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NOR-3, a donor of nitric oxide, increases intracellular Zn^{2+} concentration and decreases cellular thiol content: A model experiment using rat thymocytes, FluoZin-3, and 5-chloromethylfluorescein

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ABSTRACT

Our previous study showed that nitroprusside, a donor of nitric oxide (NO), increased intracellular Zn^{2+} concentration without affecting cellular content of glutathione (GSH) although it has been proposed that the cytotoxicity of NO is resulted from its interaction with glutathione and zinc. Nitroprusside releases not only NO but also cyanides (Fe(II)CN and Fe(III)CN), CN^-, Fe^{2+}, and Fe^{3+}. Therefore, such decomposition products may mask NO-induced action on cellular GSH content. In this study, we used NOR-3 as a donor of NO to reveal the effects of NO on intracellular Zn^{2+} concentration and cellular GSH content in a cytometric manner with fluorescent probes, FluoZin-3-AM and 5-chloromethylfluorescein diacetate. NOR-3 at 1-3 mM significantly increased intracellular Zn^{2+} concentration and decreased cellular GSH content. After the removal of extracellular Zn^{2+} by diethylenetriamine-N,N,N',N",N"-pentaacetic acid (DTPA, a chelator for Zn^{2+}), the increase in intracellular Zn^{2+} concentration by NOR-3 was still observed although DTPA significantly attenuated the increase in intracellular Zn^{2+} concentration by NOR-3. Results suggest an involvement of both intracellular Zn^{2+} release and increase in membrane Zn^{2+} permeability. It is likely that NO induces oxidative stress, leading to an increase in intracellular Zn^{2+} concentration.

Keywords: intracellular Zn^{2+}; NOR-3; cytotoxicity; nitric oxide; glutathione

1. INTRODUCTION

We have proposed that the chemicals, such as organometal compounds, decreasing cellular thiol content increase intracellular Zn^{2+} concentration (Hashimoto et al., 2009; Kawanai et al., 2009; Oyama et al., 2009). The proposal is based on a negative correlation between augmentation of FluoZin-3 fluorescence, an indicator for intracellular Zn^{2+}, and attenuation of 5-chloromethylfluorescein (5-CMF) fluorescence, an indicator for cellular nonprotein thiols when the cells are incubated with chemicals. In our previous study, however, nitroprusside, a vasodilator by donating NO, increased intracellular Zn^{2+} concentration without affecting cellular content of nonprotein thiols (Hashimoto et al., 2010). Nitric oxide (NO) reacts with superoxide anion to generate peroxynitrite that is proposed to induce Zn^{2+} release from intracellular sites or stores (Bossy-Wetzel et al., 2004; St Croix et al., 2005). Therefore, the proposal is challenged in the case of nitroprusside. Nitroprusside contains an iron molecule coordinated to five cyanide molecules and one molecule of nitric oxide. Whereas the decomposition of nitroprusside results in the production of NO, Fe(II)CN, and Fe(III)CN. Two latter molecules further produce CN^- (Meeussen et al., 1992). Thus, such decomposition products may affect NO-induced decrease in cellular content of thiols. Therefore, to test the effect of NO, we have used NOR-3 as a non-toxic donor of NO, but not nitroprusside. In this study, the effects of NOR-3 on intracellular thiol content and intracellular Zn^{2+} concentration have been examined in rat thymocytes by using a flow cytometer with propidium iodide, 5-CMF, and FluoZin-3 in order to confirm our proposal.

2. MATERIALS AND METHODS

2.1. Chemicals

NOR-3, N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN), and diethylenetriamine-N,N,N',N",N"-pentaacetic acid (DTPA), were purchased from Dojin Chemical Laboratory (Kumamoto, Japan). Fluorescent probes such as propidium iodide, FluoZin-3 pentaacetoxymethyl ester (FluoZin-3-AM), and 5-chloromethylfluorescein diacetate (5-CMF-DA) were the products of Molecular Probes Inc. (Eugene, Oregon, USA). NaCl, $CaCl_2$, $MgCl_2$, KCl, NaOH, $ZnCl_2$, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, glucose, and dimethyl sulfoxide (DMSO) were also purchased from Wako Pure Chemicals. Final concentrations of DMSO as a solvent for FluoZin-3-AM, 5-CMF-DA, and TPEN in cell suspension were 0.1 % or less. The incubation with DMSO at 0.3 % did not affect the viability of rat thymocytes during experiments.

2.2. Animals and cell preparation

This study was approved by the Committee for Animal Experiments in the University of Tokushima (No. 05279 for Y. Oyama).

The procedure to prepare cell suspension was similar to that previously reported (Chikahisa et al., 1996). In brief, thymus glands dissected from ether-anesthetized rats were sliced at a thickness of 400-500 μm with razor under an ice-cold condition (1-4ºC). 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 7.4) to dissociate thymocytes. Thereafter, the Tyrode's solution containing the cells was passed through a mesh (a diameter of 10 μm) to prepare the cell suspension (about 5×10^5 cells/ml). The beaker containing the cell suspension was water-bathed at 36ºC for 1 hr before the experiment.

2.3. Experimental protocol

NOR-3 was added to cell suspension (1 or 2 ml cell suspension in 10 ml test tube). The cell density was about 5×10^5 cells/mL. The cells were incubated with NOR-3 at 36-37°C for 2 hr under room air condition. The data acquisition of fluorescence from 2×10^3 cells by a flow cytometer required 10 sec at least.

2.4. Fluorescence measurements of cellular and membrane parameters

The method for measurement of cellular and membrane parameters using a flow cytometer equipped with an argon laser (CytoACE-150, JASCO, Tokyo, Japan) and fluorescent probe was similar to those previously described (Chikahisa et al., 1996). The fluorescence was analyzed by JASCO software (Ver.3XX, JASCO). As to chemicals used in this study, there was no fluorescence detected under our experimental condition.

To select living cells for measurement, propidium iodide was added to cell suspension to achieve a final concentration of 5 μM. Since propidium stains dead cells, the measurement of propidium fluorescence from cells provides a clue to select living cells. 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.

FluoZin-3-AM (Gee et al., 2002) was used as an indicator for intracellular Zn^{2+}. The cells were incubated with 500 nM FluoZin-3-AM for 60 min before any fluorescence measurements to estimate the change in intracellular Zn^{2+} concentration of rat thymocytes with intact membranes (Hashimoto et al., 2009; Kawanai et al., 2009; Oyama et al., 2009). FluoZin-3 fluorescence was measured from the cells that were not stained with 5 μM propidium iodide (Matsui et al., 2008). Excitation wavelength for FluoZin-3 was 488 nm and emission was detected at 530 ± 20 nm. 5-CMF-DA was used to monitor the change in cellular content of nonprotein thiols (Chikahisa et al., 1996). The cells were incubated with 1 μM 5-CMF-DA for 30 min before any fluorescence measurements. 5-CMF fluorescence was measured from the cells that were not stained with 5 μM propidium iodide. Excitation wavelength for 5-CMF was 488 nm and emission was detected at 530 ± 15 nm.

2.5. Statistics

Values were expressed as the mean ± standard deviation of 4 experiments. Statistical analysis was performed by using Tukey multivariate analysis. A P value of < 0.05 was considered significant.

3. RESULTS

3.1. Effects of NOR-3 on FluoZin-3 fluorescence of rat thymocytes

The effect of NOR-3 at concentrations ranging from 0.1 mM to 3 mM on the cells preloaded with FluoZin-3-AM was tested at 2 hr after the start of application. NOR-3 at 3 mM shifted the histogram of FluoZin-3 fluorescence monitored from the cells to a direction of higher intensity, indicating an increase in intracellular Zn^{2+} concentration (Fig. 1). The shift attained a steady level within 2 hr after the start of drug application. Dose-dependent change in the intensity of FluoZin-3 fluorescence by NOR-3 is shown in Fig. 2. The threshold concentration of NOR-3 to augment FluoZin-3 fluorescence was 1 mM when the effects of 0.1-3 mM NOR-3 were tested at 2 hr after the start of application.

Control Tyrode's solution contained 1 μM $ZnCl_2$.

In addition, the solution was contaminated with 0.1-0.2 μM zinc delivered from cells and salts during preparing cell suspension (Sakanashi et al., 2009). To study the source of Zn^{2+} in the case of NOR-3-induced increase in intracellular Zn^{2+} concentration, the effect of NOR-3 was tested in the presence of Zn^{2+} chelator (DTPA and TPEN). As shown in Fig. 3, in the presence of 10 μM DTPA, a chelator for extracellular Zn^{2+}, the control level of FluoZin-3 fluorescence was significantly attenuated. However, the augmentation of FluoZin-3 fluorescence by 1 mM NOR-3 was still observed although the treatment with DTPA significantly attenuated FluoZin-3 fluorescence (Fig. 3). TPEN, a chelator for intracellular Zn^{2+}, at 10 μM greatly attenuated the NOR-3-induced increase in intracellular Zn^{2+} concentration (Fig. 3). Removal of extracellular Ca^{2+} did not affect the effect of NOR-3 (Fig. 3).

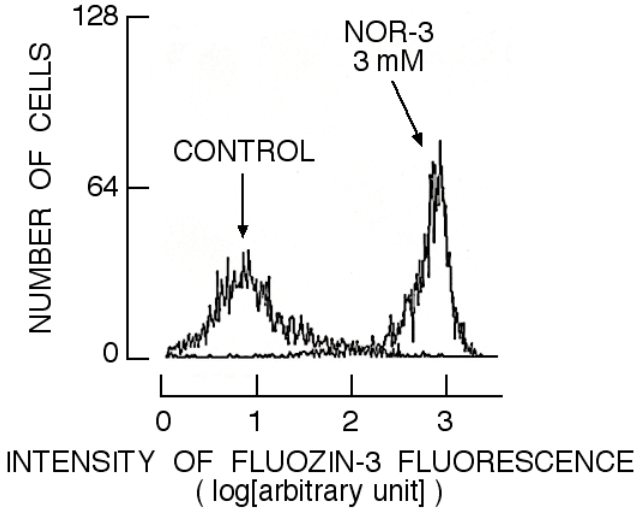

Figure 1. *Effect of NOR-3 on rat thymocytes incubated with FluoZin-3-AM. Each histogram of FluoZin-3 fluorescence was constructed from* 2×10^3 *cells. FluoZin-3 fluorescence was monitored only from the cells without exerting propidium fluorescence.*

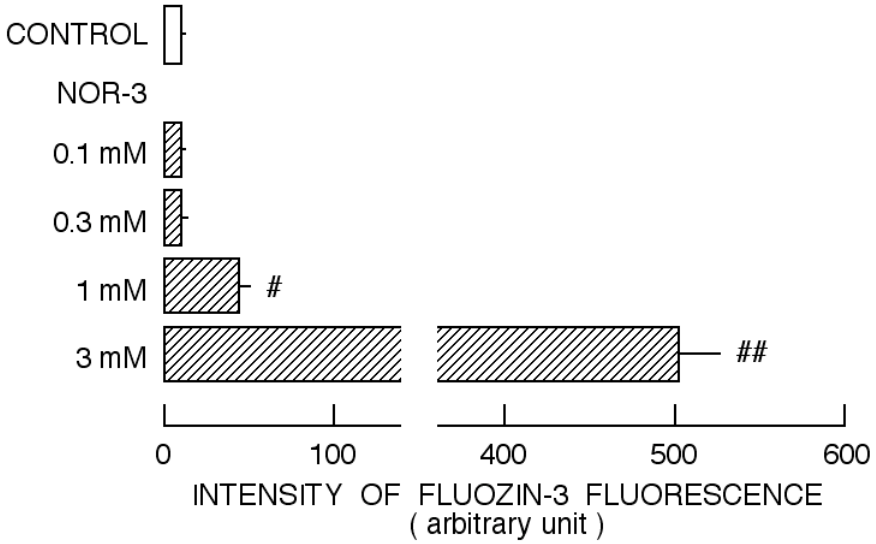

Figure 2. *Dose-dependent changes in mean intensity of FluoZin-3 fluorescence by NOR-3. Column and bar respectively indicate mean and its standard deviation of five experiments. Symbols (#, ##) indicate significant difference* ($P < 0.05$, $P < 0.01$) *to the control.*

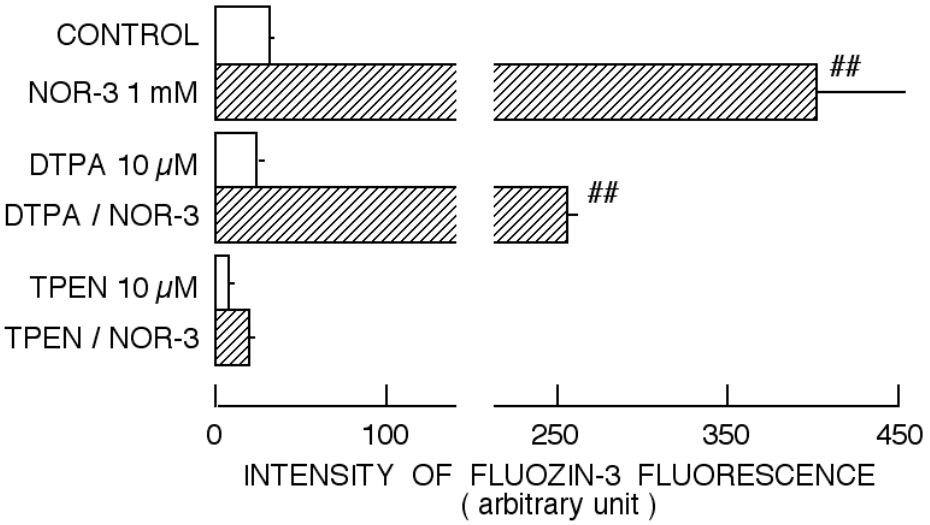

Figure 3. *Effects of Zn^{2+} chelators, DTPA and TPEN, on NOR-3-induced augmentation of FluoZin-3 fluorescence. Column and bar respectively indicate mean and its standard deviation of four experiments. Symbols (#, ##*) indicate significant difference ($P < 0.05$, $P < 0.01$) to the control.*

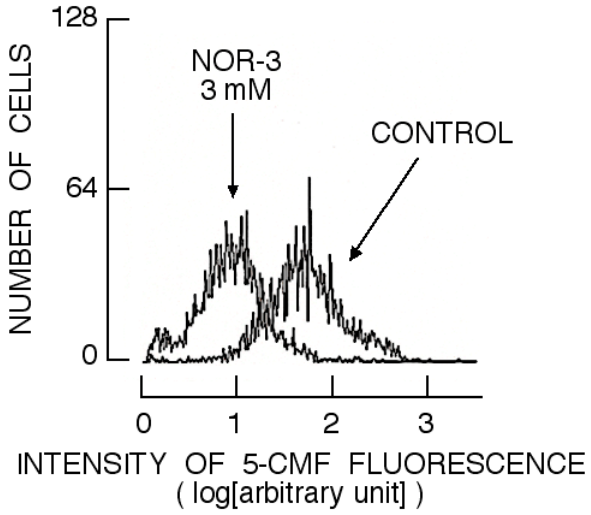

Figure 4. *Effect of NOR-3 on rat thymocytes incubated with 5-CMF-DA. Each histogram of 5-CMF fluorescence was constructed from 2.5×10^3 cells. 5-CMF fluorescence was monitored only from the cells without exerting propidium fluorescence.*

3.2. Effect of NOR-3 on 5-CMF fluorescence of rat thymocytes

The effect of NOR-3 at concentrations ranging from 0.1 mM to 3 mM on the cells preloaded with 5-CMF-DA was tested at 2 hr after the start of application. As shown in Fig. 4, NOR-3 at 3 mM shifted the histogram of 5-CMF fluorescence monitored from the cells to a direction of lower intensity, indicating a decrease in cellular GSH content. The shift of histogram attained a steady level within 2 hr after the start of NOR-3 application. Dose-dependent change in the intensity of FluoZin-3 fluorescence by NOR-3 is shown in Fig. 5. The threshold concentration of NOR-3 to attenuate 5-CMF fluorescence was 0.1 or 0.3 mM when the effects of 0.1-3 mM NOR-3 were tested at 2 hr after the start of application.

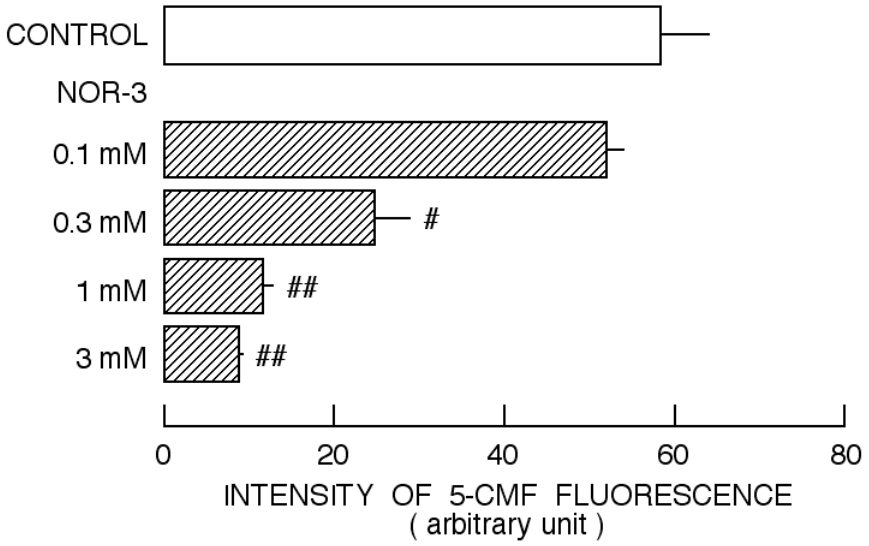

Figure 5. *Dose-dependent changes in mean intensity of 5-CMF fluorescence by NOR-3. Column and bar respectively indicate mean and its standard deviation of four experiments. Symbols (#,##) indicate significant difference ($P < 0.05$, $P < 0.01$) to the control.*

4. DISCUSSION

NOR-3 is useful for reliable generation of NO. The half-life of NO release from NOR-3 at 36-37°C is about 30 minutes in aqueous solution at pH 7.4. Therefore, the incubation with NOR-3 for 2 hr is suitable to see if NO affects cellular parameters. In this study, NOR-3 augmented FluoZin-3 fluorescence and attenuated 5-CMF fluorescence when the concentrations were millimolar (Figs. 2 and 5). Results suggest that NO increases intracellular Zn^{2+} concentration and decreases cellular GSH content. Furthermore, in the presence of DTPA, a chelator for extracellular Zn^{2+}, the NOR-3-induced increase in intensity of FluoZin-3 fluorescence was significantly attenuated, but it was still observed (Fig. 3). Results indicate that both influx of extracellular Zn^{2+} and release of intracellular Zn^{2+} are involved in the NOR-3-induced increase in intracellular Zn^{2+} concentration. Thus, NO delivered from NOR-3 may induce intracellular Zn^{2+} release and increases membrane Zn^{2+} permeability, resulting in an increased concentration of intracellular Zn^{2+}. The threshold concentration of NOR-3 to augment FluoZin-3 fluorescence was higher than that to attenuate 5-CMF fluorescence (Figs. 2 and 5). Therefore, NO-induced decrease in cellular GSH content (presumably, nonprotein thiol content) may induce an increase in intracellular Zn^{2+} concentration. Intracellular Zn^{2+} makes a complex with thiol group of protein and nonprotein (Jacob et al., 1998; Maret and Vallee, 1998; Gelinsky et al., 2002). The modification from thiol to disulfide releases Zn^{2+} from protein and nonprotein (Maret, 1994; Quesada et al., 1996). Furthermore, oxidative stress induced by H_2O_2, one of reactive oxygen species, increases membrane Zn^{2+} permeability (Matsui et al., 2010). In fact, there is a negative correlation between the augmentation of FluoZin-3 fluorescence and the attenuation of 5-CMF fluorescence (Hashimoto et al., 2009; Oyama et al., 2009; Kawanai et al., 2009).

In our previous study, nitroprusside, a donor of NO, at millimolar concentrations increased intracellular Zn^{2+} concentration without affecting cellular GSH content (Hashimoto et al., 2010). Since NO reacts with superoxide anion to generate peroxynitrite that is proposed to induce Zn^{2+} release from intracellular sites or stores (Bossy-Wetzel et al., 2004; St Croix et al., 2005), it is likely that the oxidative stress induced by peroxynitrite decreases cellular GSH content (Murphy et al., 1998). The case of nitroprusside may be exceptional since the decomposition of nitroprusside results in the production of NO, Fe(II)CN, and Fe(III)CN and two latter molecules further produce CN^-, Fe^{2+}, and Fe^{3+} (Meeussen et al., 1992). In this aspect, further study will be necessary.

The concentrations of NOR-3 were 0.1-3 mM in this study. Thus, NOR-3 probably released millimolar NO when the concentrations of 1-3 mM were used. However, millimolar concentrations of NO surrounding the cells may be not physiological

and pathological. Millimolar concentrations of nitroprusside, a donor of NO, were also required to augment FluoZin-3 fluorescence (Hashimoto et al., 2010). It may be suggested that the susceptibility to NO is dependent on the type of cells. In fact, there are large differences in the sensitivities toward the cytotoxic action of NO of different cell types (for review, Kröncke et al., 1997). Thus, neurons and islet cells are very sensitive to NO delivered from nitroprusside while keratinocytes and aorta endothelial cells are apparently much less sensitive.

REFERENCES

Bossy-Wetzel, E., Talantova, M.V., Lee, W.D., Schölzke, M.N., Harrop, A., Mathews, E., Götz, T., Han, J., Ellisman, M.H., Perkins, G.A., Lipton, S.A., 2004. Crosstalk between nitric oxide and zinc pathways to neuronal cell death involving mitochondrial dysfunction and p38-activated K^+ channels. Neuron. 41, 351-365.

Chikahisa, L., Oyama, Y., Okazaki, E., Noda, K., 1996. Fluorescent estimation of H_2O_2-induced changes in cell viability and cellular nonprotein thiol level of dissociated rat thymocytes. Jpn. J. Pharmacol. 71, 299-305.

Gee, K.R., Zhou, Z.L., Qian, W.J., Kennedy, R., 2002. Detection and imaging of zinc secretion from pancreatic beta-cells using a new fluorescent zinc indicator. J. Am. Chem. Soc. 124, 776-778.

Gelinsky, M., Vogler, R., Vahrenkamp, H., 2002. Tripodal pseudopeptides with three histidine or cysteine donors: synthesis and zinc complexation. Inorg. Chem. 41, 2560-2564.

Hashimoto, E., Kimura, K., Kawanai, T., Nishimura, Y., Oyama, Y., 2010. Nitroprusside increases intracellular Zn^{2+} concentration without affecting cellular thiol content: A model experiment using rat thymocytes and FluoZin-3. Nat. Sci. Res. Tokushima Univ. 24, 7-12.

Hashimoto, E., Oyama, T.B., Oyama, K., Nishimura, Y., Oyama, T.M., Ueha-Ishibashi, T., Okano, Y., Oyama, Y., 2009. Increase in intracellular Zn^{2+} concentration by thimerosal in rat thymocytes: Intracellular Zn^{2+} release induced by oxidative stress. Toxicol. In Vitro. 23, 1092-1099

Jacob, C., Maret, W., Vallee, B.L., 1998. Control of zinc transfer between thionein, metallothionein, and zinc proteins. Proc. Natl. Acad. Sci. U.S.A. 95, 3489-3494.

Kawanai, T., Satoh, M., Murao, K., Oyama, Y., 2009. Methylmercury elicits intracellular Zn^{2+} release in rat thymocytes: its relation to methylmercury-induced decrease in cellular thiol content. Toxicol. Lett. 191, 231-235.

Kröncke1, D., Fehsel, K., Kolb-Bachofen, V., 1997. Nitric oxide: Cytotoxicity versus cytoprotection – How, why, when, and where. Nitric Oxide: Biol. Chem. 1, 107-120.

Maret, W., 1994. Oxidative metal release from metallothionein via zinc-thiol / disulfide interchange. Proc. Natl. Acad. Sci. U.S.A. 91, 237-241.

Maret, W., Vallee, B.L., 1998. Thiolate ligands in metallothionein confer redox activity on zinc clusters. Proc. Natl. Acad. Sci. U.S.A. 95, 3478-3482.

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^{2+} permeability in rat thymocytes Possible contribution to their cytotoxicity. Toxicol. 248, 142-150.

Matsui, H., Oyama, T.M., Okano, Y., Hashimoto, E., Kawanai, T., Oyama, Y., 2010. Low micromolar zinc exerts cytotoxic action under H_2O_2-induced oxidative stress: excessive increase in intracellular Zn^{2+} concentration. Toxicol. 276, 27-32.

Meeussen, J.L., Keizer, M.G., van Riemsdijk, W.H., 1992. Dissolution behavior of iron cyanide (Prussian blue) in contaminated soils. Environ. Sci. Toxicol. 26, 1832–1838.

Murphy, M.P., Packer, M.A., Scarlett, J.L., Martin, S.W., 1998. Peroxynitrite: a biologically significant oxidant. Gen. Pharmacol. 31, 179-86.

Oyama, T.B., Oyama, K., Kawanai, T., Oyama, T.M., Hashimoto, E., Satoh, M., Oyama, Y., 2009. Tri-*n*-butyltin increases intracellular Zn^{2+} concentration by decreasing cellular thiol content in rat thymocytes. Toxicol. 262, 245-249.

Quesada, A.R., Byrnes, R.W., Krezoski, S.O., Petering, D.H., 1996. Direct reaction of H_2O_2 with sulfhydryl groups in HL-60 cells: zinc-metallothionein and other sites. Arch. Biochem. Biophys. 334, 241-250.

Sakanashi, Y., Oyama, T.M., Matsuo, Y., Oyama, T.B., Nishimura, Y., Ishida, S., Imai, S., Okano, Y., Oyama, Y., 2009. Zn^{2+}, derived from cell preparation, partly attenuates Ca^{2+}-dependent cell death induced by A23187, calcium ionophore, in rat thymocytes. Toxicol. In Vitro. 23, 338-345

St. Croix, C.M., Leelavaninchkul, K., Watkins, S.C., Kagan, V.E., Pitt, B.R., 2005. Nitric oxide and zinc homeostasis in acute lung injury. Proc. Am. Thorac. Soc. 2, 236-242.

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