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Effects of Chlorophyll-Related Compounds on Hydrogen Peroxide Induced DNA Damage within Human Lymphocytes

CHING-YUN HSU,[†,‡] CHI-MING YANG,[§] CHIAO-MING CHEN,[†] PI-YU CHAO,*[,∥] AND SHENE-PIN HU*[,⊥]

Graduate Institute of Pharmacy and School of Nutrition and Health Science, Taipei Medical University, Taipei, Taiwan 110, R.O.C., Chang-Gung Institute of Technology, Taoyuan, Taiwan 333, R.O.C., Institute of Botany and Research Center for Biodiversity, Academia Sinica, Nankang, Taipei, Taiwan, 115, R.O.C., and Department of Food and Nutrition, Chinese Culture University, Taipei, Taiwan 111, R.O.C.

Chlorophylls (Chl's) are the most abundant natural plant pigments. Four chlorophyll-related compounds (CRCs), including chlorophyllide *a* and *b* (Chlide *a* and *b*) and pheophorbide *a* and *b* (Pho *a* and *b*), were investigated for their antioxidative capacities to protect human lymphocyte DNA from hydrogen peroxide (H_2O_2) induced strand breaks and oxidative damage ex vivo. Lymphocytes exposed to H_2O_2 at concentrations of 10 and 50 μM revealed an increased frequency of DNA single-strand breaks (ssb's; as measured by the comet assay) and also an increased level of oxidized nucleoside (as measured by 8-hydroxydeoxyguanosine, 8-OHdG). All Chl's reduced the level of DNA ssb's and 8-OHdG within human lymphocytes following exposure to 10 μM H_2O_2. Only Pho *a* and *b* were able to decrease DNA ssb's and 8-OHdG following treatment of lymphocytes with 50 μM H_2O_2, in a concentration-dependent fashion. It was demonstrated herein that Pho *a* and *b* were more antioxidative than others. We applied DPPH free-radical scavenge assays in vitro, and got similar results. Pho *a* and *b* had higher ability in scavenging capacities than others. We conclude that water-extract Chl's are able to enhance the ability of human lymphocytes to resist H_2O_2-induced oxidative damage, especially for Pho *a* and *b*.

KEYWORDS: Chlorophyll; hydrogen peroxide; DNA damage; comet assay; 8-hydroxydeoxyguanosine (8-OHdG)

INTRODUCTION

Free radicals and oxygen radicals are constantly generated in vivo and cause oxidative damage to DNA that is probably a significant contributor to cancer. Epidemiological studies have shown an inverse relationship between the consumption of vegetables and the incidence of human cancer (*1*). For most cancer sites, individuals who reflect a low fruit and vegetable intake experience about twice the risk of cancer compared with those who feature a high intake of fruit and vegetables (*2*).

Chlorophylls (Chl's) are the most abundant and widely distributed green pigments found in plants, and are important in photosynthesis. For human life, Chl's are constituents of the diet, especially in the form of green vegetables and fruits (*3*). Chlorophylls or their derivatives have also been used as additives for food-coloration purposes (*4*). In the pharmaceutical sciences, Chl has been made available as an over-the-counter drug such as chlorophyllin (Chlin; a semisynthetic, water-soluble sodium−copper salt derivative of chlorophyll); such a drug type has been widely used for controlling body, fecal, and urinary odor among geriatric patients (*5*).

Chlin has been found to exhibit antioxidant activity by way of inhibition of lipid peroxidation in rat liver (*6*, *7*), and also by means of its ability to protect mitochondria from oxidative damage induced by various reactive oxygen species (ROS) (*8*). Chlin has also been shown to inhibit radiation-induced DNA and mitochondrial membrane damage (*9*), and it would also appear to be a potent protector of DNA with regard to oxidative damage (*10*). Recently, Chlin has been used in cancer-related studies (*11*).

Natural Chl's deriving from plant leaves might be degraded into different forms. For example, chlorophyll *a* and *b* may be dephytylated by chlorophyllase in vivo to form chlorophyllide (Chlide) *a* and *b*, respectively. Further, Chlide *a* and *b* may lose magnesium to form pheophorbide (Pho) *a* and *b*. These compounds might be termed chlorophyll-related compounds (CRCs). Earlier studies have focused on the digestion and absorption of Chl's (*12*, *13*). Chl's may be digested to

* To whom correspondence should be addressed. S.-P.H.: fax, 886-2-27373112; e-mail, shenepin@tmu.edu.tw. P.-Y.C: e-mail, pychao@gate.sinica.edu.tw.
[†] Graduate Institute of Pharmacy, Taipei Medical University.
[‡] Chang-Gung Institute of Technology.
[§] Academia Sinica.
[∥] Chinese Culture University.
[⊥] School of Nutrition and Health Science, Taipei Medical University.

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pheophytin and then absorbed within the micelle fraction according to the caco-2 human-cell model (*12*). Within the myeloma cell, the uptake of Chl's was converted into Pho (*13*). For the rabbit, both Chlide and Pho are the final metabolites of Chl's (unpublished data by Yang).

A number of earlier studies have indicated that vegetable extracts consisting of a greater number of Chl derivatives reveal a more positive relationship to the ability to inhibit mutations in the Ames salmonella system (*14*, *15*), and also a greater level of anitgenotoxicity in the *Drosophila* wing spot test (*16*). Furthermore, animal studies have also shown that the application of Pho was effective with regard to both antitumor and anti-inflammation activities for ICR mouse skin (*17*), as well as acting as photodynamic sensitizers against tumors for mice (*18*, *19*).

The antimutagenic effect of CRCs has been well documented. Recently, limited data appeared on the antioxidative capacity of these compounds in a tissue culture model. In this experiment, CRCs (Chlide *a* and *b* and Pho *a* and *b*) were tested for their antioxidative capacities in the context of their ability to protect (human) lymphocyte DNA from hydrogen peroxide (H_2O_2) induced DNA damage. Measurement of 8-hydroxy-2′-deoxyguanosine (8-OHdG) is the commonest method of assessing DNA damage, but there is no consensus on what the true levels are in human DNA (*20*). Here, we measured DNA oxidative damage, both by the level of 8-hydroxy-2′-deoxyguanosine (8-OHdG; test for oxidized base radical) and by way of the comet assay (test for single-strand breaks). The comet assay can be applied directly to the cell and measured DNA strand breaks.

MATERIALS AND METHODS

Chemicals. The chemicals used in these experiments were purchased from the following suppliers: agarose, Triton-100, dimethyl sulfoxide, and EDTA diodium salt dihydrate from J. T. Baker, USA; sodium chloride, hydrogen peroxide, potassium chloride, Trizma base, glucose, *N*-lauroylasarcosine, ethidium bromide (EtBr), and 1,1-diphenyl-2-picrylhydrazyl (DPPH) from Sigma, USA; low-melting-point agarose from BDH, UK. Frosted microscope slides were acquired from Fisher Scientific, USA.

Chlorophyll Related Compound Extracts. Chlorophyll-related compounds (CRCs) were obtained from spinach purchased in Taipei, Taiwan. It was prepared as previously described (*21*). Chlorophyll *a* and *b* were purified from spinach and dephytylated by the catalysis of chlorophyllase isolated from plant (*Ficus macrocarpa*) leaf, to form Chlide *a* and *b*, respectively. Chlide *a* and Chlide *b* were further magnesium (Mg) dechelated to form Pho *a* and Pho *b*, respectively.

Isolated Human Peripheral Blood Lymphocytes. Blood samples (10 mL) were obtained from healthy donors, and lymphocytes were isolated using a separation solution kit (Ficoll-Paque Plus lymphocyte isolation sterile solution; Pharmacia Biotech, Sweden.) For the experimental procedure, cells were harvested within 1 day of blood samples having been taken, and cultured with AIM V medium (serum-free lymphocyte medium; Gibco Invitrogen, USA) in a humidified atmosphere of 5% CO_2 in air at 37 °C for 24 h.

Treatment Procedures and Cell-Viability Testing. After culture, lymphocytes were exposed to one of four different CRCs (Chlide *a* and *b*, Pho *a* and *b*, with Chlin as a positive control), each of which was used at three concentrations (5, 20, or 50 μM) for 30 min at 37 °C. DNA damage was induced by exposing lymphocytes to H_2O_2 (10 or 50 μM) for 5 min on ice. Treatment on ice minimizes the possibility of cellular DNA repair after H_2O_2 injury. Cells were centrifuged (100*g* for 10 min), washed, and resuspended in the same medium for the comet assay and the assessment of 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels in DNA. All experiments were carried out in triplicate. Cell viability was tested using the MTS assay (*22*), both prior to and after either CRC or H_2O_2 treatment.

DNA Single-Strand-Break Damage Estimation Using the Comet Assay. DNA single-strand break (ssb) damage was determined using the comet assay (*23*). Cultured lymphocytes ($\sim 10^5$ cells/mL) were embedded in 75 μL of 1% low-melting-point agarose on a microscope slide (precoated with agarose) at 37 °C. The gel was allowed to set at 4 °C, and cells were lysed for a period of at least 2 h in lysis buffer at 4 °C. Cells were then alkaline-unwound, following which electrophoresis was carried out using the electrophoresis buffer at 4 °C for 15 min at 25 V with the current adjusted to 300 mA. All steps were conducted under dim light to prevent the occurrence of additional DNA damage. Following electrophoresis, slides were neutralized with neutralization buffer and stained with ethidium bromide. Under a fluorescence microscope (Nikon COOLPIX5000), the cometlike images resulting from the extension of DNA were scored as a reflection of the single-strand breaks. Duplicate slides were prepared for each experimental point sample, and 50 cometlike images selected at random per slide were evaluated to determine average DNA damage values. A computerized image analysis system (VisCOMET 1.3, Impuls, Germany) was employed to determine various comet parameters such as tail moment. The tail moment (integrated value of DNA density multiplied by the DNA migration distance) was used as the primary measure of DNA damage (*24*) (**Figure 1**).

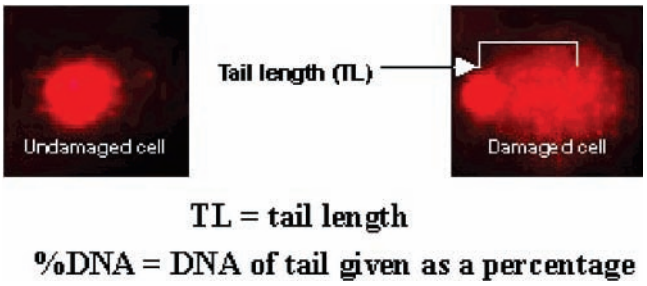

Figure 1. Under a fluorescence microscope, the cometlike images resulting from the extension of DNA were scored as a reflection of the single-strand breaks. The tail moment was defined as follows: TM = TL × % DNA.

Measurement of Oxidized Nucleoside Level by 8-Hydroxy-2′-deoxyguanosine (8-OHdG). Lymphocyte genomic DNA was purified using a genomic DNA purification kit (Bertec Enterprise, Taiwan), while 8-OHdG in lymphocyte DNA was quantified using an ELISA kit (Japan Institute for the Control of Aging, Japan). Briefly, samples or standards of 8-OHdG (0.5, 2, 8, 20, 80, and 200 ηg/mL) were placed in microtiter plates (8 × 12 wells; split type), which had been precoated with 8-OHdG. A monoclonal primary antibody recognizing 8-OHdG was added, and the plates were incubated for 1 h at 37 °C. The plates were then rinsed with PBS and subsequently incubated with an enzyme-labeled secondary antibody for a period of 1 h at 37 °C. Following this, plates were rinsed with PBS, and the chromatic substrate 3,3′,5,5′-tetramethylbenzidine was added, and incubation was carried out for 15 min at 37 °C in the dark. The reaction was terminated by the addition of phosphoric acid, and a further 3 min later, plates were read on a Microplate Reader that measured the absorbance at 450 nm. A standard curve was established by plotting the measured absorbance versus the logarithm of the concentration of 8-OHdG standards. Results were expressed as nanograms per microgram of DNA.

Measurement of DPPH Radical Scavenging. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method previously reported (*25*) was modified as follows: CRCs were dissolved in ethanol prior to reaction with a methanolic solution of DPPH (10 mM). The decrease in absorbance at 515 nm was determined with continuous data capture at 30-min intervals using a spectrophotometer (Hitachi U-3000). The DPPH scavenging capacity of CRCs was expressed as a proportional (percentage) inhibition (% inhibition), and was determined by the following expression:

$$\% \text{ inhibition} = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$$

where A_{control} is the absorbance of the sample at time = 0 and A_{sample} is the absorbance of the sample at time = 30 min.

Statistical Analyses. Data are reported as the mean ± standard deviation (SD) of triplicate determinations. Statistical analyses were

Table 1. Effect of CRCs on Human Lymphocyte Viability and DNA Damage[a]

		DNA damage	
	viability[b] (%)	comet assay (TM)[c]	8-OHdG (ηg/μg of DNA)
chlorophyllin			
5 μM	98.5 ± 2.3	110 ± 12	0.53 ± 0.08
20 μM	96.4 ± 2.2	138 ± 38	0.53 ± 0.03
50 μM	95.4 ± 6.1	113 ± 22	0.63 ± 0.05
chlorophyllide *a*			
5 μM	97.5 ± 6.4	113 ± 22	0.53 ± 0.08
20 μM	98.4 ± 5.3	136 ± 30	0.68 ± 0.08
50 μM	96.2 ± 4.3	120 ± 35	0.60 ± 0.04
chlorophyllide *b*			
5 μM	98.8 ± 2.2	120 ± 31	0.47 ± 0.10
20 μM	96.8 ± 3.5	126 ± 55	0.79 ± 0.09
50 μM	99.2 ± 4.4	104 ± 36	0.61 ± 0.08
pheophorbide *a*			
5 μM	95.8 ± 6.5	86 ± 32	0.66 ± 0.05
20 μM	96.9 ± 3.4	100 ± 25	0.55 ± 0.04
50 μM	99.4 ± 2.5	99 ± 26	0.46 ± 0.07
pheophorbide *b*			
5 μM	98.4 ± 1.6	95 ± 20	0.63 ± 0.08
20 μM	99.7 ± 3.3	91 ± 17	0.62 ± 0.08
50 μM	99.8 ± 2.6	100 ± 31	0.43 ± 0.10
control	100	100 ± 21	0.55 ± 0.06

[a] Mean ± SD. [b] Viability (measured by the MTS assay) was determined both prior to (100%) and following CRC pretreatment. [c] Mean tail moment (TM) was calculated by means of the comet assay.

Table 2. Effect of H_2O_2 on Human Lymphocyte Viability and DNA Damage[a]

		DNA damage	
	viability[b] (%)	comet assay (TM)[c]	8-OHdG (ηg/μg of DNA)
H_2O_2			
10 μM	99.4 ± 2.4	6386 ± 803[d]	2.30 ± 0.13[d]
50 μM	98.2 ± 7.1	14537 ± 1692[e]	3.98 ± 0.33[e]
control	100	100 ± 21[f]	0.55 ± 0.06[f]

[a] Mean ± SD. [b] Viability (measured by the MTS assay) was determined both prior to (100%) and following CRC pretreatment; no significant difference with regard to cell viability was apparent. [c] Mean tail moment (TM) was calculated for the comet assay. [d–f] Values featuring different letters differ significantly with regard to H_2O_2 levels (ANOVA, $p < 0.05$).

performed using a Student's *t*-test to compare differences between control and CRC-pretreated groups. One-way ANOVA was used to test for differences among the CRC groups. Post hoc comparison of means was performed by Duncan's multiple comparison, and $p < 0.05$ was considered to represent a statistically significant difference between test populations.

RESULTS

The initial viability of, and the level of DNA damage incurred by human lymphocytes from this experimental procedure, is revealed in **Table 1**. When human lymphocytes were pretreated with each of these CRCs, the subsequent cell viability did not change significantly, nor did the level of cell death or oxidative DNA damage compared with the solvent control. Following free-radical attack, the viability of and DNA damage sustained by human lymphocytes were determined, and the data are summarized in **Table 2**. The viability of human lymphocytes at the experimental procedure also did not differ significantly from control. When cells were challenged with free radicals, however, a significant increase in DNA damage was noticed compared to the control. The effect of lymphocyte exposure to 10 μM H_2O_2 was 60-fold (from 100 to 6386) greater with regard

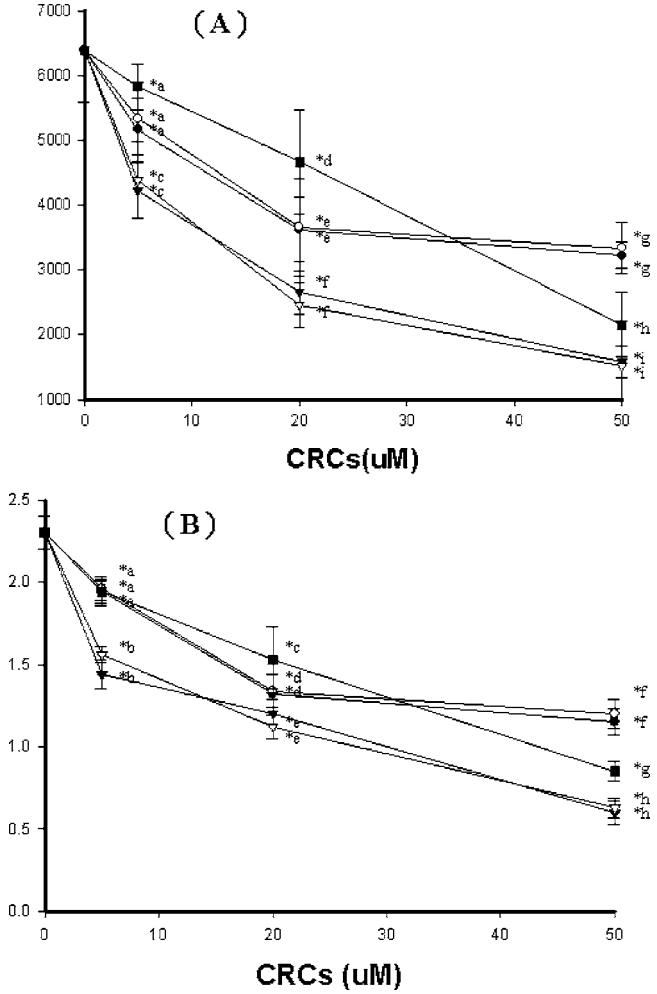

Figure 2. Effect of CRC pretreatment on 10 μM hydrogen peroxide induced DNA damage for isolated human lymphocytes. DNA oxidative damage was measured using the comet assay (**A**) and 8-OHdG levels in lymphocyte DNA (**B**). Results are the mean ± SD. (●, chlorophyllide *a*; ○, chlorophyllide *b*; ▼, pheophorbide *a*; ▽, pheophorbide *b*; ■, chlorophyllin.) Values with different letters differ significantly with regard to oxidative damage when comparing between different CRCs (ANOVA, $p < 0.05$); *$p < 0.05$ refers to differences in oxidative damage as compared with the control (without CRCs).

to DNA ssb damage than was the case for the control, and a significantly enhanced level of 8-OhdG adducts (from 0.55 to 2) was also observed. When lymphocytes were exposed to 50 μM H_2O_2, the level of oxidative damage increased significantly as revealed by the level of ssb's (from 100 to 14 537) and oxidized nucleoside formation (8-OHdG from 0.55 to 3.98), when compared to the control.

The effect of CRC pretreatment on 10 μM H_2O_2 induced DNA damage in human lymphocytes is presented in **Figure 2**. For each concentration of CRC-pretreated cells, a significantly reduced level of DNA ssb's and 8-OHdG formation following H_2O_2 exposure was observed ($p < 0.05$). It may also be seen from this figure that the protective effect on lymphocytes of pretreatment of such cells with each of the CRCs at the lower dose for each (5−20 μM) occurred in a dose-dependent manner. Those lymphocytes pretreated with Pho *a* or *b* (5−50 μM) experienced a greater level of protection against H_2O_2 exposure than did lymphocytes exposed to the other test compounds, and in a dose-dependent manner. The maximum protective effect for lymphocyte pretreatment was seen for pretreatment with 50 μM Pho *a* and *b* (respectively 75.28% and 76.17% inhibition

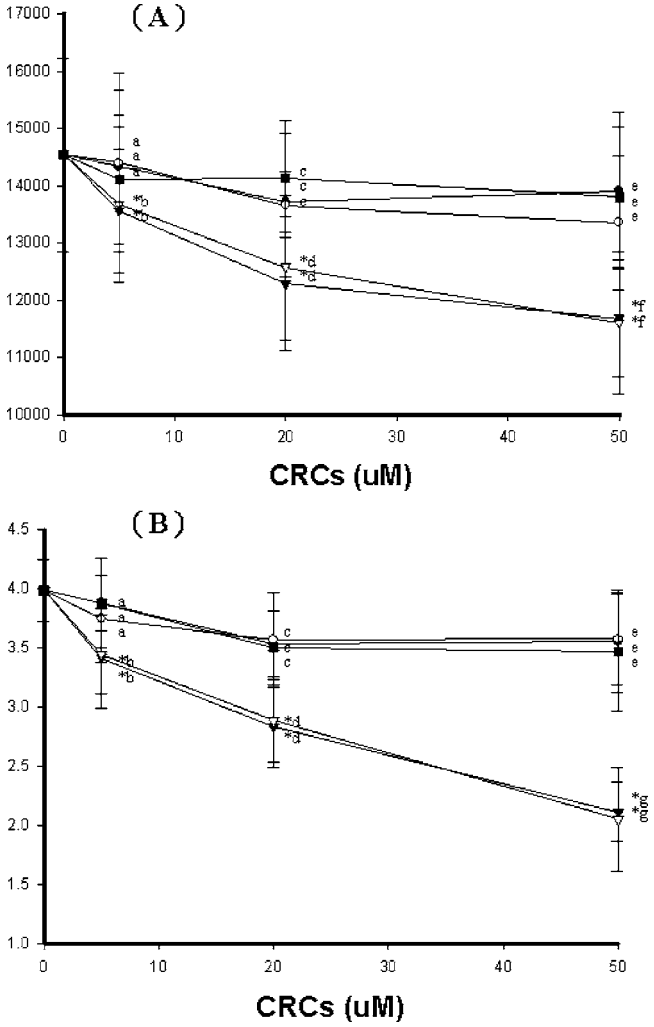

Figure 3. Effect of CRC pretreatment on 50 μM hydrogen peroxide induced DNA damage for isolated human lymphocytes. DNA oxidative damage was measured using the comet assay (**A**) and 8-OHdG levels in lymphocyte DNA (**B**). Results are the mean ± SD. (●, chlorophyllide *a*; ○, chlorophyllide *b*; ▼, pheophorbide *a*; ▽, pheophorbide *b*; ■, chlorophyllin.) Values with different letters differ significantly with regard to oxidative damage when comparing among different CRCs (ANOVA, $p < 0.05$); *$p < 0.05$ refers to differences in oxidative damage as compared with the control (without CRCs).

compared with the solvent control; **Figure 2A**). A similar result was noted when the level of Pho *a* or *b* protection to H_2O_2 exposure was assessed in the context of the production of 8-OHdG adducts (**Figure 2B**).

The protective effect of CRC pretreatment on 50 μM H_2O_2 induced DNA damage for human lymphocytes is presented in **Figure 3**. At a 50 μM H_2O_2 challenge, both Chlin and Chlide failed to demonstrate any degree of protection against DNA damage and the level of damage for both cases was similar to that of the positive control. With regard to Pho *a* and *b*, both expressed antioxidative protective effects at all of the concentrations tested: pretreatment with such agents elicited a decrease in DNA ssb's (19.63% and 20.18% inhibition compared with the solvent control) and 8-OHdG adduct formation following H_2O_2 exposure (**Figure 3**). The lymphocytes pretreated with either Pho *a* or *b* exhibited protection against not only low doses of H_2O_2 exposure, but also high doses of H_2O_2-induced DNA damage.

The free-radical scavenging capacity of CRCs is shown in **Figure 4**. Four CRCs were tested; they exhibited diverse

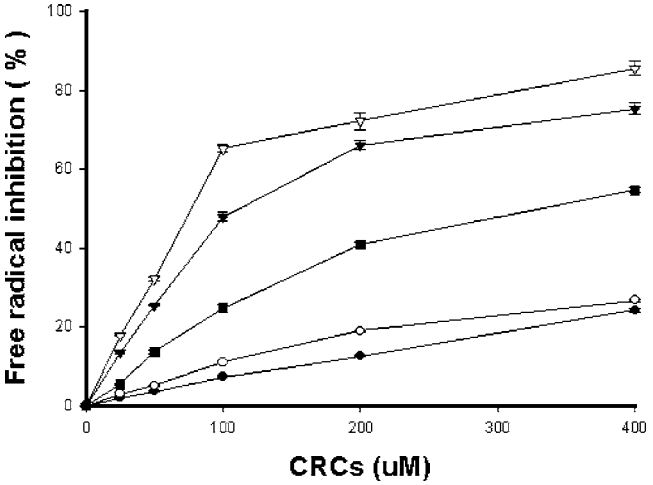

Figure 4. DPPH scavenging capacity of CRCs. The absorbance inhibition for DPPH was monitored at 515 nm. Results represent the mean ± SD. (●, chlorophyllide *a*; ○, chlorophyllide *b*; ▼, pheophorbide *a*; ▽, pheophorbide *b*; ■, chlorophyllin.)

antioxidative capacities regarding their ability to scavenge the DPPH free radical. The scavenging capacities of Pho did appear to be more pronounced than that of Chlide's. The IC_{50} value for DPPH inhibition of Pho *b*, Pho *a*, and Chlin were, respectively, 75, 120, and 360 μM, while the corresponding figures for Chlide *a* and *b* were somewhat greater than 800 μM.

From DNA damage and free-radical scavenging tests, CRCs had antioxidative effects and the composition of magnesium ion in CRCs might affect their antioxidative ability under either ex vivo or in vitro stress conditions.

DISCUSSION

Exposure to an adverse aerobic environment can elicit the generation of reactive oxygen species (ROS) resulting in oxidative stress (*26*). Excess ROS generation can significantly alter the structural and functional relationships of biomolecules and has been implicated as a cause of oxidative DNA damage (*27*). Chlin has been reported to exhibit a greater level of antimutagenic activity than the well-known suite of antioxidant vitamins including retinal, β-carotene, vitamin E, and vitamin C (*28*). Furthermore, Chlin's antioxidant ability is more substantial than that of vitamin C, glutathione, and mannitol (*8*). Natural chlorophylls, in contrast, have been little studied in this regard (*16*). Our results showed that Chl's from water extract of spinach significantly reduced single-strand-break formation and 8-OHdG adduct level; furthermore, Pho *a* and *b* experienced a greater level of protection than Chlin. The results might explain partially why vegetables containing chlorophyll derivatives would protect against and reduce the risk of ROS-related diseases.

The basic structure of CRCs contains a porphyrin-related chlorine ring skeleton, which possesses more than 10 conjugative double bonds, and appears to be a responsible chemical structure for antioxidant activity (*29*). We examined the free-radical scavenging capacity of CRCs, which quenched DPPH (10 mM) to process a stable free radical. Endo et al. (*30*) indicated that CRCs provide the hydrogen donor to reduce free radicals such as DPPH, and identified a π-cation radical of porphyrin compounds when CRCs react with free radical using electron spin resonance spectroscopy. Hydrogen peroxide is believed to cause DNA oxidative damage by generation of hydroxyl radical via the Fenton reaction. For the present study, we remain

uncertain regarding the precise quantity of ferric ions in human lymphocyte to induce hydroxyl radical generation, but we compare the composition of the water-soluble CRCs investigated in the present study: Chlin contains a copper ion in its porphyrin structure and Chlide contains magnesium ion while Pho differs markedly in that such molecules do not contain any metal ions in the porphyrin ring. It remains possible that Pho acts by way of an ion-chelation effect to reduce metal-induced hydroxyl radical generation, thus protecting DNA from oxidative damage (*31*). We speculate that both Pho *a* and *b* exert their antioxidative capacity not only by the porphyrin stabilization of ROS, but also by their inherent ion-chelation capacity.

In conclusion, the water-extract-Chl derivatives reduce the DNA damage induced by hydrogen peroxide in isolated human lymphocytes. Pho compounds were more effective than Chlide. These compounds might act as free-radical scavengers and as chelating agents to protect human lymphocyte DNA from oxidative damage.

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