thymus glands dissected from Wistar rats under ether anesthesia were sliced at a thickness of 400-500 μm with razor under chilled condition (3-4ºC). Thereafter, the slices were triturated by gently shaking in chilled Tyrode's solution (in mM: NaCl 150, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 5, HEPES 5, with an appropriate amount of NaOH to adjust pH to 7.3-7.4) to dissociate thymocytes. Then, the Tyrode's solution containing the cells was passed through a mesh (a diameter of 53 μm) to prepare cell suspension. The beaker containing the cell suspension was bathed at 36-37ºC for 1 hr before the experiment.

Rat thymocytes were used for present study because of following reasons. First, the cell membranes of thymocytes remain intact because single cells can be prepared without enzymatic treatment. Second, the process of cell death is extensively studied in murine thymocytes (McConkey et al., 1994; Quaglino and Ronchetti, 2001; Ortiz et al., 2001; Thompson et al., 2003).

2.3. Fluorescence measurements of cellular parameters

The methods for measurements of cellular parameters using a flow cytometer (CytoACE-150, JASCO, Tokyo, Japan) and propidium iodide were similar to those previously described (Chikahisa and Oyama, 1992; Chikahisa et al., 1996; Matsui et al., 2008). The fluorescence was analyzed by JASCO software (Ver.3XX, JASCO). There was no fluorescence from reagents used in this study, except for fluorescent probes, under our experimental condition. The cytogram (forward scatter *versus* side scatter) was constructed from 2×10^3 cells. Forward scatter is a parameter for cell size while side scatter represents intracellular density.

To assess cell lethality, propidium iodide was added to cell suspension to achieve a final concentration of 5 μM. Since propidium stains dead cells and the cells having compromised membranes, the measurement of propidium fluorescence from cells provides a clue to estimate the cell lethality. The fluorescence was measured at 2 min after the application of propidium iodide by a flow cytometer. Excitation wavelength for propidium was 488 nm and emission was detected at 600 ± 20 nm.

2.4. Experimental protocol

H_2O_2 (2 μl) was added to the cell suspension (2 ml). The cell density was about 5×10^5 cells/ml. To examine the effects of $ZnCl_2$ and vitamin C, the agents were respectively added to the suspension just after applying H_2O_2. The cells were incubated with respective agent and H_2O_2 at 36-37°C under room air condition. The data acquisition of propidium fluorescence from 2×10^3 cells by a flow cytometer required 10 sec at least.

Effect of H_2O_2 at concentration of 1, 3 and 10 mM on thymocytes was examined in preliminary study. Thymocytes were tolerant to oxidative stress induced by 1 mM H_2O_2 during a period of 3 hr after adding H_2O_2 into cell suspension. It was likely that H_2O_2 at 3-10 mM was lethal for thymocytes during exposure lasting for 2 hr or longer because the prolonged exposure of thymocytes to 3-10 mM H_2O_2 produced a time dependent increase in the population of cells stained with propidium, indicating a time-dependent increase in cell lethality. The time dependent change in population of cells exerting propidium fluorescence by 10 mM H_2O_2 was more rapid than that by 3 mM H_2O_2. The incubation with 3 mM H_2O_2 for 2 hr induces only small increase (less than 10%) in cell lethality that was suitable to study the actions of $ZnCl_2$ and vitamin C on H_2O_2-induced cytotoxicity.

2.5. Statistics

Values were expressed as mean ± standard deviation of 4 experiments. Statistical analysis on significance was performed with Tukey multivariate method. A P value of < 0.05 was considered significant. However, small difference (5 % or less of the control) was not applicable for statistical analysis because the control values varied within ± 2.5 % in some cases.

3. Results

3.1. Changes in cell lethality by $ZnCl_2$, vitamin C, H_2O_2, and their combinations

The cells were respectively incubated with 100 μM $ZnCl_2$, 50 μM and 500 μM vitamin C, 3 mM H_2O_2, and the combinations with H_2O_2 for 2 hr. As shown in Fig. 1, the population of cells stained with propidium (dead cells and/or the cells having compromised membranes) in the presence of $ZnCl_2$, vitamin C, and H_2O_2 alone was similar to the control. The incubation of cells with the combination of H_2O_2 with $ZnCl_2$ or vitamin C increased the population of cells stained with propidium. The results were summarized in Fig. 2. The lethality of cells incubated with the combination of H_2O_2 with $ZnCl_2$ or vitamin C was significantly higher than those of control cells and the cells respectively incubated with $ZnCl_2$, vitamin C, and H_2O_2.

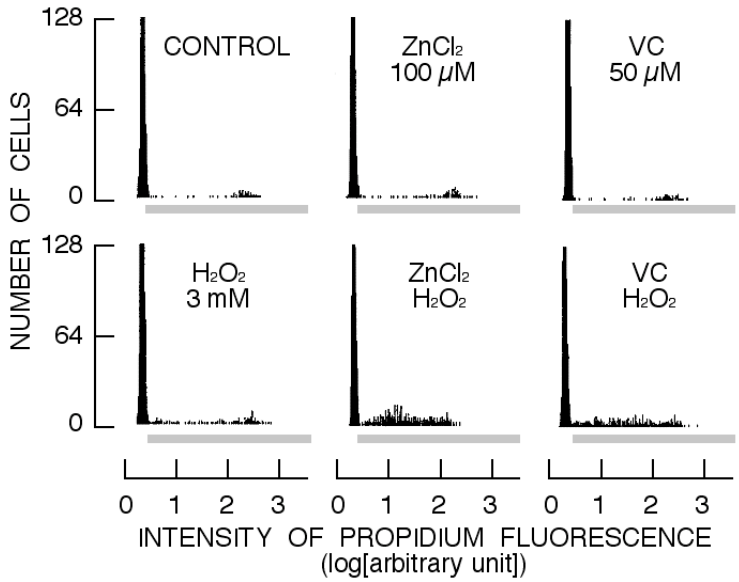

Figure 1. *Population of dead cells and/or the cells having compromised membranes accessed with cell stainability with propidium iodide. Histogram of intensity of propidium fluorescence was constructed with 2000 cells after 2 hr incubation with $ZnCl_2$, vitamin C, H_2O_2, or their combinations (H_2O_2 and $ZnCl_2$, H_2O_2 and vitamin C). Dotted line indicates the population of cells exerting propidium fluorescence in the histogram.*

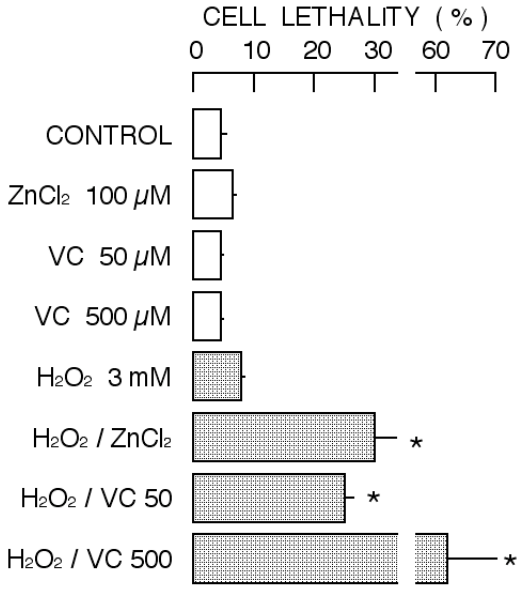

Figure 2. *Change in cell lethality accessed with cell stainability with propidium iodide. In this study, the cell lethality was percentage population of cells stained with propidium. Column and bar indicate mean and standard deviation of four experiments. Symbol (*) show significant difference ($P<0.05$) between control cell group and the group of cells treated with $ZnCl_2$, vitamin C, H_2O_2, or their combination.*

3.2. Changes in cell populations by $ZnCl_2$, vitamin C, H_2O_2, and their combinations

There was one population in the cytogram (forward scatter *versus* side scatter) under control condition (Area A of Fig. 3). However, the incubation of rat thymocytes with cytotoxic substances usually increases the population of cells with decreased intensity of forward scatter and increased intensity of side scatter (Area B of Fig. 3), resulting in two distinctive populations in the cytogram (Nakao et al., 2003; Iwase et al., 2004; Yamaguchi et al., 2005; Chikutei et al., 2006; Horimoto et al., 2006: Nishimura et al., 2006, 2008; Oyama et al., 2007; Fujimoto et al., 2009; Kinazaki et al., 2009; Sakanashi et al., 2009; Ogata et al., 2010). The increase in cell lethality induced by cytotoxic substances is usually associated with the increase in the population of shrunken cells, the cells with decreased intensity of forward scatter and increased intensity of side scatter, as shown in the cytogram obtained from the cells simultaneously incubated with 100 μM $ZnCl_2$ and 3 mM H_2O_2 (Figs. 3 and 4). However, it was not the case for the cytogram in the combination of H_2O_2 with vitamin C (Figs. 2, 3, and 4). Thus, the combination of 3 mM H_2O_2 with 50 μM or 500 μM vitamin C increased the lethality without significant increase in the population of cells belonging to Area B.

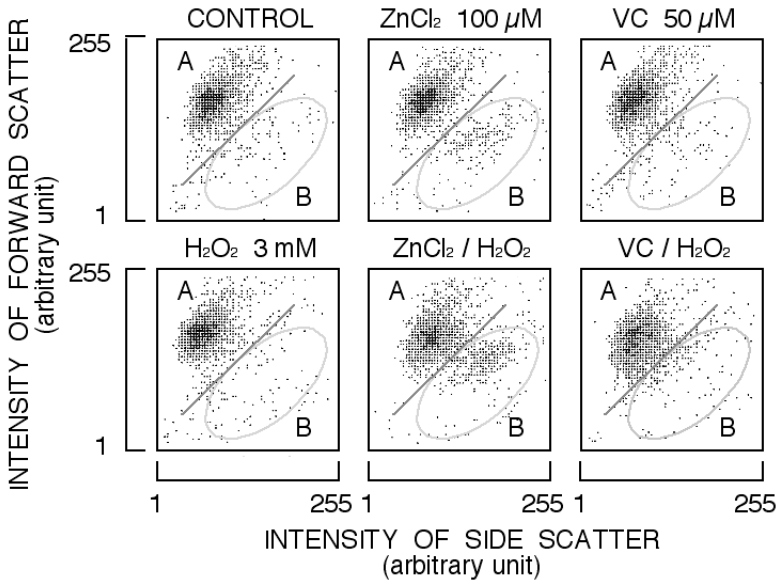

Figure 3. *Cytogram (forward scatter versus side scatter) of cells incubated with $ZnCl_2$, vitamin C, H_2O_2, or their combination for 2 hr. Cytogram was constructed with 2000 cells. Cytogram was tentatively divided to two areas, A and B, by the eye-drawn line that was based on forward and side scatters. The circle in Area B was drawn to emphasize the cell population that appeared in the case of the combination of H_2O_2 and $ZnCl_2$.*

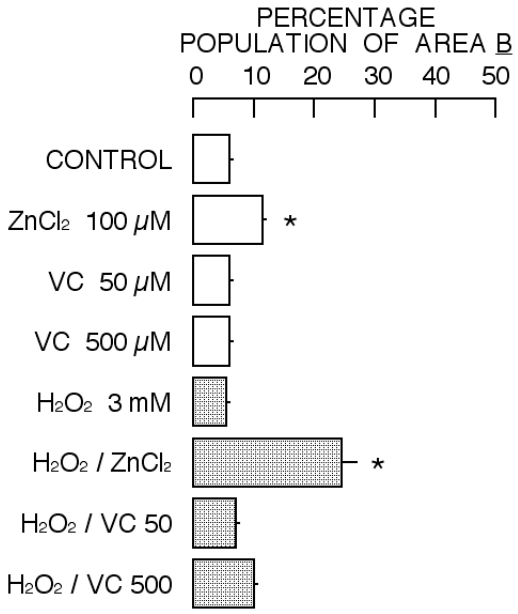

Figure 4. *Change in percentage population in Area B by 2 hr incubation with $ZnCl_2$, vitamin C, H_2O_2, or their combination. Column and bar indicate mean and standard deviation of four experiments. Symbol (*) show significant difference ($P<0.05$) between control cell group and the group of cells treated with $ZnCl_2$, vitamin C, H_2O_2, or their combination.*

3.3. Changes in forward and side scatters by $ZnCl_2$, vitamin C, H_2O_2, and their combinations

The profile of cytotoxicity induced by the combination of 3 mM H_2O_2 with 50 μM or 500 μM vitamin C seems to be against the cytometric concept described above and previously (Nakao et al., 2003; Iwase et al., 2004; Yamaguchi et al., 2005; Chikutei et al., 2006; Horimoto et al., 2006: Nishimura et al., 2006, 2008; Oyama et al., 2007; Fujimoto et al., 2009; Kinazaki et al., 2009; Sakanashi et al., 2009; Ogata et al., 2010). Therefore, the change in profile of cell population belonging to Area A of cytogram was further analyzed. As shown in Fig. 5, the simultaneous incubation of H_2O_2 and $ZnCl_2$ induced significant decrease in cell population of Area A while it was not the case for others. The intensity of forward scatter was significantly decreased by the respective combination of 3 mM H_2O_2 with 100 μM $ZnCl_2$, 50 μM vitamin C, or 500 μM vitamin C (Fig. 6). The decrease in forward scatter intensity by the combination of H_2O_2 and vitamin C was more than that by the combination of H_2O_2 and $ZnCl_2$. The intensity of side scatter was significantly increased by the combination of 3 mM H_2O_2 with 100 μM $ZnCl_2$ or 500 μM vitamin C (Fig. 7). The increase in side scatter intensity by the combination of H_2O_2 and vitamin C was less than that by the combination of H_2O_2 and $ZnCl_2$. The profile of cytogram of cells simultaneously incubated with H_2O_2 and $ZnCl_2$ was different from that with H_2O_2 and vitamin C.

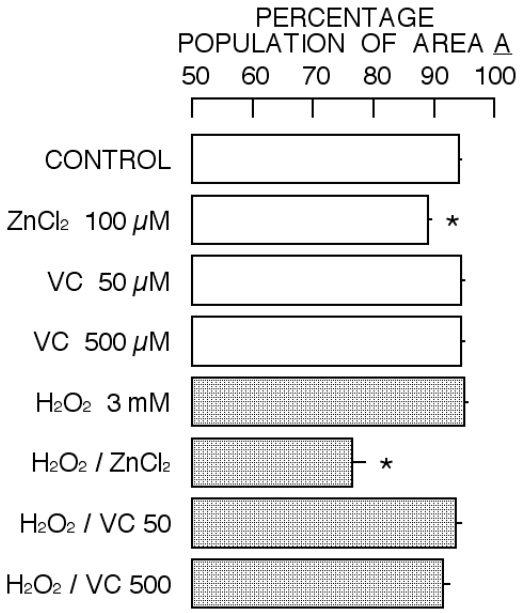

Figure 5. *Change in percentage population in Area A by 2 hr incubation with $ZnCl_2$, vitamin C, H_2O_2, or their combination. Column and bar indicate mean and standard deviation of four experiments. Symbol (*) show significant difference ($P<0.05$) between control cell group and the group of cells treated with $ZnCl_2$, vitamin C, H_2O_2, or their combination.*

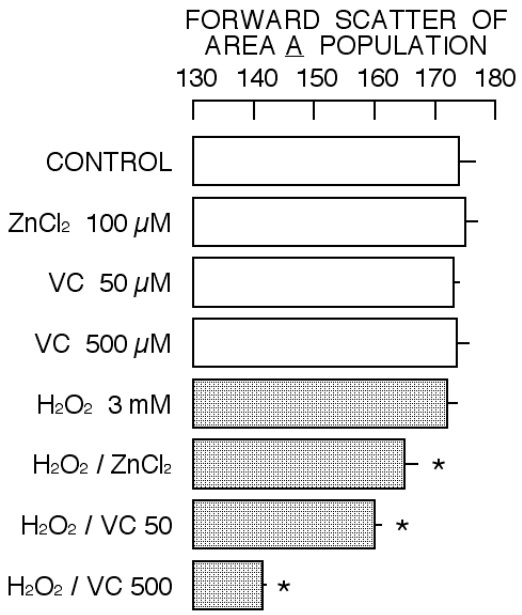

Figure 6. *Change in forward scatter, a parameter for cells size, of cells belonging to Area A by 2 hr incubation with $ZnCl_2$, vitamin C, H_2O_2, or their combination. Decrease in forward scatter indicates shrinkage. Symbol (*) show significant difference ($P<0.05$) between control cell group and the group of cells treated with $ZnCl_2$, vitamin C, H_2O_2, or their combination.*

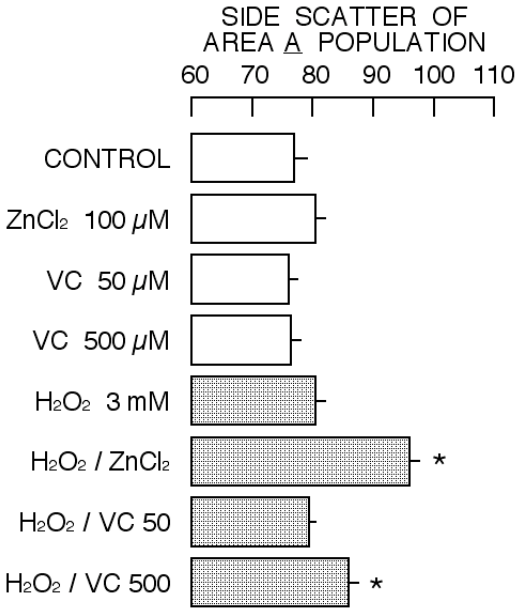

Figure 7. *Change in side scatter, a parameter for cellular density, of cells belonging to Area A by 2 hr incubation with $ZnCl_2$, vitamin C, H_2O_2, or their combination. Symbol (*) show significant difference ($P<0.05$) between control cell group and the group of cells treated with $ZnCl_2$, vitamin C, H_2O_2, or their combination.*

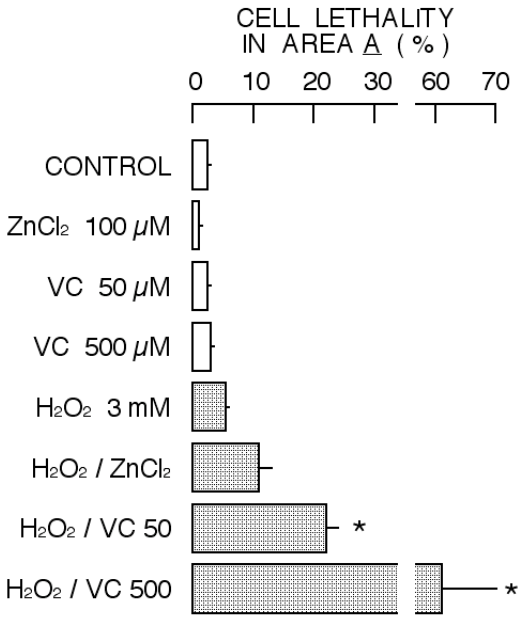

Figure 8. *Change in cell lethality in population belonging to Area A by 2 hr incubation with $ZnCl_2$, vitamin C, H_2O_2, or their combination. Column and bar indicate mean and standard deviation of four experiments. Symbol (*) show significant difference ($P<0.05$) between control cell group and the group of cells treated with $ZnCl_2$, vitamin C, H_2O_2, or their combination.*

3.4. Changes in cell lethality of populations classified with forward and side scatters

The cell lethality in Area A population is usually much less than that in Area B population as also shown in Figs. 8 and 9. The lethality of control cell population in Area A was 2.6 ± 0.2 % while it was 33.9 ± 2.5 % in Area B. Similar tendency was observed in the cases of 100 μM $ZnCl_2$, 50 μM and 500 μM vitamin C, 3 mM H_2O_2 (Figs. 8 and 9). The respective combination of H_2O_2 with $ZnCl_2$ or vitamin C increased the cell lethality in both areas. Though the increase in lethality of Area B population by the combination of H_2O_2 with $ZnCl_2$ was significantly more than that by the combination of H_2O_2 with vitamin C, it was opposite in Area A population (Figs. 8 and 9).

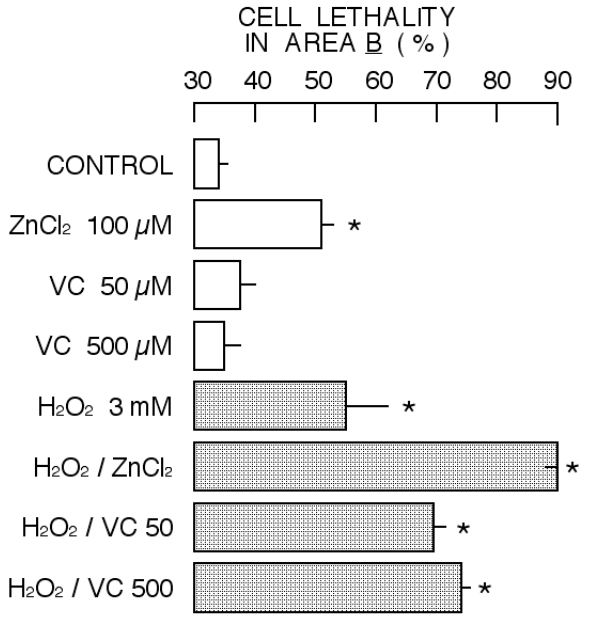

Figure 9. *Change in cell lethality in population belonging to Area B by 2 hr incubation with $ZnCl_2$, vitamin C, H_2O_2, or their combination. Column and bar indicate mean and standard deviation of four experiments. Symbol (*) show significant difference ($P<0.05$) between control cell group and the group of cells treated with $ZnCl_2$, vitamin C, H_2O_2, or their combination.*

4. Discussion

Both $ZnCl_2$ and vitamin C, known as antioxidants, potentiated the cytotoxicity of H_2O_2 (Figs. 1 and 2). While the cell lethality induced by $ZnCl_2$, vitamin C, or H_2O_2 alone was less than 10%, the combination of H_2O_2 with $ZnCl_2$ or vitamin C increased the lethality to more than 20% (Fig. 2). In the combination of 3 mM H_2O_2 and 500 μM vitamin C, the cell lethality was more than 60%. The simultaneous application of $ZnCl_2$ and vitamin C seems to synergistically potentiate the cytotoxicity of H_2O_2 in rat thymocytes when their incubation time was 2 hr. Therefore, zinc ($ZnCl_2$) and vitamin C may exert cytotoxic action under certain oxidative conditions.

The treatment of cells with cytotoxic substances usually increases the population of cells with decreased intensity of forward scatter and increased intensity of side scatter (Area B of Fig. 3), resulting in two distinctive populations in the cytogram (Nakao et al., 2003; Iwase et al., 2004; Nishimura et al., 2006, 2008; Chikutei et al., 2006; Oyama et al., 2007; Sakanashi et al., 2009; Ogata et al., 2010). Forward scatter is a parameter for cell size while side scatter reflects cellular density. Thus, cytotoxic substances increase the population of shrunken cells with increased cellular density. The cytotoxic profile in the combination of 3 mM H_2O_2 with 100 μM $ZnCl_2$ supports the 'concept' described above (Figs. 3, 4, and 5). However, the profile of cytotoxicity induced by the combination of H_2O_2 and vitamin C was different from that in the case of H_2O_2 and $ZnCl_2$. The population of shrunken cells with increased cellular density did not significantly increase in the case of H_2O_2 and vitamin C (Fig. 5). Thus, the combination of H_2O_2 and vitamin C increased the cell lethality without significantly affecting cellular density (Fig. 7). The increase in cell lethality of Area A population by the combination of H_2O_2 and vitamin C was higher than that by the combination of H_2O_2 and $ZnCl_2$ while it was opposite in Area B (Figs. 8 and 9).

The cell death induced by H_2O_2 is potentiated by $ZnCl_2$ and suppressed by TPEN, a chelator for intracellular Zn^{2+} (Matsui et al., 2009). The simultaneous application of H_2O_2 and $ZnCl_2$ greatly increases intracellular Zn^{2+} concentration (Matsui et al., 2010). Therefore, the phenomenon via the increase in intracellular Zn^{2+} concentration is involved. In the case of vitamin C, the cell death may be resulted from its interaction with H_2O_2 that produces hydroxyl radical in extracellular space (Smirnoff and Cumbes, 1989; Nappi and Vass, 2000). If so in present study, hydroxyl radical would deteriorate cell membrane, resulting in the increase in population of cells stained with propidium iodide. It may be suggested that zinc and vitamin C differently induce cytotoxic action under certain oxidative conditions although these substances are known to possess antioxidant activity.

Acknowledgement

The study was supported by the grant (2009) from the Office of Dean, Faculty of Integrated Arts and Sciences, The University of Tokushima, Tokushima, Japan.

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Received June 30, 2010
Accepted July 16, 2010