

Natural Science Research Univ. Tokushima (Peer-Reviewed Paper)  
Vol. 24, No.3 (2010) p.12-19

## ZnCl<sub>2</sub> and vitamin C, known as antioxidants, differently potentiate the cytotoxicity of H<sub>2</sub>O<sub>2</sub> 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](mailto: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<sub>2</sub> potentiates the cytotoxicity of H<sub>2</sub>O<sub>2</sub>. 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<sub>2</sub>O<sub>2</sub> has been examined to see if vitamin C potentiates the cytotoxicity of H<sub>2</sub>O<sub>2</sub>. Experiments were carried out with flow cytometer and rat thymocytes. Vitamin C also potentiated the cytotoxicity of H<sub>2</sub>O<sub>2</sub>. The increase in cell lethality induced by the combination of H<sub>2</sub>O<sub>2</sub> and ZnCl<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> and vitamin C. The profile of cytotoxicity induced by H<sub>2</sub>O<sub>2</sub> and vitamin C was different from that by H<sub>2</sub>O<sub>2</sub> and ZnCl<sub>2</sub>. 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<sub>2</sub> potentiates the cytotoxicity of H<sub>2</sub>O<sub>2</sub> in rat thymocytes (Matsui et al., 2009) by inducing excessive increase in intracellular Zn<sup>2+</sup> 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<sub>2</sub>O<sub>2</sub>, being independent of mediation by transition metals (Smirnoff and Cumbes, 1989; Nappi and Vass, 2000). Therefore, the combination of vitamin C and H<sub>2</sub>O<sub>2</sub> may induce cytotoxic action. In this study, the effects of ZnCl<sub>2</sub> and vitamin C on the cytotoxicity of H<sub>2</sub>O<sub>2</sub> were studied to see if vitamin C potentiates the

cytotoxicity of H<sub>2</sub>O<sub>2</sub> and to compare the actions of ZnCl<sub>2</sub> and vitamin C on H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> was purchased from Sumitomo Chemical Co. (Osaka, Japan). Propidium iodide was obtained from Molecular Probes Inc. (Eugene, Oregon, USA).

Other chemicals (NaCl, CaCl<sub>2</sub>, MgCl<sub>2</sub>, KCl, glucose, HEPES, NaOH, and ZnCl<sub>2</sub>) were supplied from Wako 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  $\mu\text{m}$  with razor under chilled condition (3-4°C). Thereafter, the slices were triturated by gently shaking in chilled Tyrode's solution (in mM: NaCl 150, KCl 5, CaCl<sub>2</sub> 2, MgCl<sub>2</sub> 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  $\mu\text{m}$ ) to prepare cell suspension. The beaker containing the cell suspension was bathed at 36-37°C 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  $2 \times 10^3$  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  $\mu\text{M}$ . 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  $600 \pm 20$  nm.

## 2.4. Experimental protocol

H<sub>2</sub>O<sub>2</sub> (2  $\mu\text{l}$ ) was added to the cell suspension (2 ml). The cell density was about  $5 \times 10^5$  cells/ml. To examine the effects of ZnCl<sub>2</sub> and vitamin C, the agents were respectively added to the suspension just after applying H<sub>2</sub>O<sub>2</sub>. The cells were incubated with respective agent and H<sub>2</sub>O<sub>2</sub> at 36-37°C under room air condition. The data acquisition of propidium fluorescence from  $2 \times 10^3$  cells by a flow cytometer required 10 sec at least.

Effect of H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> during a period of 3 hr after adding H<sub>2</sub>O<sub>2</sub> into cell suspension. It was likely that H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> was more rapid than that by 3 mM H<sub>2</sub>O<sub>2</sub>. The incubation with 3 mM H<sub>2</sub>O<sub>2</sub> for 2 hr induces only small increase (less than 10%) in cell lethality that was suitable to study the actions of ZnCl<sub>2</sub> and vitamin C on H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity.

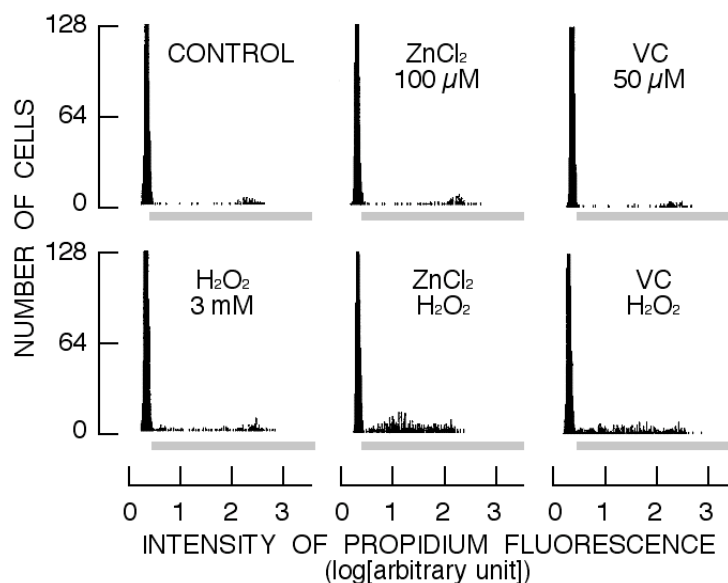
## 2.5. Statistics

Values were expressed as mean  $\pm$  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  $\pm 2.5$  % in some cases.

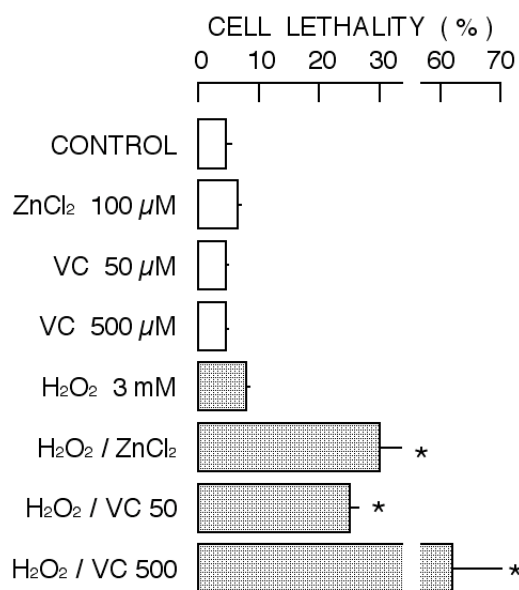
## 3. Results

### 3.1. Changes in cell lethality by ZnCl<sub>2</sub>, vitamin C, H<sub>2</sub>O<sub>2</sub>, and their combinations

The cells were respectively incubated with 100  $\mu\text{M}$  ZnCl<sub>2</sub>, 50  $\mu\text{M}$  and 500  $\mu\text{M}$  vitamin C, 3 mM H<sub>2</sub>O<sub>2</sub>, and the combinations with H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>, vitamin C, and H<sub>2</sub>O<sub>2</sub> alone was similar to the control. The incubation of cells with the combination of H<sub>2</sub>O<sub>2</sub> with ZnCl<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> with ZnCl<sub>2</sub> or vitamin C was significantly higher than those of control cells and the cells respectively incubated with ZnCl<sub>2</sub>, vitamin C, and H<sub>2</sub>O<sub>2</sub>.



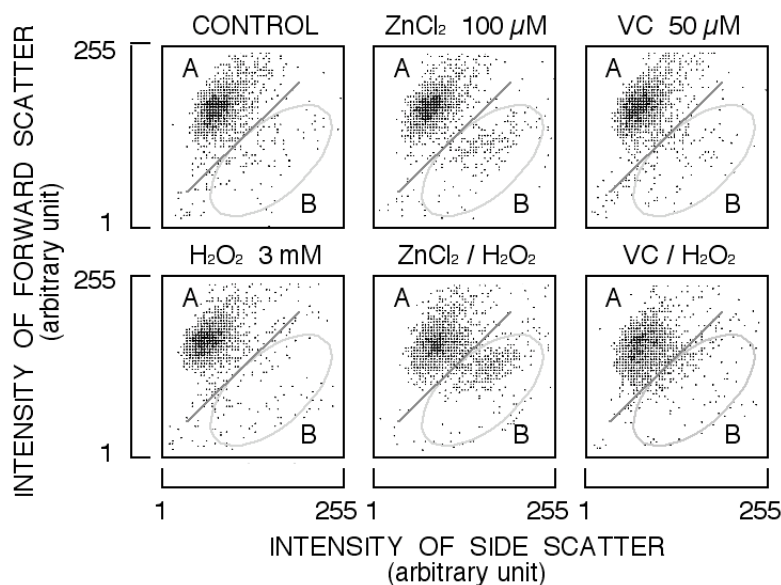
**Figure 1.** Population of dead cells and/or the cells having compromised membranes accessed with cell stainability with propidium iodide. Histogram of intensity of propidium fluorescence was constructed with 2000 cells after 2 hr incubation with ZnCl<sub>2</sub>, vitamin C, H<sub>2</sub>O<sub>2</sub>,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



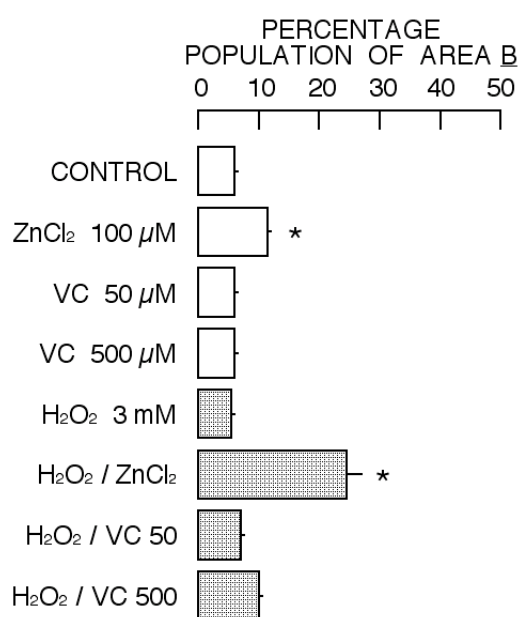
**Figure 2.** Change in cell lethality accessed with cell stainability with propidium iodide. In this study, the cell lethality was percentage population of cells stained with propidium. Column and bar indicate mean and standard deviation of four experiments. Symbol (\*) show significant difference (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

### 3.2. Changes in cell populations by ZnCl<sub>2</sub>, vitamin C, H<sub>2</sub>O<sub>2</sub>, 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<sub>2</sub> and 3 mM H<sub>2</sub>O<sub>2</sub> (Figs. 3 and 4). However, it was not the case for the cytogram in the combination of H<sub>2</sub>O<sub>2</sub> with vitamin C (Figs. 2, 3, and 4). Thus, the combination of 3 mM H<sub>2</sub>O<sub>2</sub> 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  $ZnCl_2$ .

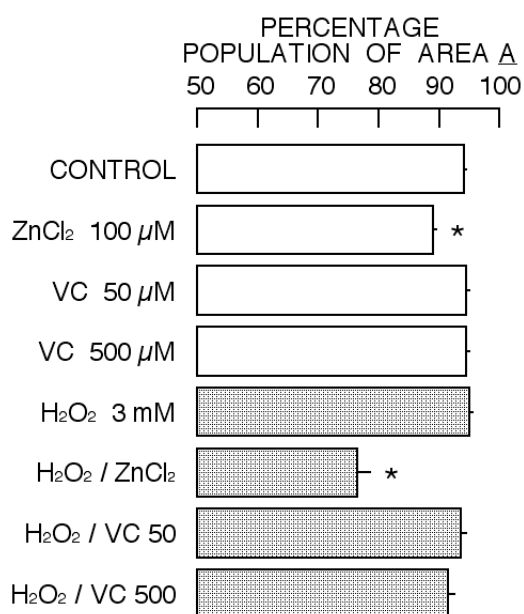


**Figure 4.** Change in percentage population in Area B by 2 hr incubation with  $ZnCl_2$ , vitamin C,  $H_2O_2$ , or their combination. Column and bar indicate mean and standard deviation of four experiments. Symbol (\*) show significant difference ( $P < 0.05$ ) between control cell group and the group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

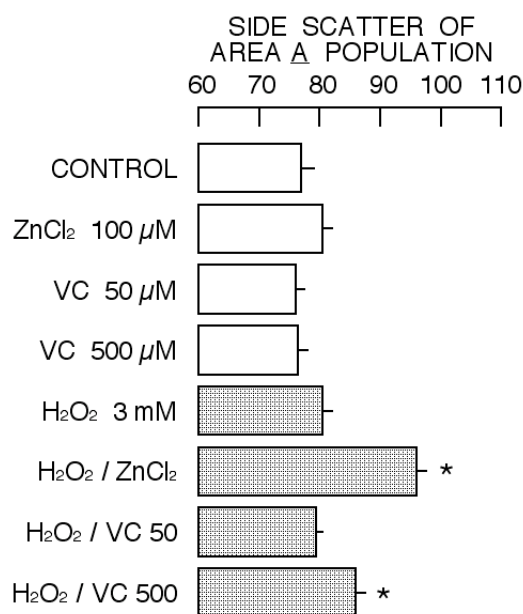
### 3.3. Changes in forward and side scatters by $ZnCl_2$ , vitamin C, $H_2O_2$ , and their combinations

The profile of cytotoxicity induced by the combination of 3 mM  $H_2O_2$  with 50  $\mu M$  or 500  $\mu M$  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  $\mu M$   $ZnCl_2$ , 50  $\mu M$  vitamin C, or 500  $\mu M$  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  $\mu M$   $ZnCl_2$  or 500  $\mu M$  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.

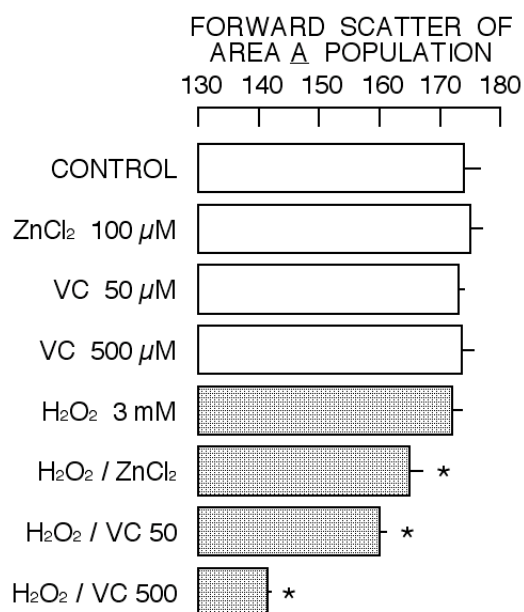
Effects of ZnCl<sub>2</sub> and vitamin C on H<sub>2</sub>O<sub>2</sub> cytotoxicity



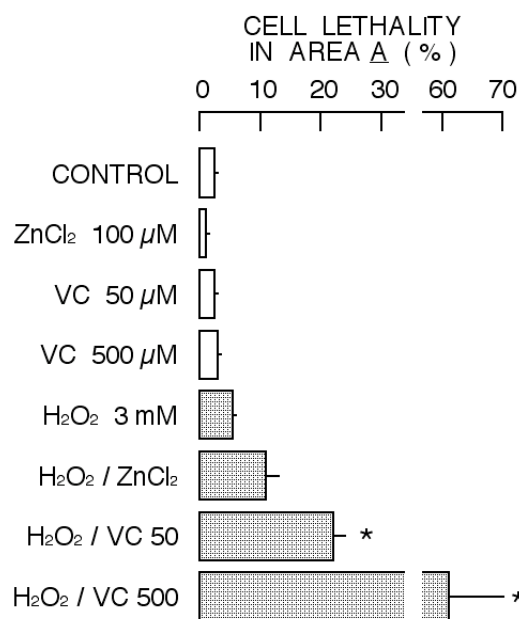
**Figure 5.** Change in percentage population in Area A by 2 hr incubation with ZnCl<sub>2</sub>, vitamin C, H<sub>2</sub>O<sub>2</sub>, or their combination. Column and bar indicate mean and standard deviation of four experiments. Symbol (\*) show significant difference (P<0.05) between control cell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

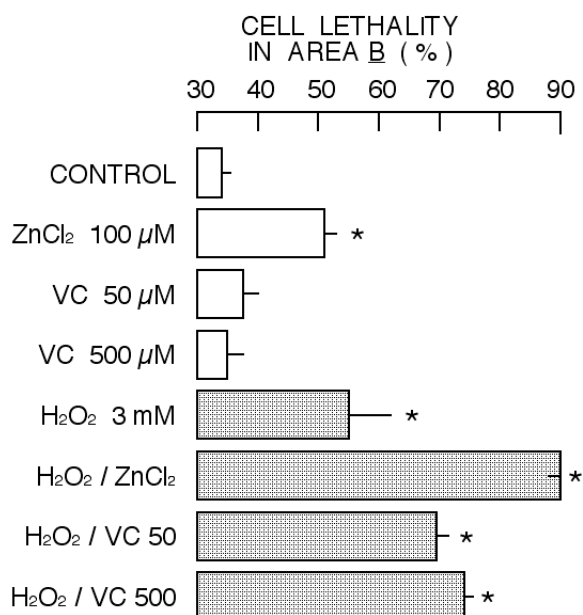


**Figure 8.** Change in cell lethality in population belonging to Area A by 2 hr incubation with ZnCl<sub>2</sub>, vitamin C, H<sub>2</sub>O<sub>2</sub>, or their combination. Column and bar indicate mean and standard deviation of four experiments. Symbol (\*) show significant difference (P<0.05) between control cell group and the group of cells treated with ZnCl<sub>2</sub>, vitamin C, H<sub>2</sub>O<sub>2</sub>, 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  $2.6 \pm 0.2$  % while it was  $33.9 \pm 2.5$  % in Area B. Similar tendency was observed in the cases of  $100 \mu\text{M}$   $\text{ZnCl}_2$ ,  $50 \mu\text{M}$  and  $500 \mu\text{M}$  vitamin C,  $3 \text{ mM}$   $\text{H}_2\text{O}_2$  (Figs. 8 and 9). The respective combination of  $\text{H}_2\text{O}_2$  with  $\text{ZnCl}_2$  or vitamin C increased the cell lethality in both areas. Though the increase in lethality of Area B population by the combination of  $\text{H}_2\text{O}_2$  with  $\text{ZnCl}_2$  was significantly more than that by the combination of  $\text{H}_2\text{O}_2$  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  $\text{ZnCl}_2$ , vitamin C,  $\text{H}_2\text{O}_2$ , 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  $\text{ZnCl}_2$ , vitamin C,  $\text{H}_2\text{O}_2$ , or their combination.

## 4. Discussion

Both  $\text{ZnCl}_2$  and vitamin C, known as antioxidants, potentiated the cytotoxicity of  $\text{H}_2\text{O}_2$  (Figs. 1 and 2). While the cell lethality induced by  $\text{ZnCl}_2$ , vitamin C, or  $\text{H}_2\text{O}_2$  alone was less than 10%, the combination of  $\text{H}_2\text{O}_2$  with  $\text{ZnCl}_2$  or vitamin C increased the lethality to more than 20% (Fig. 2). In the combination of  $3 \text{ mM}$   $\text{H}_2\text{O}_2$  and  $500 \mu\text{M}$  vitamin C, the cell lethality was more than 60%. The simultaneous application

of  $\text{ZnCl}_2$  and vitamin C seems to synergistically potentiate the cytotoxicity of  $\text{H}_2\text{O}_2$  in rat thymocytes when their incubation time was 2 hr. Therefore, zinc ( $\text{ZnCl}_2$ ) 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  $3 \text{ mM}$   $\text{H}_2\text{O}_2$  with  $100 \mu\text{M}$   $\text{ZnCl}_2$  supports the 'concept' described above (Figs. 3, 4, and 5). However, the profile of cytotoxicity induced by the combination of  $\text{H}_2\text{O}_2$  and vitamin C was different from that in the case of  $\text{H}_2\text{O}_2$  and  $\text{ZnCl}_2$ . The population of shrunken cells with increased cellular density did not significantly increase in the case of  $\text{H}_2\text{O}_2$  and vitamin C (Fig. 5). Thus, the combination of  $\text{H}_2\text{O}_2$  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  $\text{H}_2\text{O}_2$  and vitamin C was higher than that by the combination of  $\text{H}_2\text{O}_2$  and  $\text{ZnCl}_2$  while it was opposite in Area B (Figs. 8 and 9).

The cell death induced by  $\text{H}_2\text{O}_2$  is potentiated by  $\text{ZnCl}_2$  and suppressed by TPEN, a chelator for intracellular  $\text{Zn}^{2+}$  (Matsui et al., 2009). The simultaneous application of  $\text{H}_2\text{O}_2$  and  $\text{ZnCl}_2$  greatly increases intracellular  $\text{Zn}^{2+}$  concentration (Matsui et al., 2010). Therefore, the phenomenon via the increase in intracellular  $\text{Zn}^{2+}$  concentration is involved. In the case of vitamin C, the cell death may be resulted from its interaction with  $\text{H}_2\text{O}_2$  that produces hydroxyl radical in extracellular space (Smirnov 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.

## References

- Chikahisa, L., Oyama, Y., 1992. Tri-n-butyltin increases intracellular Ca<sup>2+</sup> in mouse thymocytes: a flow-cytometric study using fluorescent dyes for membrane potential and intracellular Ca<sup>2+</sup>. *Pharmacol. Toxicol.* 71, 190-195.
- Chikahisa, L., Oyama, Y., Okazaki, E., Noda, K., 1996. Fluorescent estimation of H<sub>2</sub>O<sub>2</sub>-induced changes in cell viability and cellular nonprotein thiol level of dissociated rat thymocytes. *Jpn. J. Pharmacol.* 71, 299-305.
- Chikutei, K., Oyama, T.M., Ishida, S., Okano, Y., Kobayashi, M., Matsui, H., Horimoto, K., Nishimura, Y., Ueno, S.Y., Oyama, Y., 2006. Propofol, an anesthetic possessing neuroprotective action against oxidative stress, promotes the process of cell death induced by H<sub>2</sub>O<sub>2</sub> in rat thymocytes. *Eur. J. Pharmacol.* 540, 18-23.
- Fujimoto, A., Sakanashi, Y., Matsui, H., Oyama, T., Nishimura, Y., Masuda, T., Oyama, Y., 2009. Cytometric analysis of cytotoxicity of polyphenols and related phenolics to rat thymocytes: potent cytotoxicity of resveratrol to normal cells. *Basic Clin. Pharmacol. Toxicol.* 104, 455-462.
- Horimoto, K., Nishimura, Y., Oyama, T.M., Onoda, K., Matsui, H., Oyama, T.B., Kanemaru, K., Masuda, T., Oyama, Y., 2006. Reciprocal effects of glucose on the process of cell death induced by calcium ionophore or H<sub>2</sub>O<sub>2</sub> in rat lymphocytes. *Toxicol.* 225, 97-108.
- Iwase, K., Tatsuishi, T., Nishimura, Y., Yamaguchi, J.Y., Oyama, Y., Miyoshi, N., Wada, M., 2004. Cytometric analysis of adverse action of diphenyl ditelluride on rat thymocytes: cell shrinkage as a cytotoxic parameter. *Environ. Toxicol.* 19, 614-619.
- Kinazaki, A., Sakanashi, Y., Oyama, T.M., Shibagaki, H., Yamashita, K., Hashimoto, E., Nishimura, Y., Ishida, S., Okano, Y., Oyama, Y., 2009. Micromolar Zn<sup>2+</sup> potentiates the cytotoxic action of submicromolar econazole in rat thymocytes: possible disturbance of intracellular Ca<sup>2+</sup> and Zn<sup>2+</sup> homeostasis. *Toxicol. In Vitro.* 23, 610-616.
- Maggini, S., Wenzlaff, S., Hornig, D., 2010. Essential role of vitamin C and zinc in child immunity and health. *J. Int. Med. Res.* 38, 386-414.
- Mandl, J., Szarka, A., Bánhegyi, G., 2009. Vitamin C: update on physiology and pharmacology. *Br. J. Pharmacol.* 157, 1097-1110.
- Matsui, H., Sakanashi, Y., Nishimura, Y., Kawanai, T., Oyama, Y., Ishida, Y., Okano, Y., 2009. Effects of Zn<sup>2+</sup> chelators, DTPA and TPEN, and ZnCl<sub>2</sub> on the cells treated with hydrogen peroxide: a flow-cytometric study using rat thymocytes. *Nat. Sci. Res. Univ. Tokushima* 23, 12-18. <http://web.ias.tokushima-u.ac.jp/bulletin/natj.html>
- Matsui, H., Oyama, T.M., Okano, Y., Hashimoto, E., Kawanai, T., Oyama, Y., 2010. Low micromolar zinc exerts cytotoxic action under H<sub>2</sub>O<sub>2</sub>-induced oxidative stress: Excessive increase in intracellular Zn<sup>2+</sup> concentration. *Toxicol.* (In Press) doi:10.1016/j.tox.1010.06.011
- Matsui, H., Sakanashi, Y., Oyama, T.M., Oyama, Y., Yokota, S., Ishida, S., Okano, Y., Oyama, T.B., Nishimura, Y., 2008. Imidazole antifungals, but not triazole antifungals, increase membrane Zn<sup>2+</sup> permeability in rat thymocytes Possible contribution to their cytotoxicity. *Toxicol.* 248, 142-150.
- McConkey, D.J., Jondal, M., Orrenius, S., 1994. The regulation of apoptosis in thymocytes. *Biochem. Soc. Transac.* 22, 606-610.
- Nakao, H., Umebayashi, C., Nakata, M., Nishizaki, Y., Noda, K., Okano, Y., Oyama, Y., 2003. Formaldehyde-induced shrinkage of rat thymocytes. *J. Pharmacol. Sci.* 91, 83-86.
- Nappi, A.J., Vass, E., 2000. Hydroxyl radical production by ascorbate and hydrogen peroxide. *Neurotoxic. Res.* 2, 343-355
- Nishimura, Y., Oyama, T.B., Sakanashi, Y., Oyama, T.M., Matsui, H., Okano, Y., Oyama, Y., 2008. Some characteristics of quercetin-induced cytotoxicity on rat thymocytes under in vitro condition. *Toxicol. In Vitro.* 22, 1002-1007.
- Nishimura, Y., Kanada, A., Yamaguchi, J.Y., Horimoto, K., Kobayashi, M., Tatsuishi, T., Kanemaru, K., Ueno, S.Y., Oyama, Y., 2006. Cytometric analysis of lidocaine-induced cytotoxicity: a model experiment using rat thymocytes. *Toxicol.* 218, 48-57.
- Ogata, I., Kawanai, T., Hashimoto, E., Nishimura, Y., Oyama, Y., Seo, H., 2010. Bisabololoxide A, one of the main constituents in German chamomile extract, induces apoptosis in rat thymocytes. *Arch. Toxicol.* 84, 45-52.
- Ortiz, R., Cortés, L., González-Márquez, H., Gómez, J.L., González, C., Cortés, E., 2001. Flow cytometric analysis of spontaneous and dexamethasone-induced apoptosis in thymocytes from severely malnourished rats. *Br. J. Nutr.* 86, 545-548.
- Oyama, Y., Matsui, H., Morimoto, M., Sakanashi, Y., Nishimura, Y., Ishida, S., Okano, Y., 2007. Synergic cytotoxic action induced by simultaneous application of zinc and clotrimazole in rat thymocytes. *Toxicol. Lett.* 171, 138-145.
- Padayatty, S.J., Katz, A., Wang, Y., Eck, P., Kwon, O., Lee, J.H., Chen, S., Corpe, C., Dutta, A., Dutta, S.K., Levine, M., 2003. Vitamin C as an antioxidant: evaluation of its role in disease prevention. *J. Am. Coll. Nutr.* 22, 18-35.
- Prasad, A.S., 2008. Zinc in human health: effect of zinc on immune cells. *Mol. Med.* 14, 353-357.
- Quaglino, D., Ronchetti, I.P., 2001. Cell death in the rat thymus: a minireview. *Apoptosis* 6, 389-401

- Sakanashi, Y., Oyama, T.M., Matsuo, Y., Oyama, T.B., Nishimura, Y., Ishida, S., Imai, S., Okano, Y., Oyama, Y., 2009. Zn<sup>2+</sup>, derived from cell preparation, partly attenuates Ca<sup>2+</sup>-dependent cell death induced by A23187, calcium ionophore, in rat thymocytes. *Toxicol. In Vitro.* 23, 338-345.
- Smirnoff, N., Cumbes, Q.J., 1989. Hydroxyl radical scavenging activity of compatible solutes. *Phytochem.* 28, 1057-1060.
- Stanner, S.A., Hughes, J., Kelly, C.N., Buttriss, J., 2004. A review of the epidemiological evidence for the 'antioxidant hypothesis'. *Public Health Nutr.* 7, 407-422.
- Thompson, L.F., Vaughn, J.G., Laurent, A.B., Blackburn, M.R., Van De Wiele, C.J., 2003. Mechanism of apoptosis in developing thymocytes as revealed by adenosine deaminase-deficient fetal thymic organ cultures. *Biochem. Pharmacol.* 66, 1595-1599.
- Yamaguchi, J.Y., Nishimura, Y., Kanada, A., Kobayashi, M., Mishima, K., Tatsuishi, T., Iwase, K., Oyama, Y., 2005. Cremophor EL, a non-ionic surfactant, promotes Ca<sup>2+</sup>-dependent process of cell death in rat thymocytes. *Toxicol.* 211, 179-186.

*Received June 30, 2010*

*Accepted July 16, 2010*