別紙４

研究成果の刊行に関する一覧表

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T. Fukuyama, T. Kosaka,, Y. Tajima, K. Hayashi, Y. Shutoh, and T..Harada	Detection of thymocytes apoptosis in mice induced by organochlorine pesticides methoxychlor.	Immunopharmacol Immunotoxicol	33	193-200	2011
M. Sekimoto, S. Sano, T. Hosaka, K. Nemoto and M. Degawa	Establishment of a stable human cell line, HPL-A3, for use in reporter gene assays of CYP3A inducers.	*Biol. Pharm. Bull.*			*in press.*
Y. Iwasaki, T. Hirasawa, Y. Maruyama, Y. Ishii, R. Ito, K. Saito, T. Umemura, A. Nishikawa and H. Nakazawa	Effect of interaction between phenolic compounds and copper ion on antioxidant and pro-oxidant activities.	Toxicol. in Vitro	25(7)	1320-1327	2011
Y. Iwasaki, M. Nomoto, M. Oda, K. Mochizuki, Y. Nakano, Y. Ishii, R. Ito, K. Saito, T. Umemura, A. Nishikawa and H. Nakazawa	Characterization of nitrated phenolic compounds for their anti-oxidant, pro-oxidant, and nitration activities.	Arch. Biochem. Biophys	513(1)	10-18	2011
Y. Iwasaki, K. Mochizuki, Y. Nakano, N. Maruya, M. Goto, Y. Maruyama, R. Ito, K. Saito and H. Nakazawa	Comparison of fluorescence reagents for simultaneous determination of hydroxylated phenylalanine and nitrated tyrosine by high-performance liquid chromatography with fluorescence detection.	Chromatogr.	26(1)	41-50	2012

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RESEARCH ARTICLE

Detection of thymocytes apoptosis in mice induced by organochlorine pesticides methoxychlor

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Abstract

The thymus has long been known to be vulnerable to atrophy when exposed to variety of stimuli, including hormones, immunosuppressive pharmaceuticals, and environmental chemicals. The organochlorine pesticide methoxychlor (MXC) is an immunosuppressive agent thought to affect thymic atrophy by inducing apoptosis of thymocyte T cells. We sought to develop an experimental protocol to detect *in vivo* thymocyte apoptosis induced by MXC in Balb/c mice. We treated the mice with 150–400 mg/kg MXC. We then measured thymus weight, cell counts, caspase activity (3/7, 8, and 9), annexin V labeling of phosphatidylserine (PS) and DNA fragmentation. In MXC-treated mice we observed decreases in thymus weight and cell counts and increases in caspase activity (3/7, 8, and 9), annexin V PS labeling and DNA fragmentation. These results suggest that MXC induces thymic atrophy caused by thymocyte apoptosis, and that our protocol may be useful for detecting *in vivo* thymocyte apoptosis induced by environmental chemicals in short-time.

Keywords: *Methoxychlor; apoptosis; thymus; mouse*

Introduction

The immune system is highly sensitive to the toxic effects of several types of chemicals. The thymus in particular has long been known to be vulnerable to atrophy associated with exposure to variety of substances, including hormones, immunosuppressive pharmaceuticals, and environmental chemicals.[1–5] The thymus is a complex organ that is responsible for the maturation and differentiation of most peripheral T cells.[6] Given the complexity of the thymus, compounds that cause thymic atrophy could be acting on a variety of cellular targets and causing atrophy by various mechanisms. Proposed mechanisms include inhibition of thymocyte precursors in the bone marrow or fetal liver and inhibition of intrathymic development of the thymocytes themselves. Such inhibition may result from indirect effects on the supporting stromal elements that produce growth factors and signals or direct mechanisms such as decreased cell proliferation and increased cell death through apoptosis.[5]

Apoptosis is an essential process underlying multicellular organism development and function. In the immune system, apoptosis is required for lymphocyte development and homeostasis. The dysregulation of apoptosis leads to a variety of immune disorders, including immunodeficiency, tumorogenesis, allergies, and autoimmunity.[7] Detection of *in vivo* chemical-induced thymocyte apoptosis is difficult, however, because of the rapid clearance of apoptotic cells by phagocytic cells.[8–12] In addition, it has been taken a long time to detect the apoptosis *in vivo*. Therefore, protocols are needed for the detection of *in vivo* chemical-induced thymocyte apoptosis in short time.

Some recently reported studies have found that organochlorine pesticides such as dichlorodiphenyltrichloroethane (DDT) and methoxychlor (MXC) affect the immune system[13,14] as well as the reproductive system. Developed to replace DDT,[15] MXC has a shorter half-life than DDT, lower toxicity in mammals, and greater biodegradability.[15–17] Because of their nature of persistence in the environment, bioaccumulation in the food chain, and possible health effects, the US Environmental Protection Agency restricted and banned the use of most of organochlorine pesticides during the 1970s and 1980s.

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Although organochlorine pesticides are rarely used today, measurable amounts of organochlorine pesticides or their metabolites can still be found in human tissues. Moreover, organochlorine pesticides continue to be heavily used in some developing countries, primarily for mosquito and malaria control.[18,19] Thus, the health effects of organochlorine pesticides exposure remain an important public health concern. In a previous study, we demonstrated that MXC affects the immune system, leading to atrophy of $CD4^+CD8^+$ double-positive (DP) T cells in the thymus.[20–22] In addition, Chapin et al.[13] reported that doses of 150 mg/kg/day in rats led to markedly decreased thymus weights. The mechanisms behind the immunosuppressive action of MXC are not fully understood, however.

In this issue, we attempt to detect thymocyte apoptosis induced by MXC in short time. To that end, we used our original protocol and MXC at rather high doses (about one fourth of the LD50). We sought to determine whether MXC induces thymic atrophy in young mice and whether exposure to MXC triggers *in vivo* apoptosis in thymocytes.

Materials and methods

Chemicals and reagents

MXC (more than 95% pure) was purchased from Sigma-Aldrich (St Louis, MO). Dexamethasone (DEX; more than 99% pure) and ethanol were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Corn oil was purchased from Hayashi Chemicals (Tokyo, Japan). MXC was dissolved in ethanol and diluted in corn oil. DEX was diluted in saline. DEX treatment group is referred to as the "positive control" group in this study.

Animals and housing conditions

Female BALB/c mice (age, 4 weeks) were purchased from Charles River Japan Laboratories (Kanagawa, Japan). The mice were housed individually under controlled lighting (lights on from 0700 to 1900 h), temperature (22 ± 2°C), humidity (55% ± 15%), and ventilation (at least ten 100% fresh air changes hourly). Food (Certified Pellet Diet MF, Oriental Yeast Co., Tokyo, Japan) and water were available *ad libitum*.

This study was conducted in accordance with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science.[23]

Experimental design

After a 1-week acclimatization period, mice (5 weeks old) were allocated randomly to four treatment groups and one control group ($n=8$ per group). The experimental protocol used in this study is depicted in Figure 1. As a positive control, one treatment group received a single intraperitoneal injection of DEX (10 mg/kg). The other four treatment groups received three oral doses in 8-h intervals of MXC of 0 (negative control), 150, 300, or 400 mg/kg. All dose amounts of MXC were selected to avoid death and systemic toxicity (particularly in the preliminary test) while still permitting comparisons of the immunological potencies of the chemicals. Eight hours after the last treatment, mice were anesthetized with diethyl ether and killed. Each animal's thymus was carefully removed and weighed. Approximately one-half of the thymus tissue from each mouse was used in a single-cell suspension for analysis of annexin V and caspases 3/7, 8, and 9. The remaining thymus tissue was stored at –80°C until use for the detection of DNA ladder formation.

Preparation of cell suspension

Single-cell suspensions of thymocytes in 10 mL PBS (Gibco, Carlsbad, CA) supplemented with 5% heat-inactivated fetal calf serum (Gibco) were prepared by passage through a stainless-steel screen. The number of thymocytes was determined with a Coulter counter Z2 (Beckman Coulter, Tokyo, Japan).

Caspase 3/7, 8, and 9 analysis

From each cell suspension, 100 µL (1×10^5 cells) was seeded in duplicate into opaque-walled 96-well plates (Nulge Nunc International, Tokyo, Japan). Caspases 3/7, 8, and 9 in each well were measured with a luciferin-luciferase system (caspase-Glo 3/7, 8 and 9 assay, Promega, Tokyo, Japan). We used a microplate luminometer (TR717, Berthold Japan, Tokyo, Japan) to measured the activity of each caspase in relative light units (RLU).

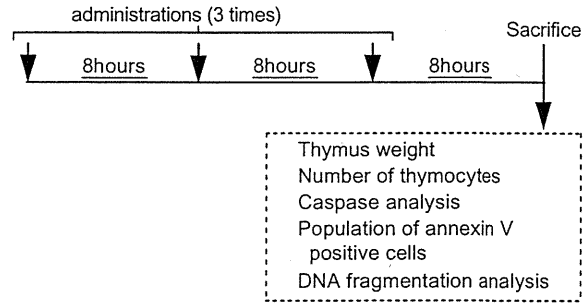

Figure 1. Experimental protocol used to administration BALB/c mice with methoxychlor (MXC). Eight hours after the last treatment, mice were anesthetized with diethyl ether and euthanized. Each animal's thymus was carefully removed and weighed. Approximately one-half of the tissues were pooled per mouse in phosphate-buffered saline (PBS, Gibco, Tokyo, Japan) analyzed for annexinV, caspase-3/7, caspase-8, and caspase-9. The other half of the thymus was kept at –80°C until used for the detection of DNA ladder formation.

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Cell staining and flow cytometric analysis

In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment.[24] Because externalization of PS occurs in the earlier stages of apoptosis, annexin V staining can enable identification of apoptosis at an earlier stage than assays that measure nuclear changes.

Flow cytometric analysis was performed by staining thymocytes with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide. To assay the thymocytes we used an annexin V: FITC Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA) in accordance with the manufacturer's protocol. Briefly, thymocytes (1×10^6 cells/animal) were pelleted by centrifugation and resuspended in 1 mL of annexin V-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$). We then incubated 100 µL of cell suspension (1×10^5 cells) with FITC-conjugated annexin V and propidium ioide for 15 min at room temperature in the dark. We added 400 µL of annexin V-binding buffer and then performed analysis with a FACSCalibur flow cytometer (BD Pharmingen) with the Cell Quest program (BD Pharmingen). For each sample, 10,000 events were collected and analyzed for the expression of antigens to annexin V and propidium ioide.

DNA fragmentation analysis

Isotissue® (Nippon Gene, Tokyo, Japan) DNA extraction kit was used to extract DNA from the frozen thymus samples. Each sample was placed in a microfuge tube with extraction buffer and proteinase K and incubated at 55°C until completely dissolved. We then added 5 M NaCl and centrifuged the mixture for 20 min (12,000*g* at 4°C. The supernatant was placed in a microfuge tube and incubated for 15 min at 55°C with RNase A (0.4 mg/mL) and then incubated for another 15 min at the same temperature with proteinase K (0.4 mg/mL). After being washed with washing buffer and 70% ethanol, DNA was eluted with Tris-EDTA (pH 8.0) buffer. The eluate was used as the DNA sample for agarose gel electrophoresis.

DNA samples were electrophoretically separated on 2% agarose gel (Mercury Heat & Pour Agarose, Pretech Instruments KB, Sollentuna, Sweden) in 90 mM Tris-boric acid-EDTA solution (Nippon Gene) at 8.5 V/cm for 2 h. After electrophoresis, the gels were stained with Vistra Green nucleic acid gel stain (GE Healthcare Bio-Sciences Corp, Piscataway, NJ), and the nucleic acids were visualized with a fluorimager (FluorImager 585, GE Healthcare UK Ltd. Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, England).

Statistical analysis

We transformed the data logarithmically to homogenize the variances and used analysis of variance (ANOVA) to evaluate the difference between test (MXC and DEX) and vehicle treatment group. When ANOVA was significant, the differences between groups were assessed by means of ANOVA followed by the Dunnett's multiple comparison test. A value of $P < 0.05$ was considered to be significant. Values are expressed as the mean ± standard deviation (SD).

Results

Thymus weights and numbers of thymocytes

In the MXC treatment groups thymus weights decreased in a dose-dependent manner, and statistically significant differences were found between the group that received a dose of 400 mg/kg and the other groups ($P < 0.01$) (Figure 2A). The total numbers of thymocytes were

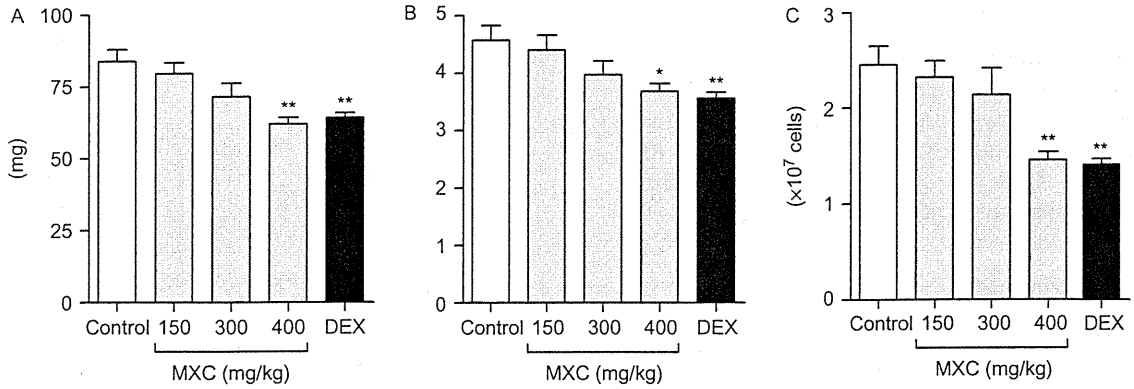

Figure 2. Absolute thymus weight (A) relative thymus weight (B) and total thymocytes count (C) of mice treated with test solution. Absolute thymus weights are expressed as means (mg/mouse) ± SD. Relative thymus weight are expressed as means (absolute thymus weigh, mg/body weight, g) ± SD. Thymocyte counts are expressed as means (×10^7 cells/mouse) ± SD. Statistical significance is marked by asterisks: **$P < 0.01$ (Dunnet's *t*-test).

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lower in the 400 mg/kg group, but the decrease was not statistically significant (Figure 2B). Absolute thymus weight and number of thymocytes in the MXC 150 mg/kg treatment group were 79.63 ± 10.8 mg and 2.32 ± 0.5 × 10^7 cells, and in the MXC 300 mg/kg treatment group were 71.50 ± 13.8 mg and 2.14 ± 0.8 × 10^7 cells. Results for both of these groups were similar to those in the control group (absolute thymus weight, 83.88 ± 12.0 mg; number of thymocytes; 2.45 ± 0.5 × 10^7 cells). In the MXC 400 mg/kg treatment group, absolute thymus weight was 62.00 ± 6.7 mg, approximately 74% of that in the control group; this is a 26% decrease, and the number of thymocytes was 1.45 ± 0.2 × 10^7 cells, approximately 60% of the cell numbers in the control; this is a 40% decrease. (Figure 2).

In the DEX treatment group, statistically significant decreases were noted in both thymus weight and total number of thymocytes ($P < 0.01$) (Figure 2). The absolute thymus weight and total number of thymocytes were 64.25 ± 4.7 mg and 2.19 ± 0.2 × 10^7 cells, approximately 75% of those for the control group (Figure 2).

Caspase 3/7, 8, and 9 analysis

Caspase 3/7, 8, and 9 activities induced by 400 mg/kg MXC treatment were significantly higher than those of the control group ($P < 0.01$) (Figure 3). Caspase 3/7, 8, and 9 activities in the MXC 150 mg/kg treatment group were 3854 ± 574, 19,603 ± 2599, and 45,325 ± 6659 RLU, and were similar to the control group values of 4241 ± 623, 22,011 ± 2443, and 50,514 ± 5406 RLU, respectively (Figure 3). In the MXC 300 mg/kg treatment group, caspase 3/7, 8, and 9 activity values were 33,378 ± 50,723, 23,285 ± 9147, and 63,915 ± 39,268 RLU, respectively, approximately 800, 105, and 130% of those of the control group (Figure 3). In the MXC 400 mg/kg treatment group, caspase 3/7, 8, and 9 activity values were 155,655 ± 47,397, 46,950 ± 11,576, and 163,973 ± 47,386 RLU, respectively, approximately 3600, 200, and 300% of those of the control group (Figure 3).

In the DEX treatment group, statistically significant increases were noted in the activity of all caspases ($P < 0.01$) (Figure 3). The activity values of caspases 3/7, 8, and 9 were 196,822 ± 13,049, 52,918 ± 3723 and 182,498 ± 14,771 RLU, approximately 4600, 240, and 360% higher, respectively, than those in the control group (Figure 3).

Flow cytometric analysis

To evaluate the state of apoptotic cells after treatment with MXC, flow cytometric analysis was performed with thymocyte staining by FITC-conjugated annexin V and propidium iodide (Figures 4 and 5). The real cell numbers of annexin V-positive thymocytes were represented. The number of annexin V-positive thymocytes

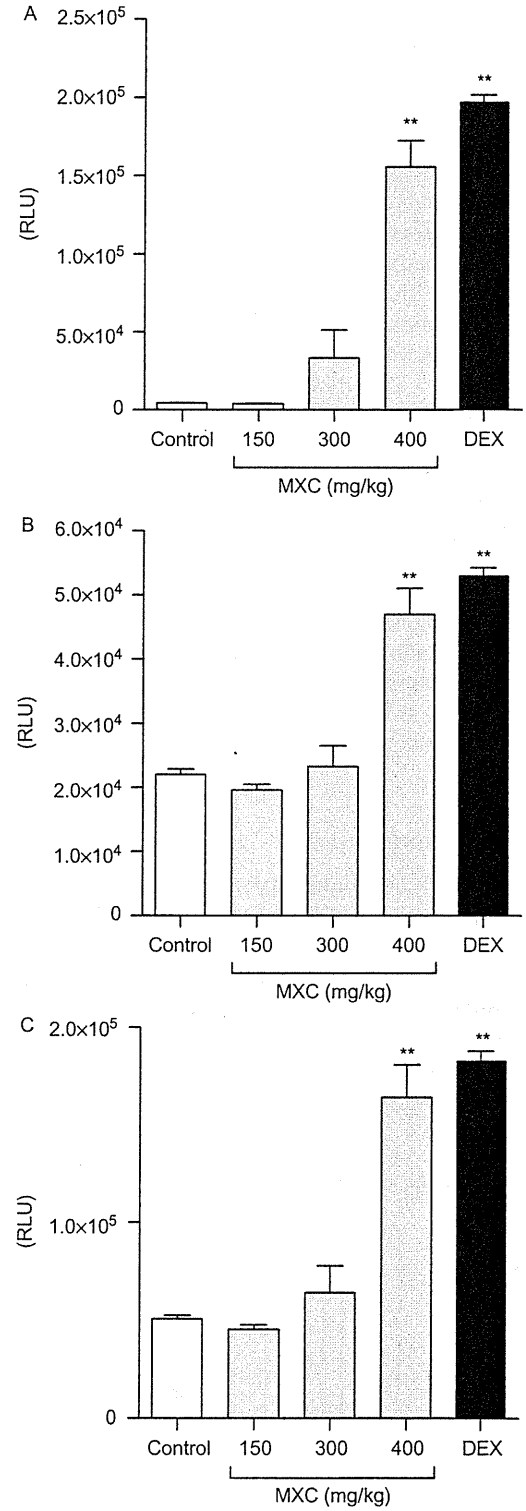

Figure 3. Caspases activities (A: 3/7; B: 8; C: 9) in the thymus of mice treated with test solution. These activities are expressed as means (RLU) ± SD. Statistical significance is marked by asterisks: $^{**}P < 0.01$ (Dunnet's *t*-test).

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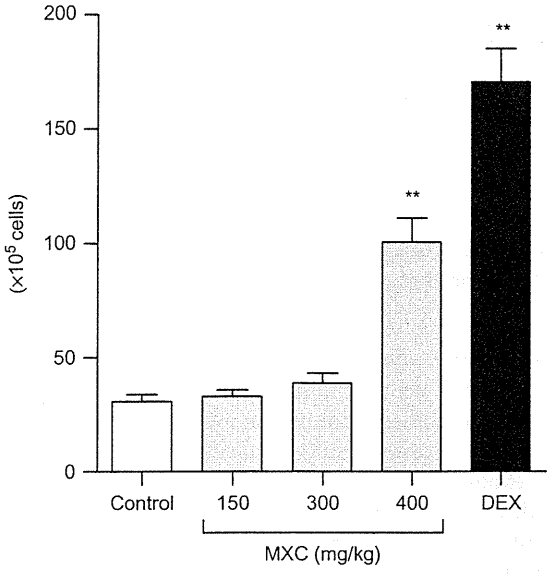

Figure 4. The populations of annexin V-positive cells (A: 3/7; B: 8; C: 9) in the thymocytes of mice treated with test solution. The populations of annexin V-positive cells are expressed as means (×10⁵ cells/mouse) ± SD. Statistical significance is marked by asterisks: $^{**}P<0.01$ (Dunnet's *t*-test).

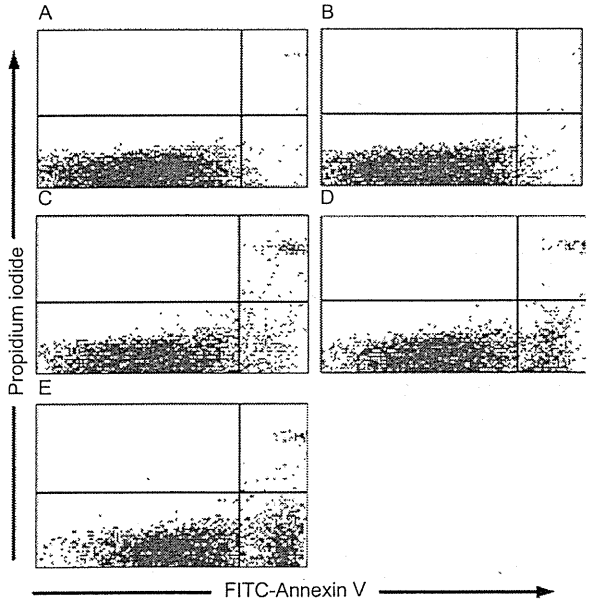

Figure 5. Characteristic exemplification of dot-plots of annexin V-positive cells (A: control; B: MXC 150 mg/kg; C: MXC 300 mg/kg; D: MXC 400 mg/kg; E: DEX) in the thymocytes of mice treated with test solution. (See colour version of this figure online at www.informahealthcare.com/ipi)

was significantly higher in the 400 mg/kg MXC-treated group than in control group ($P<0.01$) (Figure 4). In the MXC 150 mg/kg treatment group, the number of annexin V-positive thymocytes was $33.13\pm7.9\times10^5$ cells, approximately 107% of the control group value of $30.88\pm8.7\times10^5$ cells (Figures 4, 5A and 5B). In the MXC 300 mg/kg treatment group, the number of annexin V-positive thymocytes was $38.89\pm12.6\times10^5$ cells, approximately 130% that of the control group (Figures 4 and 5C). In the MXC 400 mg/kg treatment group, the number of annexin V-positive thymocytes was $100.60\pm29.8\times10^5$ cells, approximately 325% that of the control group (Figures 4 and 5D). The number of annexin V-positive thymocytes in the DEX treatment group ($170.4\pm41.3\times10^5$ cells) was approximately 550% that of the control group (Figures 4 and 5E) and this difference was highly significant ($P<0.01$).

DNA fragmentation analysis

We used agarose gel electrophoresis to assess the degradation pattern of nuclear DNA in MXC-induced cell death because it has been observed that oligonucleosomal cleavage accompanies apoptosis in most systems.[25–27] In the 300 and 400 mg/kg MXC treatment groups, we observed increase in fragmented DNA of 200 bp (Figure 6C and 6D), whereas essentially no fragmented DNA was detected in the control and MXC 150 mg/kg treatment groups (Figure 6A and 6B). In the positive control DEX treatment group, marked DNA fragmentation was noted (Figure 6E).

Discussion

Our results indicate that MXC at doses of 400 mg/kg administered three times in 8 h by oral gavage modulated the immune response in young female Balb/c mice. The indicators of apoptosis affected were thymus weight, number of thymocytes, caspase activity (3/7, 8, and 9), number of annexin V-positive thymocytes and DNA fragmentation in thymocytes (see Figures 3–6). This study is the first to demonstrate these particular effects of *in vivo* environmental chemical-induced thymocyte apoptosis in short time.

Detection of *in vivo* chemical-induced apoptosis in short time is difficult because of the rapid clearance of apoptotic cells by phagocytic cells.[8–12] To overcome this problem, we used a protocol in which mice were killed 8 h after receiving the last of three oral doses of MXC. In addition, we focused on several endpoints such as movement of PS, DNA fragmentation, and caspase activity. By using this procedure we detected more apoptosis in MXC-treated mice than in controls.

A relatively early marker of apoptosis is movement of PS residues to the external leaflet of the plasma membrane.[28] This process is thought to facilitate macrophage recognition, engulfment, and removal of dying cells.[29,30] Our results indicate that MXC increased the

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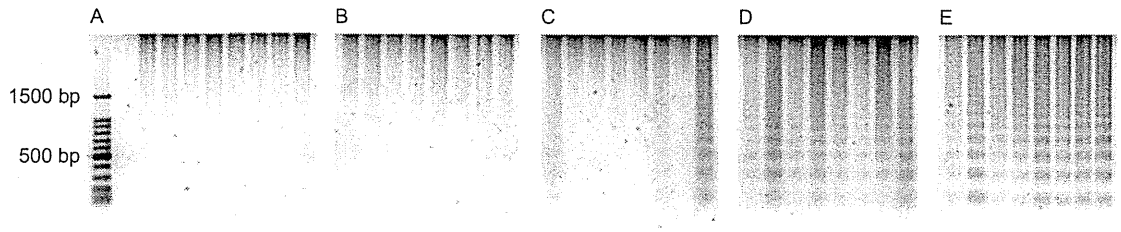

Figure 6. Agarose gel electrophoresis of DNA extracted from the thymus (A: control; B: MXC 150 mg/kg; C: MXC 300 mg/kg; D: MXC 400 mg/kg; E: DEX) of mice treated with test solution. The lanes correspond to each mouse in the experimental group. Molecular weight markers are indicated to the left.

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number of PS-exposed cells in a dose-dependent manner (see Figures 4 and 5). This upregulation of PS movement thus seems to be a useful endpoint for identifying chemical-related thymocyte apoptosis.

A later-stage marker of apoptosis is DNA fragmentation. Apoptosis has been characterized biochemically by the production of internucleosomal DNA fragments of 180–200 bp resulting from the activation of an endonuclease. We observed prominent increases in the DNA fragmentation in the 300 and 400 mg/kg MXC treatment groups (see Figure 6). This upregulation of DNA fragmentation also seems to be a useful endpoint for identifying chemical-related thymocyte apoptosis.

Caspase activation is a step in apoptosis, and measurement of caspase 3/7, 8, and 9 activity is a reliable indicator of caspase-dependent apoptosis.[31] Caspases 8 and 9 are thought to be initiator caspases, and caspases 3 and 7 effector caspases. Caspase 3, the first caspase linked to apoptosis that is upstream of DNA fragmentation, is activated by caspases 8 and 9. Caspase 3 is required for DNA degradation and chromatin condensation. Thus the dose-dependent increase in caspase 3 (see Figure 3A) indicated an increase in MXC-induced thymocyte apoptosis. Caspase 8 is the apical caspase in the tumor necrosis factor–family death-receptor or extrinsic pathway. Caspase 8 activation initiates Fas (Apo-1/CD95), tumor necrosis factor receptor and death receptor-3.[32–36] Active caspase 8 cleaves and activates the downstream effector caspase 3, which cleaves cellular targets and induces apoptosis. Furthermore, caspase 8 cleaves the BH3-only protein Bid and generates a small fragment of Bid, which translocates to the mitochondrial outer membrane and inhibits the function of anti-apoptotic proteins Bcl-2 and Bcl-x_L. This step leads to the activation of the intrinsic death pathway.[7] Caspase 9 is the apical caspase in the intrinsic or mitochondrial pathway. Active caspase 9 further activates downstream effector caspases, including caspases 3 and 7, which in turn cleave hundreds of cellular components and result in irreversible cell death. In the present study, caspase 8 and 9 activity in thymocytes treated with MXC increased significantly. This result suggests that MXC-induced thymocyte apoptosis affects both intrinsic and extrinsic apoptosis pathways.[7]

To detect *in vivo* MXC-induced thymocyte apoptosis in short time, we used our original protocol and MXC at rather high doses (about one fourth of the LD50). In recent years, several reports have suggested that oral MXC exposure suppresses immune function by inducing thymic atrophy.[13,14] Whether this thymic atrophy occurs because MXC triggers apoptosis is not clear, however. Our study is the first to demonstrate severe thymic atrophy with cellular depletion and increased thymocyte apoptosis in MXC-treated mice, findings indicating that MXC induces thymocyte apoptosis. Takeuchi et al.[21] demonstrated that morphometrical analysis revealed a significant decrease in the size of the thymic cortical area in MXC-treated rat pups. In addition, phenotyping of thymic lymphocytes revealed a significant decrease in the DP immature T cells ($CD3^{int}CD4^{+}CD8^{+}$) located in the cortex area. These reports and our results indicate that MXC may cause thymic cortical atrophy via a thymocytes apoptotic process. In general, DP cells represent the majority of thymic lymphocytes located in the cortex area and have low expression of Bcl-2, which is associated with resistance to apoptosis.[37,38] DP cells are sensitive when exposed to chemicals, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin and organotins, and tend to show apoptotic cell death in the *in vitro* and *in vivo* studies.[39–43] This suggests that immunotoxic chemicals that have the potential to cause thymic atrophy may affect DP thymocytes and result in apoptosis, as was observed with MXC.

Numerous environmental toxic chemicals such as pesticides, perfumes, organotins, and heavy metals play roles in the dysregulation of immune functions, including thymocyte apoptosis.[44–52] Such immunosuppression may predispose the highly sensitive immune system to loss of tolerance to self-antigens and subsequent increased risk for autoimmune disease and allergies. Alternatively, altered repertoires of T cells responsive to foreign antigens may cause the neonate to become more susceptible to infections.[44–46,53] From this point of view, we attempted to develop a method for detection of *in vivo* chemical-induced thymocyte apoptosis and we obtain positive reactions using MXC as a typical chemical. Whether the same events occur after administration of other chemicals

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that appear in many places in the environment remains uncertain, however. Experiments are currently under investigation in our laboratories.

In summary, we detected *in vivo* MXC-induced thymocyte apoptosis in short time by measuring thymus weight, number of thymocytes, caspase activity (3/7, 8, and 9), number of annexin V-positive thymocytes and DNA fragmentation in thymocytes.

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Declaration of interest

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References

1. Ashwell, J.D., Lu, F.W., Vacchio, M.S. Glucocorticoids in T cell development and function. Annu. Rev. Immunol. 2000, 18, 309–345.
2. Shanker, A. Is thymus redundant after adulthood? Immunol. Lett. 2004, 91, 79–86.
3. Drela, N. Xenobiotic-induced alterations in thymocyte development. APMIS 2006, 114, 399–419.
4. Zoller, A.L., Kersh, G.J. Estrogen induces thymic atrophy by eliminating early thymic progenitors and inhibiting proliferation of beta-selected thymocytes. J. Immunol. 2006, 176, 7371–7378.
5. Nohara, K., Ao, K., Miyamoto, Y., Suzuki, T., Imaizumi, S., Tateishi, Y., Omura, S., Tohyama, C., Kobayashi, T. Arsenite-induced thymus atrophy is mediated by cell cycle arrest: a characteristic downregulation of E2F-related genes revealed by a microarray approach. Toxicol. Sci. 2008, 101, 226–238.
6. Ladi, E., Yin, X., Chtanova, T., Robey, E.A. Thymic microenvironments for T cell differentiation and selection. Nat. Immunol. 2006, 7, 338–343.
7. Zhang, N., Hartig, H., Dzhagalov, I., Draper, D., He, Y.W. The role of apoptosis in the development and function of T lymphocytes. Cell Res. 2005, 15, 749–769.
8. Savill, J. Apoptosis: from worm to wonder-drug? Br. J. Rheumatol. 1995, 34, 95–98.
9. Savill, J. Apoptosis: will cell death add life to nephrology? Nephrol. Dial. Transplant. 1995, 10, 1977–1979.
10. Savill, J., Haslett, C. Granulocyte clearance by apoptosis in the resolution of inflammation. Semin. Cell Biol. 1995, 6, 385–393.
11. Kamath, A.B., Xu, H., Nagarkatti, P.S., Nagarkatti, M. Evidence for the induction of apoptosis in thymocytes by 2,3,7,8-tetrachlorodibenzo-p-dioxin in vivo. Toxicol. Appl. Pharmacol. 1997, 142, 367–377.
12. Pryputniewicz, S.J., Nagarkatti, M., Nagarkatti, P.S. Differential induction of apoptosis in activated and resting T cells by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and its repercussion on T cell responsiveness. Toxicology 1998, 129, 211–226.
13. Chapin, R.E., Harris, M.W., Davis, B.J., Ward, S.M., Wilson, R.E., Mauney, M.A., Lockhart, A.C., Smialowicz, R.J., Moser, V.C., Burka, L.T., Collins, B.J. The effects of perinatal/juvenile methoxychlor exposure on adult rat nervous, immune, and reproductive system function. Fundam. Appl. Toxicol. 1997, 40, 138–157.
14. Guo, T.L., Zhang, X.L., Bartolucci, E., McCay, J.A., White, K.L. Jr, You, L. Genistein and methoxychlor modulate the activity of natural killer cells and the expression of phenotypic markers by thymocytes and splenocytes in F0 and F1 generations of Sprague-Dawley rats. Toxicology 2002, 172, 205–215.
15. Bal, H.S. Effect of methoxychlor on reproductive systems of the rat. Proc. Soc. Exp. Biol. Med. 1984, 176, 187–196.
16. Kapoor, I.P., Metcalf, R.L., Nystrom, R.F., Sangha, G.K. Comparative metabolism of methoxychlor, methiochlor, and DDT in mouse, insects, and in a model ecosystem. J. Agric. Food Chem. 1970, 18, 1145–1152.
17. Bulger, W.H., Muccitelli, R.M., Kupfer, D. Interactions of methoxychlor, methoxychlor base-soluble contaminant, and 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane with rat uterine estrogen receptor. J. Toxicol. Environ. Health 1978, 4, 881–893.
18. Turusov, V., Rakitsky, V., Tomatis, L. Dichlorodiphenyltrichloroethane (DDT): ubiquity, persistence, and risks. Environ. Health Perspect. 2002, 110, 125–128.
19. Xu, X., Dailey, A.B., Talbott, E.O., Ilacqua, V.A., Kearney, G., Asal, N.R. Associations of serum concentrations of organochlorine pesticides with breast cancer and prostate cancer in U.S. adults. Environ. Health Perspect. 2010, 118, 60–66.
20. Fukuyama, T., Tajima, Y., Ueda, H., Hayashi, K., Shutoh, Y., Harada, T., Kosaka, T. Apoptosis in immunocytes induced by several types of pesticides. J. Immunotoxicol. 2010, 7, 39–56.
21. Takeuchi, Y., Kosaka, T., Hayashi, K., Takeda, M., Yoshida, T., Fujisawa, H., Teramoto, S., Maita, K., Harada, T. Thymic atrophy induced by methoxychlor in rat pups. Toxicol. Lett. 2002, 135, 199–207.
22. Takeuchi, Y., Kosaka, T., Hayashi, K., Ishimine, S., Ohtsuka, R., Kuwahara, M., Yoshida, T., Takahashi, N., Takeda, M., Maita, K., Harada, T. Alterations in the developing immune system of the rat after perinatal exposure to methoxychlor. *J* Toxicol Pathol 2004, 17, 165–170.
23. Japanese Association for Laboratory Animal Science. Guidelines for animal experimentation. Exp. Anim. 1987, 36, 285–288.
24. Vermes, I., Haanen, C., Steffens-Nakken, H., Reutelingsperger, C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J. Immunol. Methods 1995, 184, 39–51.
25. Brown, D.G., Sun, X.M., Cohen, G.M. Dexamethasone-induced apoptosis involves cleavage of DNA to large fragments prior to internucleosomal fragmentation. J. Biol. Chem. 1993, 268, 3037–3039.
26. Oberhammer, F., Bursch, W., Tiefenbacher, R., Fröschl, G., Pavelka, M., Purchio, T., Schulte-Hermann, R. Apoptosis is induced by transforming growth factor-beta 1 within 5 hours in regressing liver without significant fragmentation of the DNA. Hepatology 1993, 18, 1238–1246.
27. Sakahira, H., Enari, M., Ohsawa, Y., Uchiyama, Y., Nagata, S. Apoptotic nuclear morphological change without DNA fragmentation. Curr. Biol. 1999, 9, 543–546.
28. Morris, R.G., Hargreaves, A.D., Duvall, E., Wyllie, A.H. Hormone-induced cell death. 2. Surface changes in thymocytes undergoing apoptosis. Am. J. Pathol. 1984, 115, 426–436.
29. Fadok, V.A., Savill, J.S., Haslett, C., Bratton, D.L., Doherty, D.E., Campbell, P.A., Henson, P.M. Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. J. Immunol. 1992, 149, 4029–4035.
30. Fadok, V.A., Voelker, D.R., Campbell, P.A., Cohen, J.J., Bratton, D.L., Henson, P.M. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J. Immunol. 1992, 148, 2207–2216.
31. Gurtu, V., Kain, S.R., Zhang, G. Fluorometric and colorimetric detection of caspase activity associated with apoptosis. Anal. Biochem. 1997, 251, 98–102.
32. Chinnaiyan, A.M., Dixit, V.M. The cell-death machine. Curr. Biol. 1996, 6, 555–562.

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33. Chinnaiyan, A.M., Hanna, W.L., Orth, K., Duan, H., Poirier, G.G., Froelich, C.J., Dixit, V.M. Cytotoxic T-cell-derived granzyme B activates the apoptotic protease ICE-LAP3. Curr. Biol. 1996, 6, 897–899.
34. Chinnaiyan, A.M., O'Rourke, K., Yu, G.L., Lyons, R.H., Garg, M., Duan, D.R., Xing, L., Gentz, R., Ni, J., Dixit, V.M. Signal transduction by DR3, a death domain-containing receptor related to TNFR-1 and CD95. Science 1996, 274, 990–992.
35. Chinnaiyan, A.M., Tepper, C.G., Seldin, M.F., O'Rourke, K., Kischkel, F.C., Hellbardt, S., Krammer, P.H., Peter, M.E., Dixit, V.M. FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. J. Biol. Chem. 1996, 271, 4961–4965.
36. Muzio, M., Salvesen, G.S., Dixit, V.M. FLICE induced apoptosis in a cell-free system. Cleavage of caspase zymogens. J. Biol. Chem. 1997, 272, 2952–2956.
37. Gratiot-Deans, J., Merino, R., Nuñez, G., Turka, L.A. Bcl-2 expression during T-cell development: early loss and late return occur at specific stages of commitment to differentiation and survival. Proc. Natl. Acad. Sci. U.S.A. 1994, 91, 10685–10689.
38. Moore, N.C., Anderson, G., Williams, G.T., Owen, J.J., Jenkinson, E.J. Developmental regulation of bcl-2 expression in the thymus. Immunology 1994, 81, 115–119.
39. Blaylock, B.L., Holladay, S.D., Comment, C.E., Heindel, J.J., and Luster, M.I. Exposure to tetrachlorodibenzo-p-dioxin (TCDD) alters fetal thymocyte maturation. Toxicol Appl Pharmacol 1992, 112, 207–213.
40. Lai, Z.W., Fiore, N.C., Gasiewicz, T.A., Silverstone, A.E. 2,3,7,8-Tetrachlorodibenzo-p-dioxin and diethylstilbestrol affect thymocytes at different stages of development in fetal thymus organ culture. Toxicol. Appl. Pharmacol. 1998, 149, 167–177.
41. McConkey, D.J., Hartzell, P., Duddy, S.K., Håkansson, H., Orrenius, S. 2,3,7,8-Tetrachlorodibenzo-p-dioxin kills immature thymocytes by Ca2+-mediated endonuclease activation. Science 1988, 242, 256–259.
42. Raffray, M., Cohen, G.M. Bis(tri-n-butyltin)oxide induces programmed cell death (apoptosis) in immature rat thymocytes. Arch. Toxicol. 1991, 65, 135–139.
43. Wyllie, A.H. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 1980, 284, 555–556.
44. Kohler, C., Jeanvoine, G., Pierrez, J., Olive, D., Gerard, H. Modifications of the thymus and splenic thymic dependent zones after in utero exposure to phenytoin: qualitative and quantitative analysis in C3H mice. Dev. Pharmacol. Ther. 1987, 10, 405–412.
45. Pieters, R.H., Kampinga, J., Snoeij, N.J., Bol-Schoenmakers, M., Lam, A.W., Penninks, A.H., Seinen, W. An immunohistochemical study of dibutyltin-induced thymus atrophy. Arch. Toxicol. Suppl. 1989, 13, 175–178.
46. Kawashima, I., Sakabe, K., Seiki, K., Fujii-Hanamoto, H. Hormone and immune response, with special reference to steroid hormone. 3. Sex steroid effect on T-cell differentiation. Tokai J. Exp. Clin. Med. 1990, 15, 213–218.
47. Hirai, M., Ichikawa, M. Changes in serum glucocorticoid levels and thymic atrophy induced by phenytoin administration in mice. Toxicol. Lett. 1991, 56, 1–6.
48. Zelikoff, J.T., Smialowicz, R., Bigazzi, P.E., Goyer, R.A., Lawrence, D.A., Maibach, H.I., Gardner, D. Immunomodulation by metals. Fundam. Appl. Toxicol. 1994, 22, 1–7.
49. Kosuda, L.L., Hannigan, M.O., Bigazzi, P.E., Leif, J.H., Greiner, D.L. Thymus atrophy and changes in thymocyte subpopulations of BN rats with mercury-induced renal autoimmune disease. Autoimmunity 1996, 23, 77–89.
50. Staples, J.E., Fiore, N.C., Frazier, D.E. Jr, Gasiewicz, T.A., Silverstone, A.E. Overexpression of the anti-apoptotic oncogene, bcl-2, in the thymus does not prevent thymic atrophy induced by estradiol or 2,3,7, 8-tetrachlorodibenzo-p-dioxin. Toxicol. Appl. Pharmacol. 1998, 151, 200–210.
51. Nakadai, A., Li, Q., Kawada, T. Chlorpyrifos induces apoptosis in human monocyte cell line U937. Toxicology 2006, 224, 202–209.
52. Li, Q., Kobayashi, M., Kawada, T. Organophosphorus pesticides induce apoptosis in human NK cells. Toxicology 2007, 239, 89–95.
53. Camacho, I.A., Nagarkatti, M., Nagarkatti, P.S. Evidence for induction of apoptosis in T cells from murine fetal thymus following perinatal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Toxicol. Sci. 2004, 78, 96–106.

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Effect of interaction between phenolic compounds and copper ion on antioxidant and pro-oxidant activities

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ABSTRACT

Phenolic compounds are widely used in food and cosmetics to prevent undesirable oxidation. On the other hand, phenolic compounds are also strong reducing agents and under *in vitro* conditions and in the presence of copper ion, they can act as pro-oxidants. In this study, we conducted electron spin resonance (ESR) measurements for the increase in reactive oxygen species (ROS) in relation to their structure and interaction with transition metals. Moreover, the antioxidant activity was assessed with the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, and the pro-oxidant effect of phenolic compounds on DNA damage was assessed by measuring 8-hydroxy-2′-deoxyguanosine (8-OHdG), which is effectively formed during oxidative damage. In conclusion, *ortho*-dihydroxyl groups that can chelate with Cu^{2+} induce the greatest pro-oxidant activity. Moreover, the interaction between phenolic compounds and copper induced to H_2O_2. The obtained results indicated that ROS participated in oxidative DNA damage induced by phenolic compounds in the presence of Cu^{2+}.

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1. Introduction

Phenolic compounds are widely studied for their antioxidant properties. The notion of cancer chemoprevention through intervention with an antioxidant arises from the fact that fruits and vegetables contain antioxidants that are said to lower the risk of cancer in people who consume them. In contrast, a recent meta-analysis of the clinical data from 68 randomized human trials involving 232,606 subjects (385 publications) revealed that an increased risk of mortality was associated with the regular use of certain putative antioxidant supplements, such as vitamins A and E (Bjelakovic et al., 2007). They concluded that antioxidant supplements seem to increase the risk of mortality. Those studies indicate the need to assess phenolic compounds because those compounds have antioxidant activity. Antioxidant activity refers to the ability of phenolic compounds to prevent damage caused by reactive oxygen species (ROS) (such as through radical scavenging) or to prevent the generation of ROS (by binding iron) (Perron and Brumaghim, 2009). Phenolic compounds are divided into several sub-classes, including catechins, flavonols, flavones, anthocyanins, proanthocyanidins, and phenolic acids. Phenolic compounds are present in milligram quantities in one serving of green tea (Cabrera et al., 2006), black tea (Gardner et al., 2007), coffee (Nardini et al., 2002), fruits (Vinson et al., 2001), vegetables (Vinson et al., 1998), olive oil (Del Carlo et al., 2004), or red and white wine (Lodovici et al., 2001; Makris et al., 2003). The US government has acknowledged that people whose diets are rich in fruits and vegetables may consume one gram or more per day of polyphenol compounds, based on the recommendation of 5 servings/day of colored fruits and vegetables. Because it is believed that a diet rich in fruits and vegetables is consistently associated with a decreased risk of cancer, it is highly desirable to understand the biological function and mode of activity of polyphenols (Yao et al., 2004).

Copper and iron are major metal ions present in serum (Kanabrocki et al., 2008) and tissue (Al-Ebraheem et al., 2009). Furthermore, copper is an important metal ion present in chromatin and is closely associated with DNA bases, particularly guanine (Kagawa et al., 1991). Copper supplementation is believed to be a potential therapeutic tool for the treatment and prevention of involutional osteoporosis (Rico et al., 2000). Phenolic compounds are reducing agents and under *in vitro* conditions and in the presence of metal ions, such as copper or iron, they can act as pro-oxidants (Cao et al., 1997; Sugihara et al., 1999). Phenolic anti-

Abbreviations: ESR, electron spin resonance; ROS, reactive oxygen species; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; CaA, caffeic acid; ChA, chlorogenic acid; FA, ferulic acid; QA, quinic acid; Que, quercetin; Res, resveratrol; C, catechin; CG, catechin gallate; EC, epicatechin; ECG, epicatechin gallate; GC, gallocatechin; GCG, gallocatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate.

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oxidants, including quercetin (Yamashita et al., 1999), curcumin (Ahsan and Hadi, 1998), tea catechins (Hayakawa et al., 1997), salsolinol (Jung and Surh, 2001), and resveratrol (Ahmad et al., 2005), were reported to induce lipid peroxidation and/or DNA damage in the presence of cupric ions. In this reaction, Cu^{2+} is reduced to Cu^{+} by phenolic compounds and the re-oxidation of Cu^{+} to Cu^{2+} is accompanied by the formation of ROS. Therefore, it is interesting to see how an antioxidant can switch to a pro-oxidant and its biological implications.

Recent studies have demonstrated that the pro-oxidant action of various phenolic compounds is attributed to the accelerated lipid peroxidation and/or induction of DNA damage, mutagenesis, carcinogenesis, and apoptosis in cancer cells (Zheng et al., 2006; Zheng et al., 2008; Wang et al., 2008). The initial electron-transfer oxidation of phenolic compounds, particularly hydroxycinnamic acid, by Cu^{2+} generates the corresponding semiquinone radical and the radical undergoes a second electron-transfer reaction with O_2 to form *ortho*-quinone and superoxide anion ($O_2^{\cdot-}$). $O_2^{\cdot-}$ reacts with Cu^{+} to give hydrogen peroxide (H_2O_2), which is readily converted via a Fenton-type reaction into hydroxyl radical ($^{\cdot}OH$) (Fan et al., 2009). ROS, such as $^{\cdot}OH$ and $O_2^{\cdot-}$, is generated by these mechanisms.

In this study, we conducted electron spin resonance (ESR) measurements for the increase in ROS in relation to its structure and interaction with transition metals. Moreover, the antioxidant activity was assessed with the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, and the pro-oxidant effect of phenolic compounds on DNA damage was assessed by measuring 8-hydroxy-2′-deoxyguanosine (8-OHdG), which is effectively formed during oxidative damage.

2. Materials and methods

2.1. Reagents and chemicals

Caffeic acid (CaA), *trans*-4-hydroxy-3-methoxycinnamic acid (Ferulic acid, FA), quercetin dihydrate (Que), rutin, (−)-epigallocatechin (EGC), (−)-epicatechin (EC), and gallocatechin (GC) were obtained from Wako Pure Chemical Industries (Tokyo, Japan). Chlorogenic acid hydrate (ChA), D-(−)-quinic acid (QA), resveratrol (Res), and (+)-catechin (C) were provided by Tokyo Chemical Industry (Tokyo, Japan). (−)-Epigallocatechin gallate (EGCG), (−)-epicatechin gallate (ECG), (−)-gallocatechin gallate (GCG), and (−)-catechin gallate (CG) were purchased from Sigma (Tokyo, Japan).

5,5′-Dimethyl-1-pyrroline-*N*-oxide (DMPO) and α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) were purchased from Labotech Co. (Tokyo, Japan). Dimethyl sulfoxide (DMSO), hydrogen peroxide (30%), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Wako Pure Chemical Industries (Tokyo, Japan). Copper(II) sulfate pentahydrate was purchased from Kanto Chemical (Tokyo, Japan). Ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid, disodium salt (EDTA), and bathocuproinedisulfonic acid, disodium salt were obtained from Dojindo Laboratories (Kumamoto, Japan). Catalase and superoxide dismutase (SOD) were purchased from Sigma (Tokyo, Japan).

Deoxyribonucleic acid sodium salt from calf thymus, phosphatase alkaline from bovine intestinal mucosa, deoxyguanosine (dG), and 8-hydroxy-2′-deoxyguanosine (8-OHdG) were obtained from Sigma (Tokyo, Japan). Nuclease P1 and a DNA extractor kit were purchased from Wako Pure Chemical Industries (Tokyo, Japan).

2.2. DPPH radical-scavenging activity

The modified DPPH method was used for the determination of antioxidant activity (Szydłowska-Czerniak et al., 2010). DPPH radical solution (2 mM) was prepared in methanol and the phenolic compounds were diluted in methanol to 0.02, 0.1, 0.2, and 0.4 mM. In a 1.5 mL disposable tube, the prepared DPPH (250 μL) solution was added to a sample of diluted phenolic compound (250 μL). After 37 °C for 30 min incubation, the absorbance was read at 540 nm and scavenging activity was determined with the following equation: % scavenging activity = $[A_{control} - A_{sample}]$ $A_{control} \times 100$. The data presented are means ± SD of three determinations.

2.3. Electron spin resonance measurement of hydroxyl radical

The analysis of $^{\cdot}OH$ was carried out with an ESR spectrometer (JES-RE1X, JEOL Co., Tokyo, Japan). The ESR spectrum was measured at a microwave frequency of 9.43 GHz, a magnetic field of 335.5 ± 5 mT, a microwave power of 9 mW, a modulation of 100 kHz, a time constant of 0.03 s, and a sweep time of 30 s, using the ESR spectrometer. The spectra of the samples were scanned to record the signal intensities (peak-to-peak heights).

A typical incubation consisted of phosphate buffer (50 mM, pH 7.4), POBN (10 mM), DMSO (10%), phenolic compounds (2 mM), and copper(II) sulfate pentahydrate (1 mM) in a final volume of 0.3 mL. Samples were taken at a reaction at 37 °C for 30 min.

2.4. DNA digestion and determination of dG and 8-OHdG

In order to prevent the formation of oxidative by-products during DNA isolation, DNA was digested by using the slightly modified method (Ishii et al., 2007). Calf thymus DNA (2 mg/mL) 250 μL was incubated at 37 °C for 30 min after adding 50 μL of phenolic compounds (2 mM) and 50 μL of copper(II) sulfate pentahydrate (1 mM) in 0.15 mL of phosphate buffer (50 mM, pH 7.4). Treated calf thymus DNA was immediately centrifuged at 10,000*g* for 5 min at 10 °C after the total volume was adjusted to 1.5 mL by adding NaI and 2-propanol. After washing with ethanol, the pellet was dissolved in 0.2 mL of 20 mM sodium acetate buffer, pH 4.8. DNA was enzymatically hydrolyzed by adding 5.0 μL of nuclease P1 to obtain a concentration of 500 units/mL. The mixture was incubated at 60 °C for 15 min. After the addition of 20 μL of 1.0 M Tris–HCl buffer (pH 8.0), 5.0 μL of alkaline phosphatase was added to give a final concentration of 20 units/mL. The mixture was passed through a 3000 NMWL filter (Millipore, Tokyo, Japan) after incubating at 37 °C for 60 min. Then, the digested solution was injected into the LC-UV-ECD instrument for 8-OHdG and dG analysis.

2.5. HPLC-UV-ECD conditions for dG and 8-OHdG analysis

In the dG and 8-OHdG assay, the UV detector and the ECD used were a Shimadzu SPD10A (Tokyo, Japan) and an ESA Coulochem III (Tokyo, Japan), respectively. A Shimadzu 10Avp pump (Tokyo, Japan) was used to induce flow through the analytical column. An Inertsil ODS3 (4.6 mm × 150 mm, 3.0 μm, GL Sciences, Tokyo, Japan) was used for separation. An aliquot (20 μL) of the sample was injected into the ODS column whose temperature was maintained at 40 °C. The mobile phase was a mixture of 10 mM sodium phosphate/methanol (97/3, v/v). The compounds were eluted isocratically at a flow rate of 1.0 mL/min. The wavelength of the UV detector was set at 290 nm for the detection of dG. The Coulochem III ECD was used with a guard cell (Model 5020; -350 mV), and an analytical cell (Model 5011; electrode 1, 150 mV; electrode 2, 300 mV).

2.6. Statistical analysis

All results are expressed as mean ± SD. Statistics were analyzed using one-way analysis of variance (ANOVA), and if statistically significant, *post hoc* analysis using the Dunnett method was fol-

lowed as a multiple comparison among groups. Values of $P < 0.05$ and 0.01 were considered statistically significant.

3. Results

3.1. Radical scavenging activity of phenolic compounds

The antioxidant activities of the phenolic compounds were measured by the DPPH method, which is one of the oldest and the most frequently used methods for evaluating antioxidant activity. The scavenging effects of phenolic compounds on DPPH radicals are shown in Fig. 1. All the phenolic compounds excepted QA exhibited antioxidant activities. In catechin and its related compounds, the chemical structure was a main contributor to the antioxidant activity. The DPPH radical scavenging activities of gallate groups, such as CG, ECG, GCG, and EGCG, were stronger than those of non-gallate groups. The pyrogallol group (benzene-1,2,3-triol) has stronger DPPH radical scavenging activity than the catechol group (1,2-dihydroxybenzene). Finally, epicatechin precursors, such as EC, ECG, EGC, and EGCG, exhibited antioxidant activities that were similar to or greater than those of epicatechin epimers, namely C, CG, GC, and GCG.

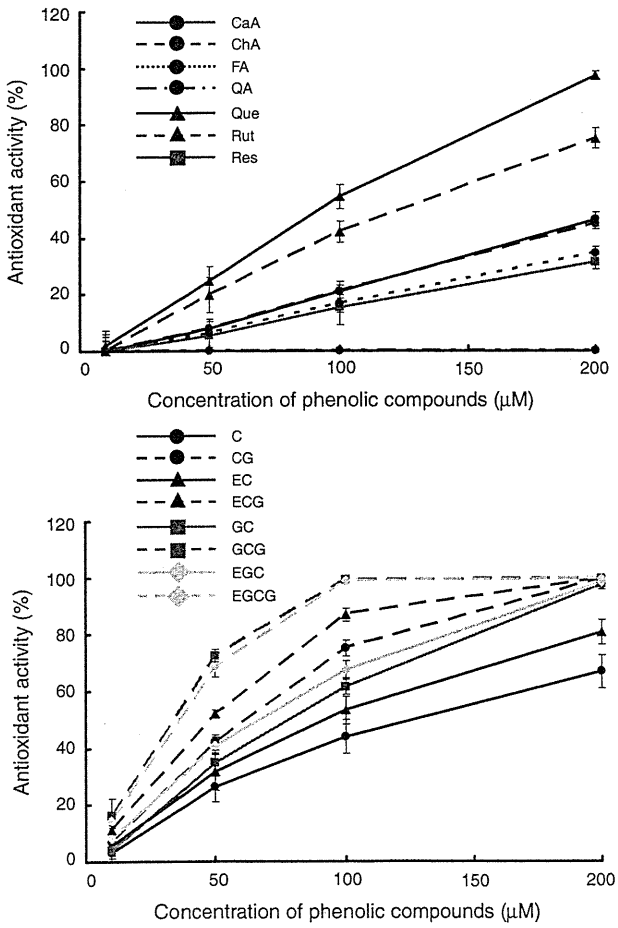

Fig. 1. Antioxidant activities of phenolic compounds as assessed by DPPH assay. DPPH concentration was 1 mM. Data points represent means ± SD ($n = 3$). CaA, caffeic acid; ChA, chlorogenic acid; FA, ferulic acid; QA, quinic acid; Que, quercetin; Res, resveratrol; C, catechin; CG, catechin gallate; EC, epicatechin; ECG, epicatechin gallate; GC, gallocatechin; GCG, gallocatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate.

3.2. Assessment of POBN as spin trapping reagent

·OH is one of the most aggressive ROS. It can be generated by the interaction of metal ion and H_2O_2 (Fenton reaction) and/or O_2^- with H_2O_2 (Haber Weiss reaction), especially in the presence of catalytic metal ions. It is important to measure such ROS as ·OH because ROS induces DNA damage, mutagenesis, and carcinogenesis. However, it is difficult to detect ·OH because of its extremely short lifetime. The most direct means to demonstrate the formation of ·OH is to quantify its radical products by ESR measurement, using either fast flow systems or spin traps that combine with reactive radicals to form more stable adducts. DMPO is used widely as a spin trapping reagent. The Fenton reaction can generate ·OH, which can be trapped by DMPO and give a typical four line ESR spectrum with intensity 1:2:2:1 ($\alpha^N = \alpha^H = 1.49$ mT). ·CH_3, which adduct of DMPO generates ESR spectrum with hyperfine splitting constants of $\alpha^N = 1.64$ mT, $\alpha^H = 2.35$ mT (Barr et al., 1996) was generated by the interaction of DMSO and ·OH (Fig. 2). When copper ion is coexistent to DMPO, DMPO–OH signal appears. If ·OH has been generated, ·CH_3 could be generated by the interaction of DMSO and ·OH. However, there is no DMPO–CH_3 signal (Fig. 3). A number of studies have suspected artificial reactions, because DMPO is affected by copper ion, and susceptible to decomposition by light and oxygen.

The most common spin trap used to trap ·CH_3 that is produced in the *in vitro* or *in vivo* reaction of DMSO with ·OH is the nitrone compound POBN. ·OH is added to DMSO and the resulting alkoxyl radicals decompose to ·CH_3, which is efficiently scavenged by POBN. The rate constant between DMSO and ·OH has been utilized to increase ESR spin trapping detection of ·OH ($k = 7 \times 10^9$ M^{-1} s^{-1}) (Babbs and Steiner, 1990). The ·CH_3 adduct of POBN generates a six line ESR spectrum with hyperfine splitting constants of $\alpha^N = 1.57$ mT, $\alpha^H = 0.28$ mT (Yue Qian et al., 2005). Fig. 4 shows the ESR spectra and correlation of DMPO–OH with POBN–CH_3 using

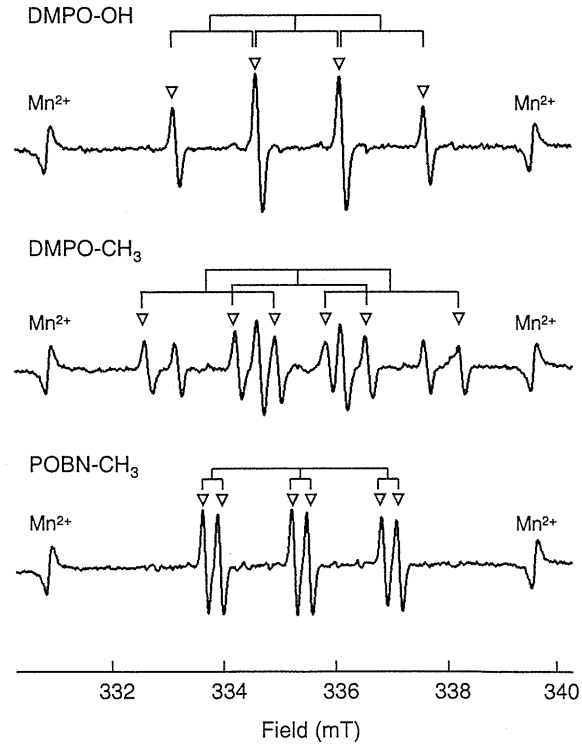

Fig. 2. ESR spectra of DMPO–OH, DMPO–CH_3, and POBN–CH_3.

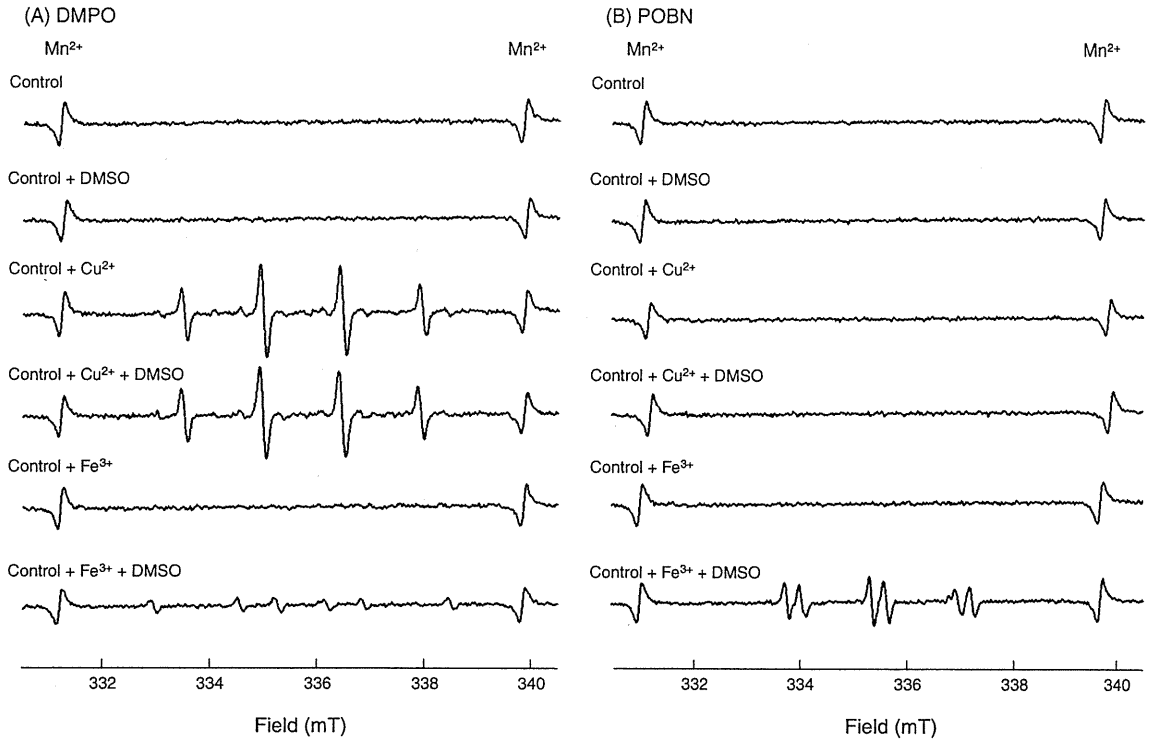

Fig. 3. ESR spectra from the reaction of spin trapping reagent with DMSO in the presence of a metal ion. (A) DMPO and (B) POBN.

the Fenton reaction. The result indicates that instead of directly trapping ·OH, this method actually traps and measures ·CH_3 that is formed during the interaction of DMSO with ·OH. Moreover, this method could detect ·OH, which is not affected by copper ion (Fig. 3).

3.3. Measurement of hydroxyl radical by ESR using POBN as spin trapping reagent

In order to quantify DNA damage induced by phenolic compounds and copper ion, ESR analysis was conducted to determine ·OH. Fig. 5 shows that no POBN–CH_3 signal is detected in solutions containing no CaA or ChA, and that the POBN–CH_3 signal intensity was increased by the interaction of CaA or ChA with Cu^{2+}. Moreover, POBN–CH_3 signal intensity decreased when CaA or ChA concentration was increased further.

The formation of Cu^{2+} chelate with phenolic compounds was confirmed in the reaction with EDTA, a well-known chelating reagent for metal ions. Bathocuproinedisulfonic acid (a specific Cu^{+} chelator) and catalase (H_2O_2 scavenger) decreased ·OH generation induced by phenolic compounds in the presence of Cu^{2+} (Fig. 6).

In addition, the pro-oxidant activities of the interaction between phenolic compounds and copper are shown in Fig. 7. ROS was not detected without Cu^{2+} for the phenolic compounds alone. However, the results indicate that CaA, ChA, Que and catechin groups generated ROS in the presence of Cu^{2+}. Catechin groups that possessed gallate groups showed decreased pro-oxidant activity. On the other hand, FA, QA, and Res were either very small or not generated ROS at all in the presence of Cu^{2+}.

3.4. Oxidative damage and formation of 8-OHdG in calf thymus DNA

The hydroxylation of dG to form 8-OHdG adduct is an important biomarker of oxidative DNA damage. When calf thymus DNA was treated with phenolic compounds in the presence of $CuSO_4$, 8-OHdG was effectively formed. Using the same ESR conditions, we examined the effect of phenolic compounds on the copper-dependent 8-OHdG formation. Phenolic compounds alone were not increased 8-OHdG without Cu^{2+}. Catechin groups considerably increased 8-OHdG levels in DNA treated with phenolic compounds/copper (Fig. 8). Particularly, *ortho*-dihydroxyl groups that can chelate with Cu^{2+} induce the greatest pro-oxidant activity. Other compounds such as FA, QA, and Res cannot be oxidized DNA by Cu^{2+}.

4. Discussion

Phenolic compounds are widely studied for their antioxidant activity. Antioxidant activity refers to both the ability of phenolic compounds to prevent damage from ROS (through radical scavenging) and/or to prevent generation of ROS (by binding metal ion). All the phenolic compounds in our experiments showed antioxidant activity. Meanwhile, the scavenging activities of catechin and its related compounds were significantly different. It was said that the pyrogallol structure in the B-ring play an important role in the rapid scavenging ability (Kondo et al., 2001). The presence of C-ring a gallate group and/or B-ring a pyrogallol group in their structures increased their ability to scavenge free radicals (Fig. 1).

On the other hand, if phenolic compounds and copper were absorbed by the body, they would interact with each other to generate ROS. There are several reports indicating that various phenolic compounds, such as resveratrol and catechin, promote DNA damage by Cu^{2+}. Most of the reports assessed DNA damage by analyzing plasmid DNA strand breaks. This method is widely used as a model system to induce DNA strand breaks. We clearly saw the effects of ROS on DNA but could not quantify the amount of ROS produced. One study that estimated the production of ROS and/or the pro-oxidant activity used 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Labieniec and Gabryelak, 2007). Fluorogenic

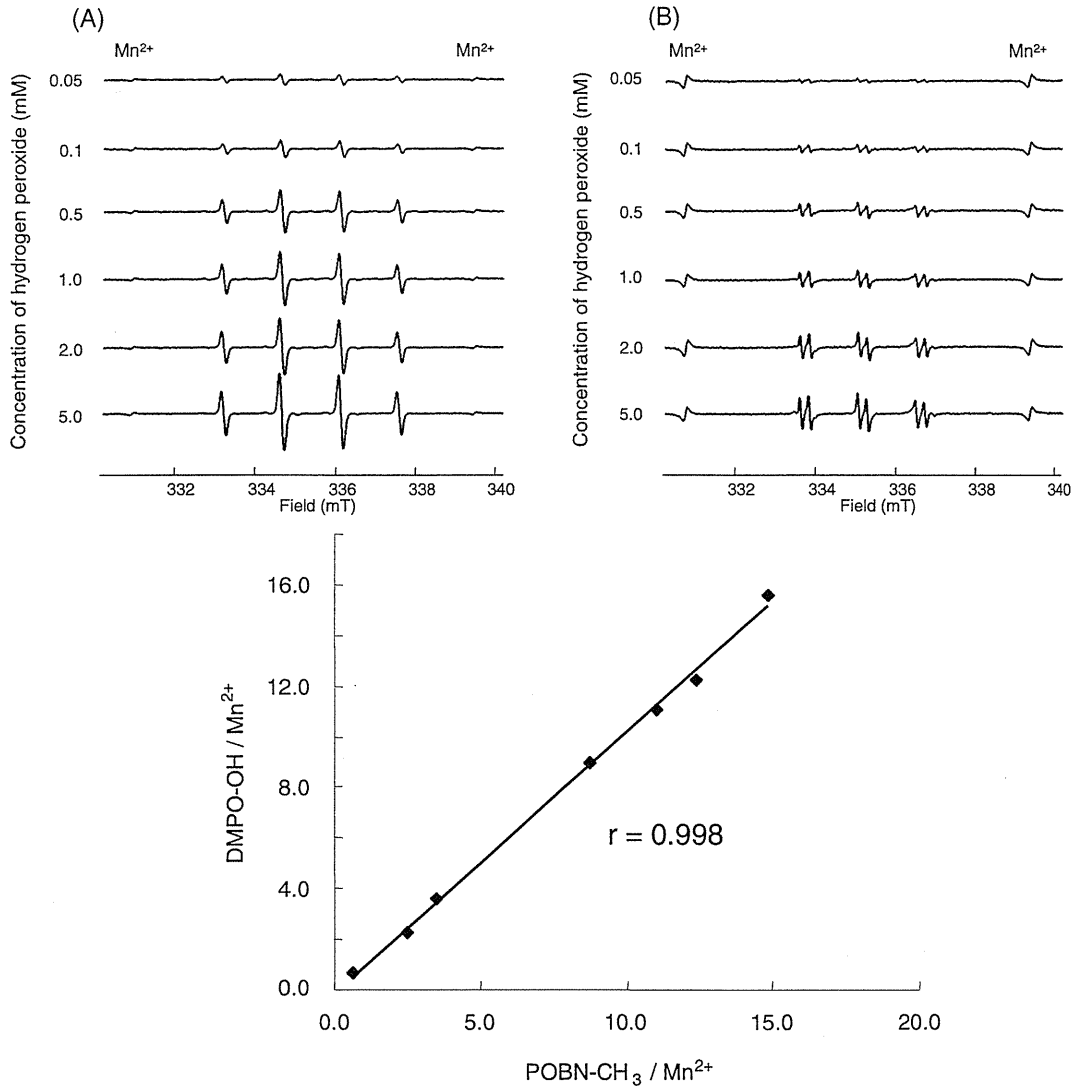

Fig. 4. Comparison of ESR spectra between (A) DMPO–OH and (B) POBN–CH_3. Correlation of DMPO–OH and POBN–CH_3 signal.

probes are widely employed to monitor oxidative activity in cells. DCFH-DA is converted into DCFH by intracellular esterases, and the non-fluorescent DCFH is oxidized to DCF by reacting with ROS. Specifically, the oxidation of DCFH is a result of the reaction with H_2O_2 or peroxynitrite (Rota et al., 1999). Although there remain many controversies particularly about the specificity, $DCFH_2$-DA/$DCFH_2$ are useful probes for oxidative studies in cell-free and biological systems. However, the oxidation of $DCFH_2$ is non-specific (Chen et al., 2010). For this reason, ROS assessment should be carefully conducted to avoid misinterpretation of results.

Free radicals have unpaired electrons that can be detected selectively by ESR measurement. The spin trapping method takes advantage of the rapid reaction of many radicals with certain chemical acceptor molecules (spin trapping reagents) to produce more stable secondary radicals. Spin trapping reagents are diamagnetic compounds that rapidly scavenge transient radicals to form stable paramagnetic spin adduct radicals. Because this secondary radical retains an unpaired electron, it can often be detected by ESR measurement. Many spin trapping reagents are available. One example is DMPO, which has been used for decades to trap oxygen radicals in biological systems. However, the use of the spin trapping technique for direct measurements in functioning biological systems is limited by the poor stability of the spin trapped adducts. On the other hand, POBN and α-phenyl-*N*-*tert*-butyl nitrone (PBN), which are nitrone spin reagents, are frequently used in *in vivo* and *in vitro* studies (Awasthi et al., 1997; Yue Qian et al., 2005) because stable adducts with carbon-centered radicals are formed. In this study, we were able to measure ROS production and pro-oxidant activity using POBN reagent instead of DMPO reagent (Fig. 4).

Phenolic compounds can switch from antioxidants to pro-oxidants in the presence of Cu^{2+} to induce ROS production and subsequently DNA damage. There are several reports that clarify the mechanism of the pro-oxidant activity. Phenolic compounds can reduce Cu^{2+} to Cu^{+} via electron transfer. In addition, chelated and/or free Cu^{+} can be oxidized to Cu^{2+}. It is said that *ortho*-dihydroxyl groups that can chelate with Cu^{2+} induce the greatest pro-oxidant activity. Actually, our results also indicated the possible mechanisms of ROS production induced by phenolic compounds in the presence of Cu^{2+}. Other compounds such as FA, QA, and Res cannot be induced ROS by Cu^{2+}, because they have no *ortho*-dihydroxyl groups. Moreover, the interaction between phenolic

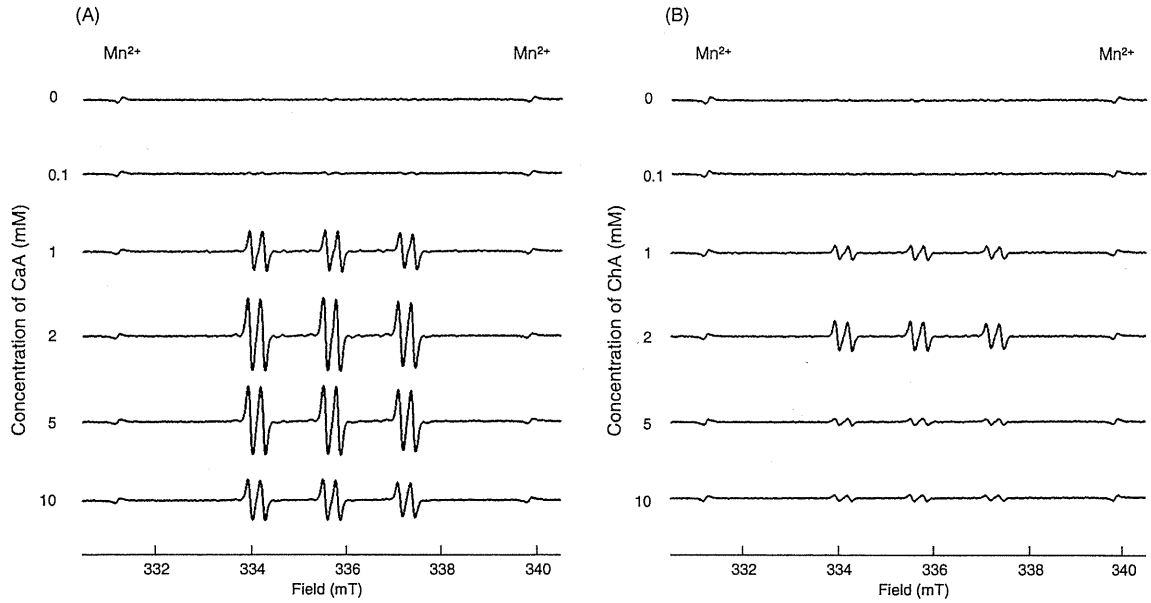

Fig. 5. Representative ESR spectra of the interaction between CaA (A) or ChA (B) and copper.The reaction mixture consisted of copper (1 mM), phenolic compound, DMSO (10%), and POBN (10 mM) in phosphate buffer (50 mM, pH 7.4), and the ESR spectra were recorded after reacting for 30 min at 37 °C.

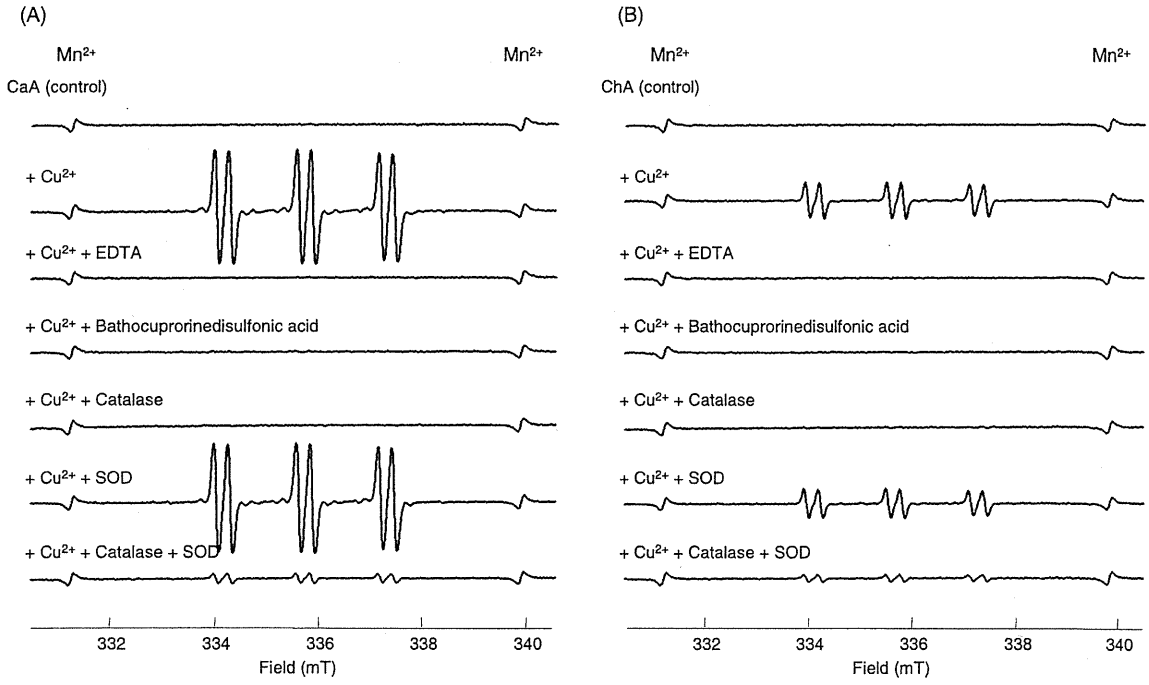

Fig. 6. Effect of chelator and antioxidant enzyme on ROS generation. The reaction mixture consisted of copper (1 mM), phenolic compound (2 mM), DMSO (10%), and POBN (10 mM) in phosphate buffer (50 mM, pH 7.4), and the ESR spectra were recorded after reacting for 30 min at 37 °C.

compounds and copper induced to H_2O_2 (Fig. 6). Both O_2 uptake and ·OH formation were suppressed by the Cu^+ chelator bathocuproine and catalase, confirming the involvement of Cu^+ and H_2O_2 in a metal catalyzed Fenton reaction. In addition, these ESR results were correlated with 8-OHdG that is effectively formed for the assessment of oxidative damage (Figs. 7 and 8).

In conclusion, we conducted ESR measurement for the increase in ROS in relation to its structure and interaction with transition metals. Moreover, we demonstrated that ROS participated in oxidative DNA damage induced by phenolic compounds in the presence of Cu^{2+}. The obtained results indicate one possibility *in vitro*. However, they are not sufficient evidence to claim that phenolic compounds are dangerous. Oral and intravenous dosing experiments revealed that phenolic compounds, such as catechin, had very low bioavailability. According to Manach et al., (2004), important inter-individual differences in the capacity to metabolize phenolic compounds originate from differences in the activities of cytochrome P450 and carrier systems that may be influenced by

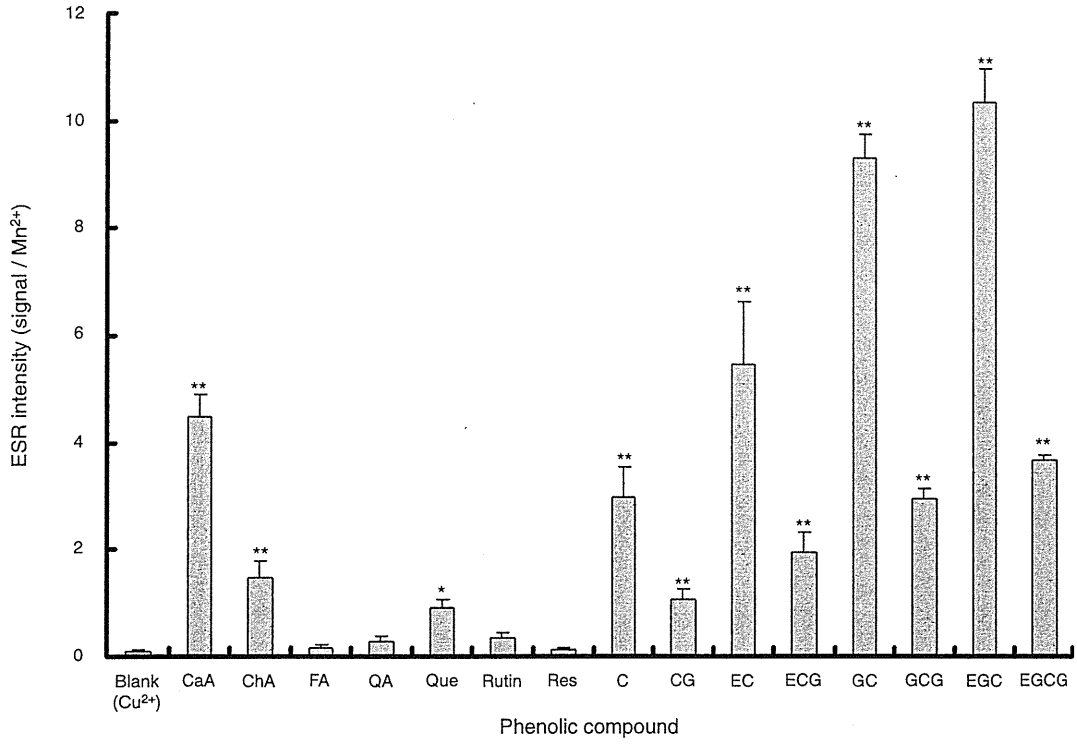

Fig. 7. Pro-oxidant activities in the interaction between phenolic compounds and copper. The concentration of each phenolic compound was 2 mM and copper concentration was 1 mM. Data points represent means ± SD ($n = 6$). * and **: statistically significant vs blank ($P < 0.05$ and $P < 0.01$ by ANOVA). CaA, caffeic acid; ChA, chlorogenic acid; FA, ferulic acid; QA, quinic acid; Que, quercetin; Res, resveratrol; C, catechin; CG, catechin gallate; EC, epicatechin; ECG, epicatechin gallate; GC, gallocatechin; GCG, gallocatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate.

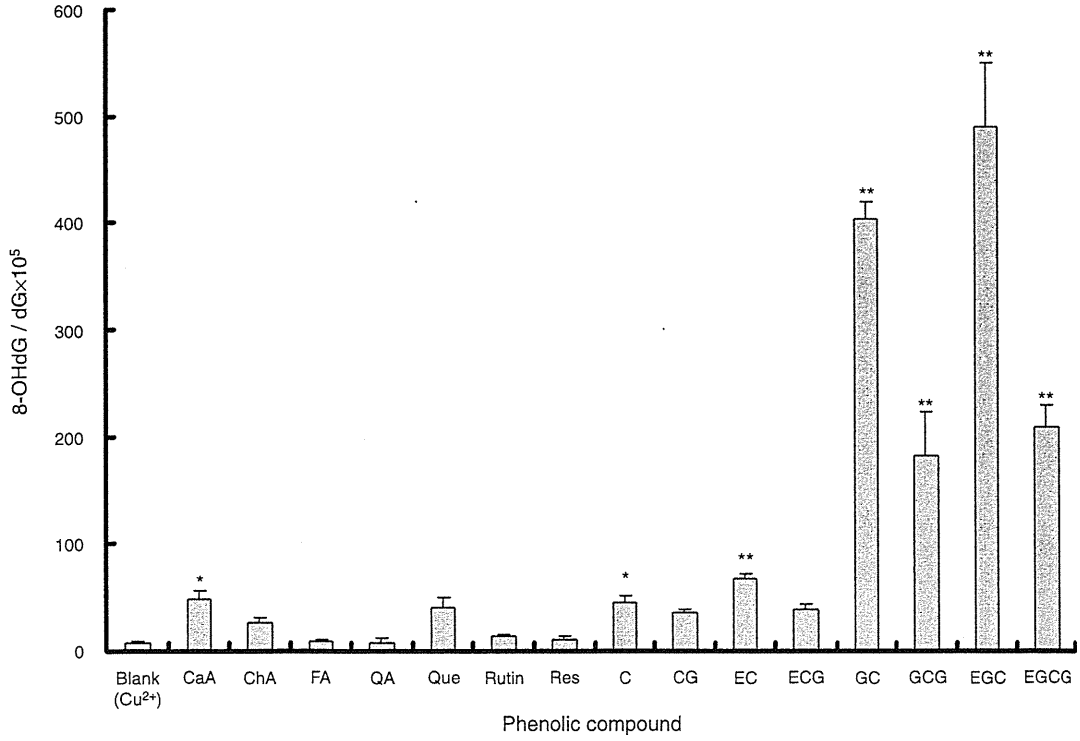

Fig. 8. Changes of 8-OHdG levels in the interaction between phenolic compounds and copper. The concentration of each phenolic compound was 0.2 mM and copper concentration was 0.1 mM. Data points represent means ± SD ($n = 6$). * and **: statistically significant vs blank ($P < 0.05$ and $P < 0.01$ by ANOVA). CaA, caffeic acid; ChA, chlorogenic acid; FA, ferulic acid; QA, quinic acid; Que, quercetin; Res, resveratrol; C, catechin; CG, catechin gallate; EC, epicatechin; ECG, epicatechin gallate; GC, gallocatechin; GCG, gallocatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate.

genetic polymorphisms. In contrast to the pharmacokinetic profiles of flavonoids, such as catechin (Masukawa et al., 2006) and isoflavones (Pascual-Teresa et al., 2006), various plasma concentration peaks were observed from 0.5 h to 8 h after the consumption of green coffee extract, suggesting a complex and dynamic process underlying absorption and metabolism (Farah et al., 2008). Therefore, it seems reasonable to conclude that the most important consideration is the disturbance of the pro-oxidant/antioxidant system in favor of the former. Further studies are required to reveal the chemical mechanisms of the antioxidant activity and the pro-oxidant activity of phenolic compounds.

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References

Ahmad, A., Syed, F.A., Singh, S., Hadi, S.M., 2005. Prooxidant activity of resveratrol in the presence of copper ions: mutagenicity in plasmid DNA. Toxicol. Lett. 159, 1–12.

Ahsan, H., Hadi, S.M., 1998. Strand scission in DNA induced by curcumin in the presence of Cu(II). Cancer Lett. 124, 23–30.

Al-Ebraheem, A., Farquharson, M.J., Ryan, E., 2009. The evaluation of biologically important trace metals in liver, kidney and breast tissue. Appl. Radiat. Isot. 67, 470–474.

Awasthi, D., Church, D.F., Torbati, D., Carey, M.E., Pryor, W.A., 1997. Oxidative stress following traumatic brain injury in rats. Surg. Neurol. 47, 575–581.

Babbs, C.F., Steiner, M.G., 1990. Detection and quantitation of hydroxyl radical using dimethyl sulfoxide as molecular probe. Methods Enzymol. 186, 137–147.

Barr, D.P., Martin, M.V., Guengerich, F.P., Mason, R.P., 1996. Reaction of cytochrome P450 with cumene hydroperoxide: ESR spin-trapping evidence for the homolytic scission of the peroxide O–O bond by ferric cytochrome P450 1A2. Chem. Res. Toxicol. 9, 318–325.

Bjelakovic, G., Nikolova, D., Gluud, L.L., Simonetti, R.G., Gluud, C., 2007. Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. JAMA 297, 842–857.

Cabrera, C., Artacho, R., Giménez, R., 2006. Beneficial effects of green tea – a review. J. Am Coll. Nutr. 25, 79–99.

Cao, G., Sofic, E., Prior, R.L., 1997. Antioxidant and prooxidant behavior of flavonoids: structure–activity relationships. Free Radic. Biol. Med. 22, 749–760.

Chen, X., Zhong, Z., Xu, Z., Chen, L., Wang, Y., 2010. 2′,7′-Dichlorodihydrofluorescein as a fluorescent probe for reactive oxygen species measurement: forty years of application and controversy. Free Radic. Res. 44, 587–604.

Del Carlo, M., Sacchetti, G., Di Mattia, C., Compagnone, D., Mastrocola, D., Liberatore, L., Cichelli, A., 2004. Contribution of the phenolic fraction to the antioxidant activity and oxidative stability of olive oil. J. Agric. Food Chem. 52, 4072–4079.

Fan, G.J., Jin, X.L., Qian, Y.P., Wang, Q., Yang, R.T., Dai, F., Tang, J.J., Shang, Y.J., Cheng, L.X., Yang, J., Zhou, B., 2009. Hydroxycinnamic acids as DNA-cleaving agents in the presence of Cu(II) ions: mechanism, structure–activity relationship, and biological implications. Chemistry 15, 12889–12899.

Farah, A., Monteiro, M., Donangelo, C.M., Lafay, S., 2008. Chlorogenic acids from green coffee extract are highly bioavailable in humans. J. Nutr. 138, 2309–2315.

Gardner, E.J., Ruxton, C.H., Leeds, A.R., 2007. Black tea-helpful or harmful? A review of the evidence. Eur. J. Clin. Nutr. 61, 3–18.

Hayakawa, F., Kimura, T., Maeda, T., Fujita, M., Sohmiya, H., Fujii, M., Ando, T., 1997. DNA cleavage reaction and linoleic acid peroxidation induced by tea catechins in the presence of cupric ion. Biochim. Biophys. Acta 1336, 123–131.

Ishii, Y., Ogara, A., Okamura, T., Umemura, T., Nishikawa, A., Iwasaki, Y., Ito, R., Saito, K., Hirose, M., Nakazawa, H., 2007. Development of quantitative analysis of 8-nitroguanine concomitant with 8-hydroxydeoxyguanosine formation by liquid chromatography with mass spectrometry and glyoxal derivatization. J. Pharm. Biomed. Anal. 43, 1737–1743.

Jung, Y., Surh, Y., 2001. Oxidative DNA damage and cytotoxicity induced by copper-stimulated redox cycling of salsolinol, a neurotoxic tetrahydroisoquinoline alkaloid. Free Radic. Biol. Med. 30, 1407–1417.

Kagawa, T.F., Geierstanger, B.H., Wang, A.H., Ho, P.S., 1991. Covalent modification of guanine bases in double-stranded DNA. The 1.2-A Z-DNA structure of d(CGCGCG) in the presence of $CuCl_2$. J. Biol. Chem 266, 20175–20184.

Kanabrocki, E.L., Sothern, R.B., Ryan, M.D., Kahn, S., Augustine, G., Johnson, C., Foley, S., Gathing, A., Eastman, G., Friedman, N., Nemchausky, B.A., Kaplan, E., 2008. Circadian characteristics of serum calcium, magnesium and eight trace elements and of their metallo-moieties in urine of healthy middle-aged men. Clin. Ter. 159, 329–346.

Kondo, K., Kurihara, M., Fukuhara, K., 2001. Mechanism of antioxidant effect of catechins. Methods Enzymol. 335, 203–217.

Labieniec, M., Gabryelak, T., 2007. Antioxidative and oxidative changes in the digestive gland cells of freshwater mussels Unio tumidus caused by selected phenolic compounds in the presence of H_2O_2 or Cu^{2+} ions. Toxicol. In Vitro 21, 146–156.

Lodovici, M., Guglielmi, F., Casalini, C., Meoni, M., Cheynier, V., Dolara, P., 2001. Antioxidant and radical scavenging properties in vitro of polyphenolic extracts from red wine. Eur. J. Nutr. 40, 74–77.

Makris, D.P., Psarra, E., Kallithraka, S., Kefalas, P., 2003. The effect of polyphenolic composition as related to antioxidant capacity in white wines. Food Res. Int. 36, 805–814.

Manach, C., Scalbert, S., Morand, C., Rémésy, C., Jiménez, L., 2004. Polyphenols: food sources and bioavailability. Am. J. Clin. Nutr. 79, 727–747.

Masukawa, Y., Matsui, Y., Shimizu, N., Kondou, N., Endou, N., Kuzukawa, M., Hase, T., 2006. Determination of green tea catechins in human plasma using liquid chromatography-electrospray ionization mass spectrometry. J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 834, 26–34.

Nardini, M., Cirillo, E., Natella, F., Scaccini, C., 2002. Absorption of phenolic acids in humans after coffee consumption. J. Agric. Food Chem. 50, 5735–5741.

Pascual-Teresa, S.T., Hallund, J., Talbot, D., Schroot, J., Williams, C.M., Bugel, S., Cassidy, A., 2006. Absorption of isoflavones in humans: effects of food matrix and processing. J. Nutr. Bioch. 17, 257–264.

Perron, N.R., Brumaghim, J.L., 2009. A review of the antioxidant mechanisms of polyphenol compounds related to iron binding. Cell Biochem. Biophys. 53, 75–100.

Rico, H., Roca-Botran, C., Hernández, E.R., Seco, C., Paez, E., Valencia, M.J., Villa, L.F., 2000. The effect of supplemental copper on osteopenia induced by ovariectomy in rats. Menopause 7, 413–416.

Rota, C., Chignell, C.F., Mason, R.P., 1999. Evidence for free radical formation during the oxidation of 2′-7′-dichlorofluorescin to the fluorescent dye 2′-7′-dichlorofluorescein by horseradish peroxidase: Possible implications for oxidative stress measurements. Free Radic. Biol. Med. 27, 873–881.

Sugihara, N., Arakawa, T., Ohnishi, M., Furuno, K., 1999. Anti- and pro-oxidative effects of flavonoids on metal-induced lipid hydroperoxide-dependent lipid peroxidation in cultured hepatocytes loaded with alpha-linolenic acid. Free Radic. Biol. Med. 27, 1313–1323.

Szydłowska-Czerniak, A., Trokowski, K., Karlovits, G., Szłyk, E., 2010. Determination of antioxidant capacity, phenolic acids, and fatty acid composition of rapeseed varieties. J. Agric. Food Chem. 58, 7502–7509.

Vinson, J.A., Hao, Y., Su, X., Zubik, L., 1998. Phenol antioxidant quantity and quality in foods: vegetables. J. Agric. Food Chem. 46, 3630–3634.

Vinson, J.A., Su, X., Zubik, L., Bose, P., 2001. Phenol antioxidant quantity and quality in foods: fruits. J. Agric. Food Chem. 49, 5315–5321.

Wang, T., Chen, L.X., Long, Y., Wu, W.M., Wang, R., 2008. DNA damage induced by caffeic acid phenyl ester in the presence of Cu(II) ions: potential mechanism of its anticancer properties. Cancer Lett. 263, 77–88.

Yamashita, N., Tanemura, H., Kawanishi, S., 1999. Mechanism of oxidative DNA damage induced by quercetin in the presence of Cu(II). Mutat. Res. 425, 107–115.

Yao, L.H., Jiang, Y.M., Shi, J., Tomás-Barberán, F.A., Datta, N., Singanusong, R., Chen, S.S., 2004. Flavonoids in food and their health benefits. Plant Foods Hum. Nutr. 59, 113–122.

Yue Qian, S., Kadiiska, M.B., Guo, Q., Mason, R.P., 2005. A novel protocol to identify and quantify all spin trapped free radicals from in vitro/in vivo interaction of $HO^{\cdot}$ and DMSO: LC/ESR, LC/MS, and dual spin trapping combinations. Free Radic. Biol. Med. 38, 125–135.

Zheng, L.F., Wei, Q.Y., Cai, Y.J., Fang, J.G., Zhou, B., Yang, L., Liu, Z.L., 2006. DNA damage induced by resveratrol and its synthetic analogues in the presence of Cu (II) ions: mechanism and structure–activity relationship. Free Radic. Biol. Med. 41, 1807–1816.

Zheng, L.F., Dai, F., Zhou, B., Yang, L., Liu, Z.L., 2008. Prooxidant activity of hydroxycinnamic acids on DNA damage in the presence of Cu(II) ions: mechanism and structure–activity relationship. Food Chem. Toxicol. 46, 149–156.

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Characterization of nitrated phenolic compounds for their anti-oxidant, pro-oxidant, and nitration activities

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ABSTRACT

Coffee is one of the most widely consumed beverages worldwide. Evidence of the health benefits and the important contribution of coffee brew to the intake of anti-oxidants in the diet has increased coffee consumption. Chlorogenic acid (ChA) and caffeic acid (CaA) are the major phenolic compounds in coffee. However, phenolic compounds, which are generally effective anti-oxidants, can become pro-oxidants in the presence of Cu^{2+} to induce DNA damage under certain conditions. On the other hand, sodium nitrite ($NaNO_2$) is widely used as a food additive to preserve and tinge color on cured meat and fish. It is possible that phenolic compounds react with $NaNO_2$ under acidic conditions, such as gastric juice. In this study, we identified compounds produced by the reaction between ChA or CaA in coffee and $NaNO_2$ in artificial gastric juice. The identified phenolic compounds and nitrated phenolic compounds were assessed for their anti-oxidant, pro-oxidant, and nitration activities by performing an *in vitro* assay. The nitrated phenolic compounds seemed to show increased anti-oxidant activity and decreased pro-oxidant activity. However, one nitrated CaA compound that has a furoxan ring showed the ability to release NO_2^- in the neutral condition.

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Introduction

Coffee is one of the most widely consumed beverages worldwide. During the past few years, evidence of the health benefits [1] and the important contribution of coffee brew to the intake of anti-oxidants in the diet [2–4] has increased coffee consumption. Many studies have examined the association between coffee consumption and health, particularly cardiovascular morbidity. The question of whether or not coffee intake increases the risk of coronary heart disease remains unanswered. Several studies have shown that caffeine in coffee induces various acute cardiovascular effects, including effects on blood pressure, circulating catecholamines, arterial stiffness, and endothelium-dependent vasodilation [5,6]. On the other hand, coffee consumption is also associated with the decreased risk of type 2 diabetes [7], Alzheimer's disease [8], and cancer [9]. It is said that these effects are produced by polyphenols, which have anti-oxidant activity. Anti-oxidant activity refers to the ability of polyphenol compounds to prevent damage caused by reactive oxygen species (ROS) (such as by radical scavenging) or to prevent the generation of these species (by binding iron) [10]. Chlorogenic acid (ChA) and caffeic acid (CaA) are the major phenolic compounds in coffee and act as anti-oxidants. However, phenolic compounds, which are generally effective anti-oxidants, can become pro-oxidants in the presence of Cu^{2+} to induce DNA damage under certain conditions. It has been demonstrated that compounds bearing *o*-dihydroxyl groups (i.e., ChA and CaA) are the most active in inducing plasmid pBR322 DNA strand breakage in the presence of Cu^{2+} [11].

The major dietary source of nitrite includes cured meat and cereals, but approximately 90% of nitrite ingested by humans is accounted for by the reduction of nitrate in the oral cavity via the action of nitrate reductase produced by microorganisms present in the oral cavity [12]. Sodium nitrite ($NaNO_2$) is widely used as a food additive to preserve and tinge color on cured meat and fish [13]. Nitrite concentrations *in vivo* are 0.5–3.6 μM in plasma [14], 15 μM in respiratory tract lining fluid [15], and 30–210 μM in saliva [16], making nitrous acid formation likely in many tissue compartments. Excessive nitrite production is noted particularly in inflammation. Humans, therefore, ingest nitrite from both exogenous and endogenous sources. At the acidic pH of the stomach, nitrite yields nitric oxide (NO) and NO-derived species that may exert a biological impact locally in terms of antimicrobial effects, blood flow, mucus secretion, and gastric motility. It has been reported that the co-administration of catechol and $NaNO_2$ to rats increased 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels in forestomach epithelium DNA [17].

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Some dietary phenolics with anti-oxidant activity can reduce nitrite to NO under acidic conditions. As coffee contains ChA and CaA, it is possible that these compounds reduce nitrous acid to NO when $NaNO_2$ is mixed with gastric juice after drinking coffee. In fact, it has been reported that ChA and CaA were nitrated by $NaNO_2$ under acidic conditions, and the nitration could give rise to several nitrated ChA and CaA compounds *in vitro* [18]. However, there are no reports of the characteristics of the nitrated compounds.

In the present study, we identified compounds produced by the reaction between ChA or CaA in coffee and $NaNO_2$ in artificial gastric juice. The identified phenolic compounds and the nitrated phenolic compounds were assessed for their anti-oxidant, pro-oxidant, and nitration activities by performing an *in vitro* assay.

Materials and methods

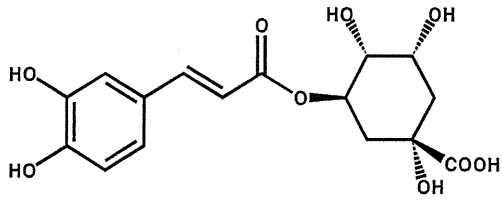
Reagents and chemicals

Chlorogenic acid (ChA) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Caffeic acid (CaA), nuclease P1, and DNA Extractor Kit were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Copper(II) sulfate pentahydrate and sodium nitrite ($NaNO_2$) were purchased from Kanto Chemical (Tokyo, Japan). α-(4-Pyridyl-1-oxide)-*N*-*tert*-butylnitrone (POBN) was purchased from Labotech Co. (Tokyo, Japan). Deoxyribonucleic acid sodium salt from calf thymus, phosphatase alkaline from bovine intestinal mucosa, deoxyguanosine (dG), and 8-hydroxy-2′-deoxyguanosine (8-OHdG) were obtained from Sigma (Tokyo, Japan). Water was purified using a Milli-Q gradient A10 system (Millipore, MA, USA). Other chemicals and solvents were obtained from Wako Pure Chemical Industries.

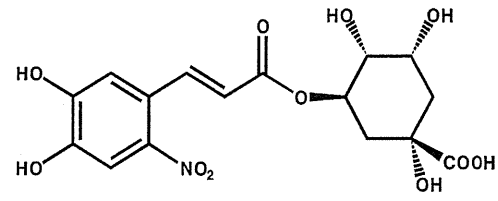
Synthesis of nitrated chlorogenic acid and caffeic acid

Nitrated ChA and CaA were synthesized according to the method reported by Napolitano and d'Ischia [18]. Briefly, ChA or CaA (5 mmol) was dissolved in 0.05 M acetate buffer, pH 4 (500 mL) and $NaNO_2$ (25 mmol) and the mixture was continuously stirred at room temperature. Then, the mixture was extracted with ethyl acetate (3 × 150 mL) and the combined organic layers were dried over sodium sulfate to dryness. The target substance was removed from the reaction solution by silica gel chromatography using toluene–ethyl acetate 80:20 (eluent A), 60:40 (eluent B) or 40:60 (eluent C). Purified nitrated ChA and CaA were identified by comparison of their spectral properties (^1H NMR, ^{13}C NMR, and LC-PDA-ESI-MS) with those reported [18,19]. Their chemical structures are shown in Fig. 1.

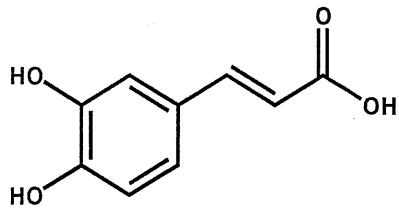
Nitrochlorogenic acid (ChA-NO). UV: λ_{max} (MeOH) 276, 339, 430 nm. ^1H NMR (CD_3OD) δ (ppm): 2.12 (m, 4H), 3.74 (dd, J = 10.0, 3.2 Hz, 1H), 4.20 (m, 1H), 5.42 (m, 1H), 6.31 (d, J = 15.6 Hz, 1H), 7.05 (s, 1H), 7.57 (s, 1H), 8.15 (d, J = 15.6 Hz, 1H).



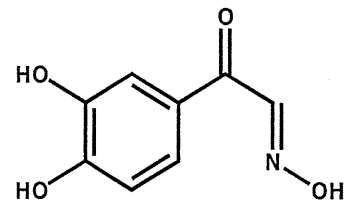
Chlorogenic acid (ChA)



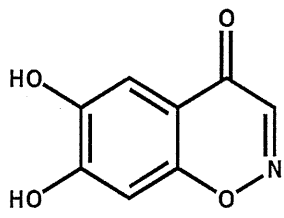
Nitrochlorogenic acid
(ChA-NO)



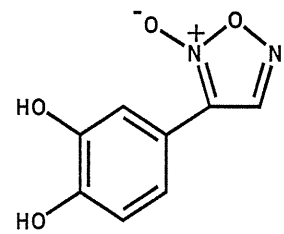
Caffeic acid (CaA)



2-(3,4-Dihydroxyphenyl)-2-oxoethanaloxime
(CaA-NO-1)



6,7-Dihydroxy-1,2-(4*H*)-benzoxazin-4-one
(CaA-NO-2)



2-Oxy-3-(3,4-dihydroxyphenyl)-1,2,5-oxadiazole
(CaA-NO-3)

Fig. 1. Structures of ChA, CaA, and nitrated phenolic compounds.