

Involvement of NO Generation in Aluminum-Induced Cell Death

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Previously, we have reported that the exposure of PC12 cells to the aluminum–maltolate complex (Al(maltol)₃) results in decreased cell viability *via* the apoptotic cell death pathway. In this study, we have used several nitric oxide synthase (NOS) inhibitors and the NO generator diethylenetriamine NONOate (DETA NONOate) to examine whether or not intracellular nitric oxide (NO) generation is involved in the onset mechanism of Al(maltol)₃-induced cell death. Cell viability was assessed by measuring lactate dehydrogenase (LDH) release and caspase-3 activity. Treatment of the cells with 150 μM Al(maltol)₃ for 48 h resulted in intracellular NO generation. Exposure of the cells to DETA NONOate also induced a marked decrease in cell viability. Pre-treatment of the cells with a general NOS inhibitor or with a selective inducible NOS (iNOS) inhibitor effectively prevented Al(maltol)₃-induced cell death. However, a neuronal NOS (nNOS) inhibitor did not exhibit any protective effect against Al(maltol)₃-induced cell death. In addition, ascorbic acid markedly inhibited Al(maltol)₃- and DETA NONOate-induced cell death. Based on these results, we discussed the involvement of intracellular NO generation in the onset mechanisms of Al(maltol)₃-induced cell death.

Key words aluminum toxicity; nitric oxide; caspase; ascorbic acid

Aluminum (Al) is the third most abundant element in the earth's crust and has been implicated as an etiologic factor in neurological disorders including Alzheimer's disease,^{1,2)} Parkinson's dementia syndrome, and dialysis encephalopathy syndrome.^{3,4)} In fact, some evidence supports the selective accumulation of Al within neurons containing neurofibrillary tangles in patients with Alzheimer's disease and within the aging human brain.^{1,5)} Meiri *et al.*, have also reported that brain Al concentrations reach submicromolar levels in some encephalopathies.⁶⁾ Several lines of research that use cultured cells and the aluminum–maltolate complex (Al(maltol)₃), which is a membrane permeable, lipophilic complex of Al, also showed that the exposure of cells such as the Neuro-2a murine neuroblastoma cells,⁷⁾ human NT2 neuroblastoma cells,⁸⁾ and PC12 cells^{9,10)} to the Al complex results in a decrease in cell viability *via* the apoptotic cell death pathway. Recently, we have reported that the treatment of PC12 cells with Al(maltol)₃ causes a decrease in the levels of the intracellular reduced glutathione depending on the amount of Al(maltol)₃ accumulated in the cells.¹¹⁾ These findings strongly suggested that Al accumulation in tissues is closely related to the development of neurodegenerative disorders although a causal relationship between Al and neurodegenerative disorders remains unclear.

NO is considered to be a modulator and a simple and diffusible free radical.¹²⁾ It is believed to play an important role in physiological and pathophysiological events in many cellular systems.^{13–15)} Furthermore, it has also been reported that NO concentration increased in the brain during the course of ischemia, Alzheimer's disease, and other degenerative conditions.^{16–18)} Numerous studies in several cell systems have demonstrated that NO is closely related to cell death mechanisms and plays the role of a mediator.^{19–23)} A recent study has reported that NO is produced in the mitochondria *via* Ca²⁺-dependent mitochondrial NO synthases (mtNOS).^{24–26)} The NO produced in the mitochondria by mtNOS plays the role of a modulator of mitochondrial oxygen consumption and transmembrane potential *via* a reversible reaction with cytochrome c oxidase. It is well-known

that NO rapidly reacts with superoxide anion radicals to form peroxynitrite, which is an oxidant substance producing cytotoxic effects in many cells.^{14,20)}

Previously, we have reported that accumulation of Al(maltol)₃ in PC12 cells results in apoptotic cell death depending on the intracellular generation of reactive oxygen species (ROS).¹⁰⁾ Therefore, it would be interesting to determine if intracellular NO generation is involved in the onset mechanism of Al-mediated-cytotoxicity. Therefore, in the present study, we examined the effects of a NO generator and NO synthase inhibitors on Al(maltol)₃-induced cell death. Our results suggest that intracellular NO generation may play an important role in the development of cell toxicity associated with Al(maltol)₃ treatment.

MATERIALS AND METHODS

Chemicals 3-Hydroxy-2-methyl-4-pyrone (maltol), N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), *n*-heptyl-β-D-thioglucoside, and dimethyl sulfoxide were obtained from Wako Pure Chemical (Osaka, Japan). Diaminofluorescein-2 diacetate (DAF-2 DA) was purchased from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC), 7-nitroindazole (7-NI), and β-nicotinamide adenine dinucleotide (reduced form, β-NADH) were purchased from Sigma (St. Louis, MO, U.S.A.). 2'-(4-Hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi-1H-benzimidazole (Hoechst 33528) was obtained from Molecular Probes, Inc. (Eugene, OR, U.S.A.). 2-Methyl-2-thiourea sulfate (SMT) and diethylenetriamine NONOate (DETA NONOate) were obtained from Ardrich (St. Louis, MO, U.S.A.) and Cayman (Ann. Arbor, MI, U.S.A.), respectively. All chemicals used were of the purest grade commercially available.

Preparation of Al(maltol)₃ Al(maltol)₃ was prepared according to the procedure described by Finnegan *et al.*²⁷⁾ A stock solution (25 mM) of Al(maltol)₃ was prepared in deion-

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ized water and sterilized using a 0.22-mm filter.

Cell Culture The PC12 cells were mainly cultured in 35-mm dishes coated with poly-D-lysine at a density of approximately 3.5×10^5 cells/ml; the medium used was Dulbecco's modified Eagle medium supplemented with 5% fetal bovine serum and 5% horse serum at 37 °C under 95% air/5% CO₂. The cells were allowed to develop for 24 h before exposure to Al(maltol)₃ (150 μM) for 48 h or DETA NONOate (250 μM) for 18 h. On the other hand, the control cells were cultured in the presence of maltol in quantities that was three times the quantity of Al(maltol)₃ employed. Morphological changes in the cells were examined throughout the course of the experiment using a phase-contrast microscope (Olympus IX 70-S8F microscope).

Determination of NO Production NO was detected using the fluorescence dye DAF-2 DA, according to a previously published paper.²⁸⁾ Cells were incubated with 10 μM DAF-2 DA for 30 min at 37 °C. The reaction was terminated by the addition of L-NAME at a final concentration of 5 mM. The cells were washed twice and resuspended with in Ca, Mg free-phosphate buffered saline (CMF-PBS) containing 5 mM L-NAME, respectively, and the fluorescence of the cell suspension was measured. The excitation and emission wavelengths were 495 and 515 nm, respectively. The fluorescence intensity (FI; arbitrary unit) was expressed as the value per mg of protein.

Cell Viability Measurement Cell viability was assessed by lactate dehydrogenase (LDH) release measurement. The reaction was initiated by the addition of an aliquot (250 μl) of the culture medium to an assay medium (750 μl) containing β-NADH (88 μg/ml), pyruvate (100 μM), and 100 mM phosphate buffer (pH 7.5). The rate of change in absorbance of NADH at 340 nm (ΔA_{340}) was measured at 1 min using the Hitachi spectrophotometer U2810. The data were expressed as values relative (%) to the total LDH that could be released using 1% *n*-heptyl-β-D-thioglucoside.¹⁰⁾

Caspase Activity Assay The PC12 cells (1×10^7) were washed twice with CMF-PBS and then suspended in an ice-cold 50-mM potassium phosphate buffer (pH 7.5). To prevent the non-specific cleavage of proteins, cell lysis was performed by two cycles of freezing and thawing at 4 °C. The mixture was centrifuged at 13000×*g* for 10 min at 4 °C, and the supernatant obtained was stored at -80 °C until use in the caspase activity assay. The reaction was initiated by the addition of Ac-DEVD-AMC (at a final concentration of 50 μM) to the reaction mixture containing 50 mM Tris/HCl buffer (pH 7.5), 0.1% CHAPS, 10 mM dithiothreitol, and the cell lysate (50 μg protein) at 37 °C. The total volume of the assay medium was 500 μl. After 30 min, the reaction was terminated by the addition of the stop solution (50 μl) comprising 175 mM acetic acid and 1% sodium acetate. The AMC levels were measured by using the Hitachi fluorescence spectrophotometer F-4500 with the excitation and emission wavelengths at 380 and 400 nm, respectively. The enzyme activity was expressed as FI/mg protein/min.

Assay for Nuclear Condensation The cells were fixed using a 10% formalin neutral phosphate buffer solution (pH 7.4) for 5 min at room temperature. After washing with distilled water, the cells were stained for 5 min with Hoechst 33258 at a concentration of 8 μg/ml. The cells were washed again with distilled water. Dye fluorescence was measured

using the Olympus IX 70 fluorescence microscope with the excitation and emission wavelengths at 340 and 510 nm, respectively.

Protein Determination Protein concentration was determined by the procedure described by Lowry *et al.* using bovine serum albumin as the standard.²⁹⁾

Statistical Analysis Data are presented as the mean ± S.E.M. values of three different experiments. The data were analyzed by an ANOVA Scheffe's multiple *t* test.

RESULTS AND DISCUSSION

NO Generation by Al(maltol)₃ Treatment To determine the relationship between cell death by Al(maltol)₃ treatment and intracellular NO generation, we measured the fluorescence of cells labeled with the fluorescent dye DAF-2 DA.

As shown in Fig. 1, a 48-h exposure of the cells to 150 μM Al(maltol)₃ resulted in an increase in fluorescence intensity of the dye incorporated into the cells. Development of dye fluorescence depends on the formation of a fluorescent product as a result of the interaction of the dye with the NO generated.²⁸⁾ Hence, this result indicates that treatment of the cells with Al(maltol)₃ caused intracellular NO generation. This possibility was further confirmed by a complete inhibition of Al(maltol)₃-induced fluorescence increase in the presence of 5 mM L-NAME. On the other hand, the dye fluorescence of the control cell was not affected by the addition of L-NAME (FI/mg protein of control without and with L-NAME were 0.53 ± 0.02 and 0.55 ± 0.01 , respectively).

Effect of NO on Cell Viability The PC12 cells were exposed to 250 μM DETA NONOate to assess the possible toxic effects of NO on cell viability.

As shown in Table 1, treatment of the cells with an exogenous NO donor for 18 h facilitated LDH release and resulted in an increase in caspase-3 activity.

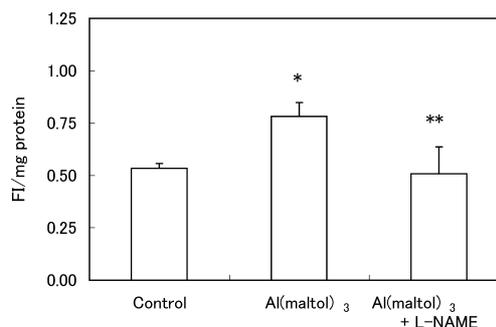


Fig. 1. Effect of Al(maltol)₃ Treatment on NO Generation in PC12 Cells

The cells were exposed to 150 μM Al(maltol)₃ for 48 h in the absence and presence of 5 mM L-NAME. Values are expressed as means ± S.E.M. for 4–8 independent measurements. **p* < 0.05 vs. Control. ***p* < 0.05 vs. Al(maltol)₃ treatment.

Table 1. Changes in LDH and Caspase-3 Activities by NONOate Treatment

	LDH release (%)	Caspase-3 activity (FI/min/mg protein)
Control cells	8.7 ± 0.5	67.0 ± 1.0
Treated cells	31.8 ± 3.5*	851 ± 102.7*

The cells were exposed to 250 μM DETA NONOate for 18 h. Values are expressed as means ± S.E.M. for three independent measurements. **p* < 0.05 vs. control.

Effects of NOS Inhibitors on Al(maltol)₃-Induced Cell Death Next, we examined the effects of several NOS inhibitors, such as L-NAME and SMT, on cell death by treating the cells with 150 μ M Al(maltol)₃ for 48 h.

As shown in Fig. 2, a 48-h exposure to 150 μ M Al(maltol)₃ resulted in marked facilitation of LDH release from the cells (Fig. 2A) and activation of caspase-3 activity (Fig. 2B). In contrast, pretreatment of the cells with L-NAME, which is a general competitive inhibitor of NOS,³⁰ effectively inhibited caspase-3 activation and Al(maltol)₃-induced LDH release in a concentration-dependent manner. However, the extents of inhibitory effect of L-NAME against LDH release and caspase-3 activity were different in each other. This suggests the possibility that mechanisms of response reflection in these parameters are different, although the exact reason for the discrepancy is unclear at present. On the other hand, treatment of the cells with L-NAME alone did not affect these parameters.

Furthermore, it is clear that L-NAME also protected Al(maltol)₃-induced changes in cell and nuclear morphology (Figs. 3A, B). These results also suggest that NO is involved in the onset mechanism of Al(maltol)₃-induced cell death. Next, we examined the effects of SMT, a selective iNOS inhibitor,³¹ and 7-NI, a selective nNOS inhibitor,³² on the cell viability.

As shown in Fig. 4, pretreatment of the cells with SMT resulted in effective protection against LDH release (Fig. 4A) and caspase-3 activity (Fig. 4B). Treatment of the cells with only SMT under the same conditions did not affect these parameters. On the other hand, the extent of LDH release (%) in Al(maltol)₃-treated cells without and with 100 μ M 7-NI was 50.8 ± 3.4 and 55.2 ± 1.4 , respectively; this indicates that 7-NI did not protect against Al(maltol)₃-induced cell damage.

In a preliminary experiment, we found that Al(maltol)₃ treatment of the cells did not induce an appreciable increase

of iNOS protein expression (data not shown). Szabo *et al.* have demonstrated that SMT plays as a direct competitive inhibitor of the activity of iNOS.³¹ From these findings, it

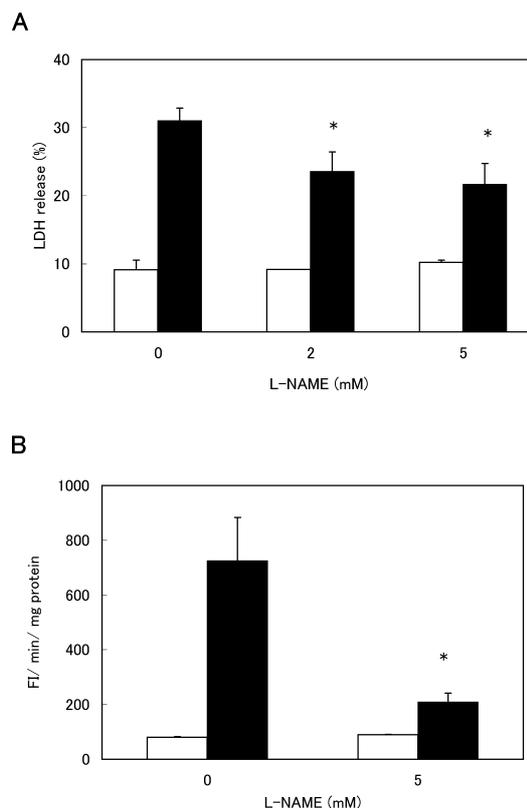
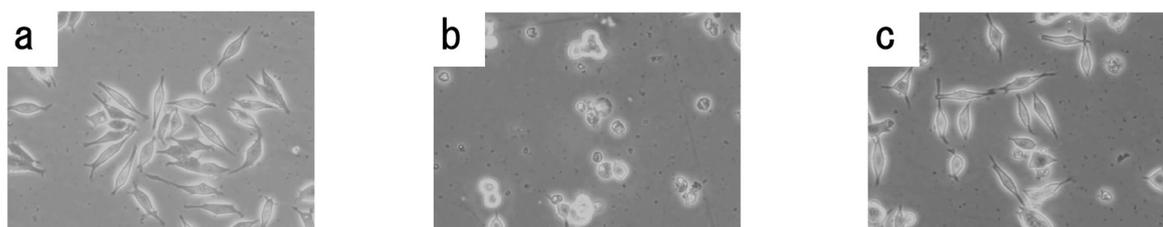


Fig. 2. Effects of L-NAME on Al(maltol)₃-Induced LDH Release (A) and Caspase-3 Activation (B)

The cells were preincubated with L-NAME (2 and 5 mM in A and 5 mM in B) for 30 min before 48-h exposure to Al(maltol)₃ (150 μ M). Symbols: □, control; ■, Al(maltol)₃ treatment. Values are expressed as means \pm S.E.M. for three independent measurements. * $p < 0.05$ vs. Al(maltol)₃ treatment.

A



B

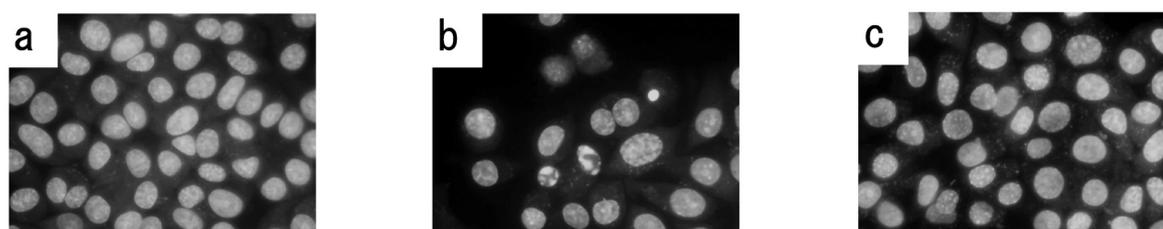


Fig. 3. Effect of L-NAME on Cell Morphology (A) and the Fluorescence Image of Hoechst-Stained Cells (B)

The experimental conditions are the same as those described in the legend of Fig. 2. a, control; b, Al(maltol)₃ treated; c, Al(maltol)₃ + 5 mM L-NAME treated.

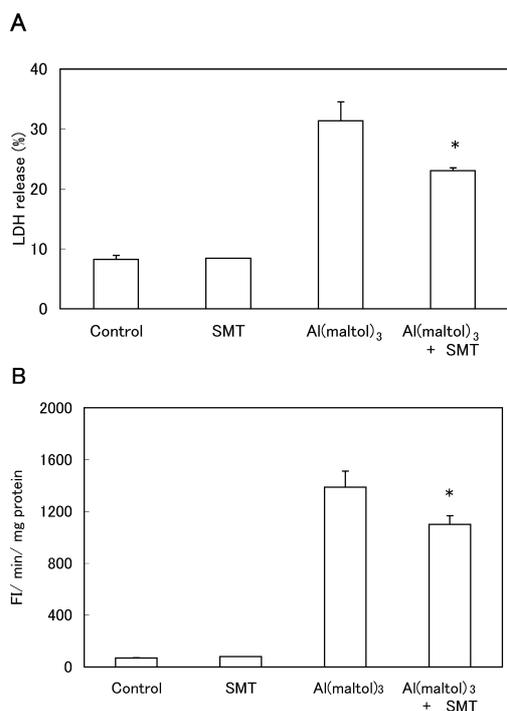


Fig. 4. Effect of SMT on Al(maltol)₃-Induced LDH Release and Caspase-3 Activation

The experimental conditions are the same as those described in the legend of Fig. 2, except for the use of SMT (5 mM) instead of L-NAME. (A) LDH release and (B) Caspase-3 activity. Values are expressed as means \pm S.E.M. for three independent measurements. * $p < 0.05$ vs. Al(maltol)₃ treatment.

seems likely that the inhibitory effect of SMT against Al(maltol)₃-induced cell death may be due to inhibition of iNOS activity rather than inhibition of the protein expression. Together these results and findings, we speculated that iNOS, but not nNOS, plays an important role in the onset mechanisms of Al(maltol)₃-induced cell death.

Effects of Ascorbic Acid Previously, we have reported that NAC effectively protected Al(maltol)₃-induced cell death by increasing the concentration of intracellular reduced glutathione.¹¹⁾ To further confirm the protective effects of an antioxidant on Al(maltol)₃-induced cell death, we employed ascorbic acid in the present study.

Figures 5 and 6 show the effects of ascorbic acid on Al(maltol)₃- and DETA NONOate-induced cell death and cell morphology. As shown in Fig. 5, A and B, it is clear that both Al(maltol)₃- and DETA NONOate-induced LDH release are almost completely prevented by ascorbic acid present in the culture medium. A similar protective effect of ascorbic acid was also observed against Al(maltol)₃- and NO-induced cell morphological changes (Fig. 6).

Yamamoto *et al.* have also reported that ascorbic acid effectively protects against NOR3-induced cell death of PC12 cells; this protection is better than that provided by reduced glutathione and cysteine.²²⁾ In addition, Desole *et al.* have demonstrated that manganese-induced apoptosis of PC12 cells, which is related to oxidative stress, is completely inhibited by ascorbic acid.³³⁾ These results and findings suggest that cell death induced by Al(maltol)₃ and DETA NONOate is involved in intracellular ROS generation. In a previous paper, we have reported that the cytotoxic effect of Al(maltol)₃ depends on the concentration of intracellular

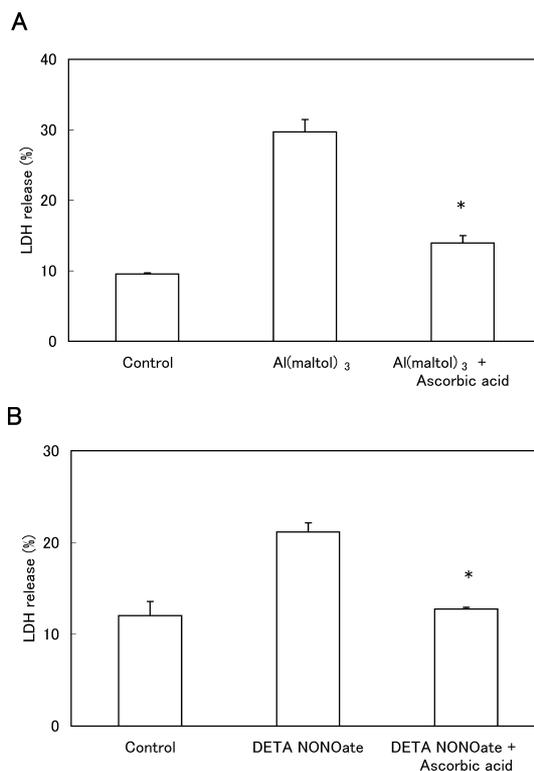


Fig. 5. Protective Effect of Ascorbic Acid against Al(maltol)₃- and NO-Induced LDH Release

The cells were preincubated with 1 mM ascorbic acid for 30 min before a 48-h exposure to Al(maltol)₃, or an 18-h exposure to DETA NONOate. The concentrations of Al(maltol)₃ and DETA NONOate were 150 and 250 μ M, respectively. Values are expressed as means \pm S.E.M. for three independent measurements. * $p < 0.05$ vs. Al(maltol)₃ treatment.

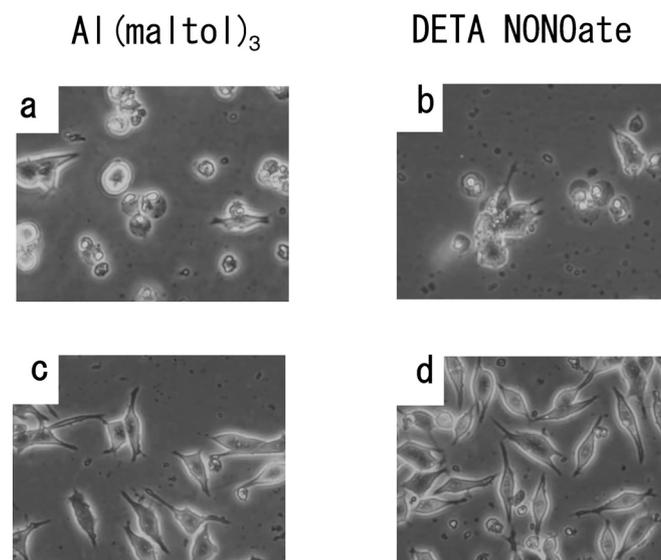


Fig. 6. Protective Effects of Ascorbic Acid on Cell Morphological Changes

The experimental conditions are the same as those described in the legend of Fig. 5. a and b, without ascorbic acid; c and d, with 1 mM ascorbic acid.

Al(maltol)₃ incorporated into the cells.¹⁰⁾

Based on these findings, it is speculated that intracellular NO generation that is related to Al(maltol)₃ accumulation in cells plays an important role in the onset mechanism of Al(maltol)₃-induced cell death. Further, it seems that these data give us an important clue for the analyzing mechanisms

concerning the onset of Al-mediated neurodegenerative diseases.

Acknowledgments This work was supported by a grant from the special research fund of Hokuriku University.

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