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# Differential cytoprotective effect of copper- and iron-containing chlorophyllins against oxidative stress-mediated cell death

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**Abstract**

Chlorophyllin (CHL), a copper-containing chlorophyll derivative, was previously reported to possess reactive oxygen species (ROS) scavenging activity. However, the antioxidant property of iron-containing chlorophyllin, iron chlorin e6 (FeCe6), has not been explored in detail. This study systematically investigated the antioxidant capacity of two chlorophyllins, CHL and FeCe6, and their cytoprotective effects against ROS toxicity in human Jurkat T-cells. Both CHL and FeCe6 exhibited a wide range of antioxidant activities to ABTS radical, singlet oxygen, peroxynitrite, and peroxyl radical. Notably, FeCe6 exerted a significant suppressive effect on hydrogen peroxide-dependent cytotoxicity and apoptosis in Jurkat T-cells, while negligible or much weaker inhibition was observed by CHL. This differential cytoprotective effect is likely due to the different hydrogen peroxide scavenging capacity of two chlorophyllins. Moreover, FeCe6 attenuated menadione-induced cell death and tumour necrosis factor (TNF)-α-induced IκB phosphorylation via scavenging of intracellular ROS. Taken together, the results suggest that FeCe6 is a promising cytoprotective antioxidant against oxidative stress-mediated cellular toxicity.

**Keywords:** *Chlorophyll derivative, chlorophyllin, iron chlorin e6, antioxidant, hydrogen peroxide, cytoprotection*

**Abbreviations:** *CHL, chlorophyllin; FeCe6$^{+}$, iron (III) chlorin e6; Ce6, chlorin e6; MnTMPyP$^{5+}$, manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin; FeTMPyP$^{5+}$, iron (III) tetrakis (1-methyl-4-pyridyl) porphyrin; MnTBAP$^{3-}$, manganese (III) tetra (4-benzoic acid) porphyrin; BHT, butylated hydroxytoluene; ABTS, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid; TMP, 2,2-6,6-tetramethyl-4-piperidinol; AAPH, 2,2′-azobis (2-amidinopropane) dihydrochloride; DHR, dihydrorhodamine; PI, propidium iodide; JC-1, 5,5′,6,6′,-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; TNF, tumour necrosis factor; NF-κB, nuclear factor kappa B; IκB, inhibitory kappa B*

## Introduction

Reactive oxygen species (ROS) are inevitably produced during the normal metabolic process such as cellular respiration or diverse oxidase reactions [1]. Extracellular ROS from a membrane-bound NADPH oxidase in the activated phagocytes are beneficial for host immune defense against invading micro-organisms; however, intracellular ROS are able to damage or process cellular components due to their intrinsic highly-reactive properties [1]. To keep cellular homeostasis from the toxic effects of ROS, cells are equipped with diverse intracellular ROS scavenging substances from antioxidant enzymes to small antioxidant compounds [2].

Several redox-active metal-containing compounds have been investigated as potent antioxidants based on their core transition metal mimicking the active site of superoxide dismutase (SOD) and catalase [3,4]. Much attention has lately been paid to metallporphyrins as a novel class of catalytic antioxidants that scavenge a broad range of ROS such as superoxide, hydrogen peroxide ($H_2O_2$), peroxynitrite ($ONOO^-$) and lipid peroxyl radicals [5,6]. Accumulating evidences have demonstrated that metalloporphyrins have been very

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effective in mitigating the toxic effects of reactive oxygen or nitrogen species, indicating that they are promising antioxidants for clinical applications [5,7–10].

Chlorophyllin (CHL), a mixture of copper chlorin e4 (CuCe4) and copper chlorin e6 (CuCe6), is comprised of redox-active copper ion and chlorin ring [11], which is similar to metalloporphyrins such as MnTMPyP and MnTBAP (Figure 1). As a substitute for water-insoluble chlorophyll, earlier studies on the biological functions of CHL mainly focused on its anti-mutagenic and anti-carcinogenic properties [12,13]. CHL, however, is also increasingly recognized as a potent antioxidant compound. A line of studies have revealed that CHL not only scavenges ROS such as superoxide, hydroxyl radical and singlet oxygen ($^1O_2$) but also prevents lipid peroxidation from oxidative damage [14–18]. Moreover, metal chelation of chlorophyll is known to strengthen the antioxidant activity of tetrapyrroles [19]. These findings raise the possibility that metal-containing chlorophyll derivatives may be a potent antioxidant compound.

On the other hand, there is preliminary evidence suggesting that iron chlorin e6 (FeCe6), an iron-containing chlorophyllin (Figure 1), is able to scavenge superoxide and hydroxyl radical [14,20]. However, it has not been determined whether FeCe6 is able to act as a potent intracellular antioxidant in mammalian cells under oxidative stress conditions. The cytoprotective antioxidant activity of these two chlorophyllins, CHL and FeCe6, was thus examined in this study. We here provide evidence that FeCe6 is a potent antioxidant compound to attenuate oxidative stress-mediated cell death.

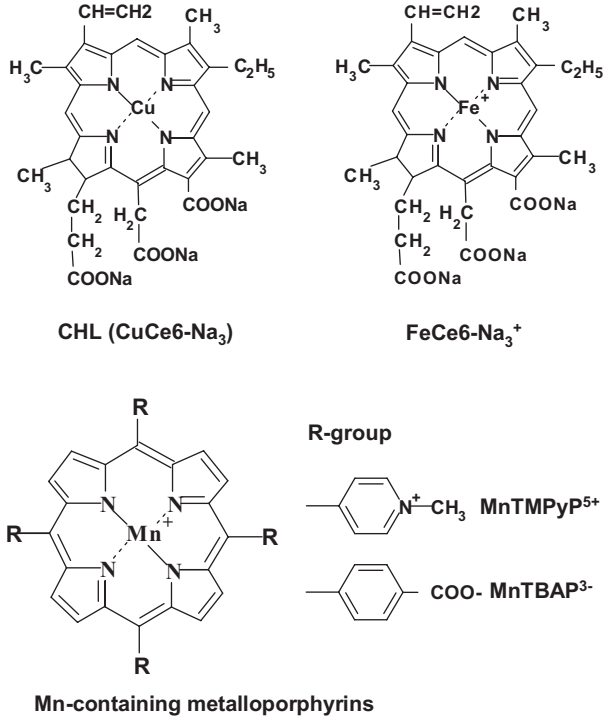


Figure 1. Structures of chlorophyllins and metalloporphyrins.

## Materials and methods

### *Chemicals*

FeCe6, chlorin e6 (Ce6) and manganese (III) tetrakis(1-methyl-4-pyridyl) porphyrin (MnTMPyP) were purchased from Porphyrin Products Inc. (Logan, UT). Iron (III) tetrakis(1-methyl-4-pyridyl) porphyrin (FeTMPyP) was obtained from Calbiochem (San Diego, CA). $H_2O_2$, chlorophyll, CHL, Trolox, butylated hydroxytoluene (BHT), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 2,2-6,6-tetramethyl-4-piperidinol (TMP), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), dihydrorhodamine123 (DHR123), sodium azide ($NaN_3$), glucose oxidase, propidium iodide (PI), 3-[4,5-dimethylthiazol-2-yl]-2.5-diphenyltetrazolium bromide (MTT) and menadione sodium bisulphite were purchased from Sigma-Aldrich (St. Louis, MO). Apoptosis assay kit, 5,5′,6,6′,-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Molecular Probes (Eugene, OR). Recombinant human TNF-α was purchased from Calbiochem (San Diego, CA, USA). Anti-phospho-IκB (Ser 32/36) antibody was purchased from Cell Signaling Technology (Beverly, MA).

### *$ABTS^{\bullet+}$ radical scavenging assay*

$ABTS^{\bullet+}$ radical was generated by incubation of 7 mM ABTS with 2.5 mM potassium persulphate in the dark for 14 h, and then diluted to 60 μM using a molar extinction coefficient of $ABTS^{\bullet+}$ at 734 nm ($\varepsilon$ = 15 $mM^{-1}$ $cm^{-1}$) [21]. The $ABTS^{\bullet+}$ solution was then mixed with the test compound and 5 min later the absorbance of $ABTS^{\bullet+}$ was measured at 734 nm. Relative ABTS radical scavenging activity (%) was determined as $[(A_0 - A_i)/A_0] \times 100$, where $A_0$ and $A_i$ indicate the absorbance in the absence ($A_0$) or presence ($A_i$) of the test compound. $IC_{50}$ values were calculated from the slope, obtained from a plot of $(A_0/A_i) - 1$ vs the compound concentration, at $(A_0/A_i) - 1 = 1$.

### *Singlet oxygen scavenging assay*

Singlet oxygen quenching activity was determined using a TMP as a spin trapping agent by electron spin resonance (ESR) spectrometry [22]. The generation of singlet oxygen was initiated by the addition of $H_2O_2$ at a final concentration of 39 mM to a reaction mixture containing 0.4 M NaOCl and 12.6 mM TMP in the absence or presence of the test compound in phosphate-buffered saline (PBS, pH 7.4). The reaction mixture was then scanned using an ESR spectrometer (JEOL, Tokyo, Japan). Instrumental settings of the ESR spectrometer were standardized as follows: microwave power, 4 mW; microwave frequency, 9.43 GHz; modulation frequency, 100 kHz; modulation

width, 0.1 mT; scan rage, 10 mT; sweep time, 1 min; and time constant, 0.1 s.

*Peroxynitrite scavenging assay*

Peroxynitrite was synthesized in a quenched flow reactor according to the previous method and its concentration was determined using a molar extinction coefficient at 302 nm ($\varepsilon$ = 1670 $M^{-1}$ $cm^{-1}$) [23]. Peroxynitrite scavenging activity was determined by the relative inhibition of oxidation of non-fluorescent DHR123 to fluorescent rhodamine 123 by peroxynitrite. Briefly, peroxynitrite at a final concentration of 40 μM was added to a reaction mixture containing 10 μM DHR123 and the test compound in PBS, pH 7.4. After 5 min incubation, the fluorescence of rhodamine 123 was measured using a fluorescence spectrophotometer at excitation and emission wavelengths of 500 nm and 528 nm, respectively.

*Breakage of plasmid DNA*

To induce peroxyl radical-induced DNA breakage, pcDNA3 was incubated with 4 mM AAPH in the presence of the test compound at 37°C for 1 h in 50 mM potassium phosphate buffer, pH 7.4 [24]. The mixture was then analysed by 1% agarose gel electrophoresis.

*Cell culture and cell viability assay*

Human Jurkat T-cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum (FBS). Human umbilical vein endothelial cells (HUVEC) and human lung epithelial A549 cells were grown in DMEM containing 10% FBS. Jurkat T-cell death was determined by measuring the uptake of propidium iodide (PI). In brief, after the designated treatment to induce cell death, cells were washed with PBS, incubated with PI (1 μg/ml) and then analysed by a FACSCalibur flow cytometer (BD) using FL-2 channel. To determine the cell viability of HUVEC and A549 cells, MTT colourimetric assay was conducted as described previously [14]. Cells were grown in 96-well plates and pre-treated with the test compound for 6 h. After washing with PBS, the medium was then replaced by the fresh medium containing glucose oxidase or $H_2O_2$. MTT stock solution (5 mg/ml) was added to each well and incubated for 4 h. After removal of cultural medium, formed MTT formazan was solubilized with DMSO and the absorbance was measured at 540 nm.

*Measurement of apoptosis*

Apoptotic cell death was measured by using a Vybrant™ apoptosis assay kit (Molecular Probes) according to the manufacturer's manual. Cells were harvested after the designated treatment, washed with cold PBS and stained with Annexin V-FITC and PI. Fluorescence was then detected by flow cytometric analysis, measuring the fluorescence emission at 530 nm (FL-1) and 610 nm (FL-2), and the data were analysed by CellQuest software (BD). Mitochondrial membrane potential was determined as described previously [25]. After the designated treatment, cells were stained with JC-1 (10 μg/ml) at room temperature for 10 min and analysed by flow cytometry with FL-1 and FL-2 channels.

*Measurement of intracellular ROS*

Intracellular ROS was measured by using an oxidant-sensitive fluorescent probe, DCFH-DA. Cells were pre-treated with FeCe6 for 1 h, washed with cold PBS and incubated with 20 μM DCFH-DA at 37°C for 20 min. Cells were then treated with menadione for 1 h and analysed by flow cytometry (FL-1). To measure time-dependent formation of intracellular ROS, DCFH-DA-loaded cells were transferred into 96-well plates. After treatment with menadione, fluorescence was monitored by a Varian Cary Eclipse fluorescence spectrophotometer at excitation wavelength of 488 nm and emission wavelength of 523 nm.

*$H_2O_2$ scavenging assay*

$H_2O_2$-dismutating activity was determined according to the previous method [26]. $H_2O_2$ (final concentration of 19 mM) was added to a reaction mixture containing the test compound in 50 mM potassium phosphate buffer, pH 7.0, and the decrease in $H_2O_2$ absorbance was then monitored for 4 min at 240 nm. Decomposition of $H_2O_2$ was calculated as follows: $H_2O_2$ decomposed (M) = $\Delta A_{240}/(43.6 \times 1\ \text{cm})$ using a molar extinction coefficient of $H_2O_2$ at 240 nm ($\varepsilon$ = 43.6 $M^{-1}$ $cm^{-1}$). One unit of catalase-like activity was defined as micromole of $H_2O_2$ decomposed per minute. The rate constant for the reaction of sample with $H_2O_2$ was determined by the horseradish peroxidase (HRP)-mediated phenol red oxidation assay [27]. HRP (final concentration of 30 U/ml) was added to a reaction mixture containing 0.1 mg/ml phenol red, 60 μM $H_2O_2$ and the test compound in 10 mM potassium phosphate buffer, pH 7.0, and 5 min later the absorbance at 610 nm was measured. The rate constant was calculated as follows: $k_{\text{sample}} = k_{\text{HRP}} \cdot [\text{HRP}] \cdot (I_0/I - 1)/[\text{sample}]$, where $k_{\text{HRP}}$ is the rate constant for HRP, 3.7 $\times$ $10^6$ $M^{-1}$ $s^{-1}$. $I_0$ and $I$ represent the absorbance of reaction mixture in the absence and presence of sample, respectively.

*Measurement of IκB phosphorylation*

Jurkat T-cells were pre-treated with FeCe6, washed with PBS and treated with TNF-α (20 ng/mL) for

5 or 10 min. Cells were lysed in 10 mM Tris-HCl (pH 7.8), 0.5% Triton X-100, 50 mM KCl, 0.1 mM dithiothreitol and protease inhibitors cocktail (Roche). Cell lysates were then immunoblotted with anti-phospho-IκB antibody.

### *Statistical analysis*

All values were expressed as means ± SD of *n* observations. Data were statistically analysed by unpaired Student's *t*-test after analysis of variance. *p*-values of 0.05 or less were considered significant.

## Results

### *Antioxidant activity of chlorophyllins*

It has been previously reported that CHL is capable of scavenging ROS, but antioxidant function of FeCe6 has not been examined in detail. Thus, we first compared the antioxidant abilities of CHL and FeCe6 with other established antioxidants using an ABTS assay. The two chlorophyllins demonstrated dose-dependent quenching activity to $ABTS^{\bullet+}$ as efficient as standard antioxidant Trolox (Figure 2A). $IC_{50}$ values of two chlorophyllins in ABTS assay were estimated to be 12.00 μM of FeCe6 and 24.60 μM of CHL, which is much lower than that of BHT (159.24 μM), but slightly higher than that of Trolox (10.75 μM). Similar results were also observed in 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay as FeCe6 and CHL showed stronger DPPH radical scavenging activity than BHT, but weaker than Trolox (data not shown).

Next, we evaluated the antioxidant potential of two chlorophyllins to scavenge singlet oxygen ($^1O_2$), which is known to induce genotoxic and cytotoxic damage [28]. TMP was employed for detection of singlet oxygen, which reacted with TMP to form TMP-$^1O_2$ adducts as detected by ESR spectrometry (Figure 2B, 1st row). At 100 μM of concentration, FeCe6 and CHL completely abolished the formation of TMP-$^1O_2$ (Figure 2B, 2nd and 3rd row), whereas Ce6, a metal-deficient chlorophyllin, showed no inhibition (Figure 2B, 4th row). This finding indicates that the core metal of chlorophyllins is necessary for scavenging

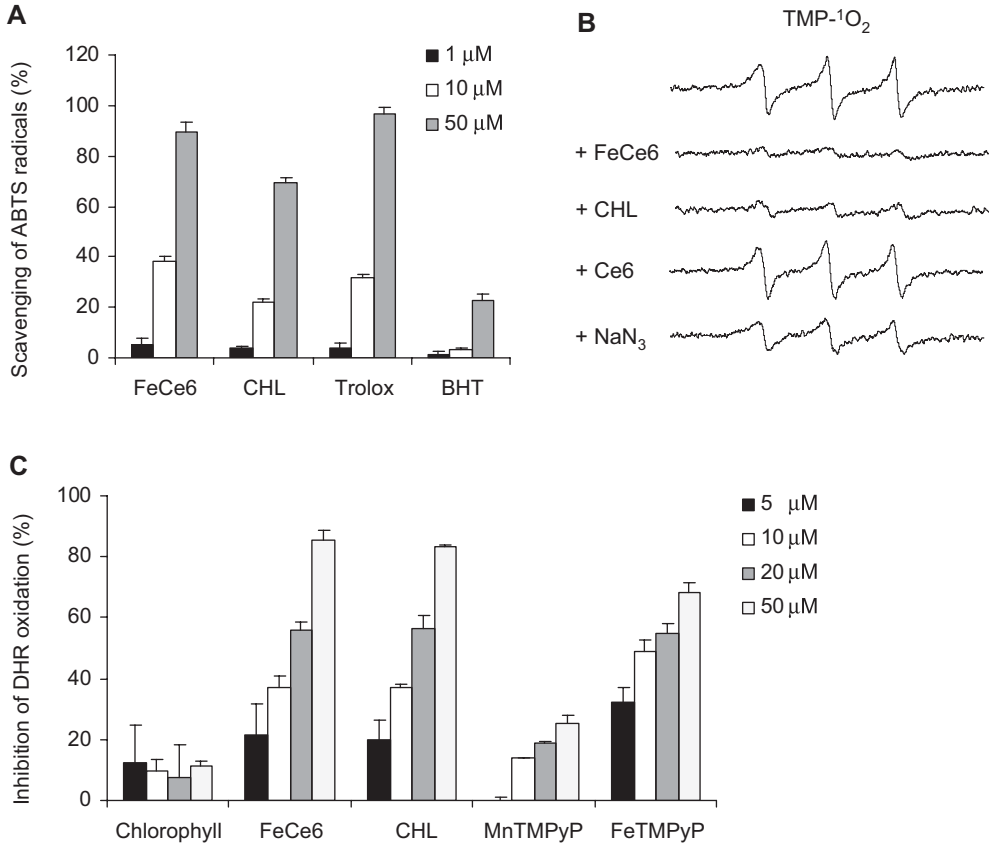


Figure 2. *In vitro* antioxidant activities of chlorophyllins. (A) ABTS radicals were incubated with the indicated amount of FeCe6, CHL, Trolox or BHT for 5 min and the absorbance of $ABTS^{\bullet+}$ was determined at 734 nm. ABTS radical scavenging activity was calculated as described in Materials and methods ($n$ = 3). (B) ESR spectra of TMP-$^1O_2$ adducts. Singlet oxygen ($^1O_2$) was generated in the reaction mixture containing FeCe6, CHL, Ce6 or $NaN_3$ (100 μM) as indicated and analysed by ESR spectrometry. (C) Peroxynitrite-mediated DHR123 oxidation. DHR123 was incubated with peroxynitrite (40 μM) in the reaction mixture containing chlorophyll, FeCe6, CHL, MnTMPyP or FeTMPyP as indicated and the fluorescence was measured. Data were presented as the relative inhibition of DHR 123 oxidation as compared to control in the absence of added test compound ($n$ = 3).

of singlet oxygen. Singlet oxygen quenching activity of CHL was consistent with the previous findings [15,18], but we, here, provide first evidence that iron-containing chlorophyllin is also able to scavenge singlet oxygen. Interestingly, singlet oxygen scavenging capacity of two chlorophyllins was superior to that of a well-known singlet oxygen quencher, $NaN_3$ (Figure 2B, 5$^{th}$ row).

As several metalloporphyrins such as manganese- or iron-porphyrins have been shown to decompose peroxynitrite [5,29–31], two chlorophyllins were tested on the peroxynitrite scavenging ability using DHR123 as a probe for peroxynitrite. As shown in Figure 2C, both FeCe6 and CHL exhibited dose-dependent inhibitions on peroxynitrite-mediated DHR123 oxidation as determined by the fluorescence of rhodamine 123, but magnesium-containing chlorophyll showed negligible effect. Since the two chlorophyllins did not reduce the fluorescence of rhodamine 123 induced by peroxynitrite (data not shown), both FeCe6 and CHL are likely to possess peroxynitrite scavenging ability. Considering that metalloporphyrins catalyse the decomposition of peroxynitrite by recycling two valence states [29], the transition metal moiety of chlorophyllins may be essential for their efficient scavenging of peroxynitrite. To compare the peroxynitrite scavenging activity of two chlorophyllins with other peroxynitrite scavengers, we examined the inhibiton of peroxynitrite-mediated DHR123 oxidation by MnTMPyP and FeTMPyP, which have been shown to decompose peroxynitrite [30]. The peroxynitrite scavenging activities of two chlorophyllins were stronger than that of MnTMPyP but slightly weaker than FeTMPyP (Figure 2C). According to the previous report, rate constants of two metalloporphyrins-catalszed peroxynitrite decomposition was $4.8 \times 10^4$ $M^{-1}$ $s^{-1}$ for MnTMPyP and $7.9 \times 10^5$ $M^{-1}$ $s^{-1}$ for FeTMPyP in the absence of reductant, respectively [30]. Our result thus indicates that Fe- and Cu-chlorophyllins are able to decompose peroxynitrite as efficiently as other peroxynitrite scavengers. These multifunctional *in vitro* antioxidant abilities of two chlorophyllins led us to investigate their cytoprotective potential against ROS toxicity.

### *Inhibition of peroxyl radical-induced plasmid DNA cleavage by chlorophyllins*

To test whether these chlorophyllins could protect biomolecules against free radical toxicity, plasmid DNA cleavage assay was performed using an AAPH as a peroxyl radical generator. AAPH-induced peroxyl radicals have been widely shown to induce lipid peroxidation and DNA damage, which involves both strand scission and base modification [32,33]. As shown in Figure 3, supercoiled form of plasmid DNA (I) was sequentially cleaved into open circular form (II) and linear form (III) by AAPH-induced peroxyl radical (lane 2). In the presence of FeCe6, plasmid

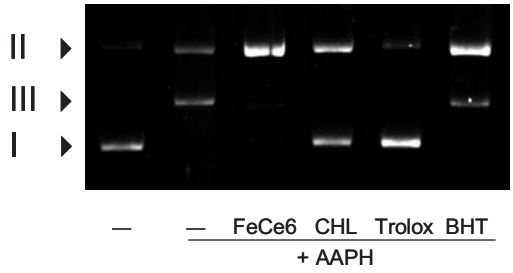


Figure 3. Protective effect of chlorophyllins on peroxyl radical-induced plasmid DNA cleavage. pcDNA3 was incubated with AAPH (4 mM) in the presence of FeCe6, CHL, Trolox or BHT (50 μM) for 1 h and analysed by agarose gel electrophoresis. Form I, II and III indicates supercoiled, open circular and linear form, respectively.

DNA was partially cleaved into open circular form, but not into linear form by AAPH (lane 3). On the other hand, CHL showed more efficient protection than FeCe6 against peroxyl radical, as half of the supercoiled form was still intact from DNA nicking (lane 4). The reference antioxidant, Trolox, exhibited the strongest protection against the peroxyl radical-induced DNA breakage (lane 5), while BHT showed the weakest inhibition (lane 6), similar to the result in ABTS assay. Taken together with our previous findings that CHL and FeCe6 protected plasmid DNA breakage from hydroxyl radical [14], this result confirms their antioxidant capacity to prevent oxidative stress-mediated damage on the cellular component.

### *Inhibition of $H_2O_2$-dependent cytotoxicity by FeCe6*

Although the antioxidant activity of CHL has been already reported, its cytoprotective potential against $H_2O_2$-dependent mammalian cell death is still questionable [14]. Thus, here we examined the cytoprotective role of CHL and FeCe6 against $H_2O_2$ toxicity in human Jurkat T lymphocytes. Lymphocytes are continuously exposed to ROS derived from activated macrophages and neutrophils during an inflammatory response against microbial infection. As shown in Figure 4A, treatment with 0.5 mM $H_2O_2$ caused a marked cytotoxicity to Jurkat T-cell as 39.5% of cells treated were PI-positive, which represents dead cells, but pre-treatment with FeCe6 efficiently decreased $H_2O_2$-induced Jurkat T-cell death by 14.6%. On the other hand, neither CHL nor Trolox (data not shown) exhibited considerable inhibitory effects against $H_2O_2$ cytotoxicity in spite of its prominent *in vitro* antioxidant property as shown earlier. This failure of cytoprotection by CHL on $H_2O_2$-dependent cell death was inconsistent with our previous study in human endothelial cells [14].

To mimic more physiological oxidative stress conditions, glucose oxidase was used as a continuing source of $H_2O_2$. As expected, glucose oxidase induced an evident cytotoxicity to HUVEC cells at a higher dose than 1 mU/mL (data not shown).

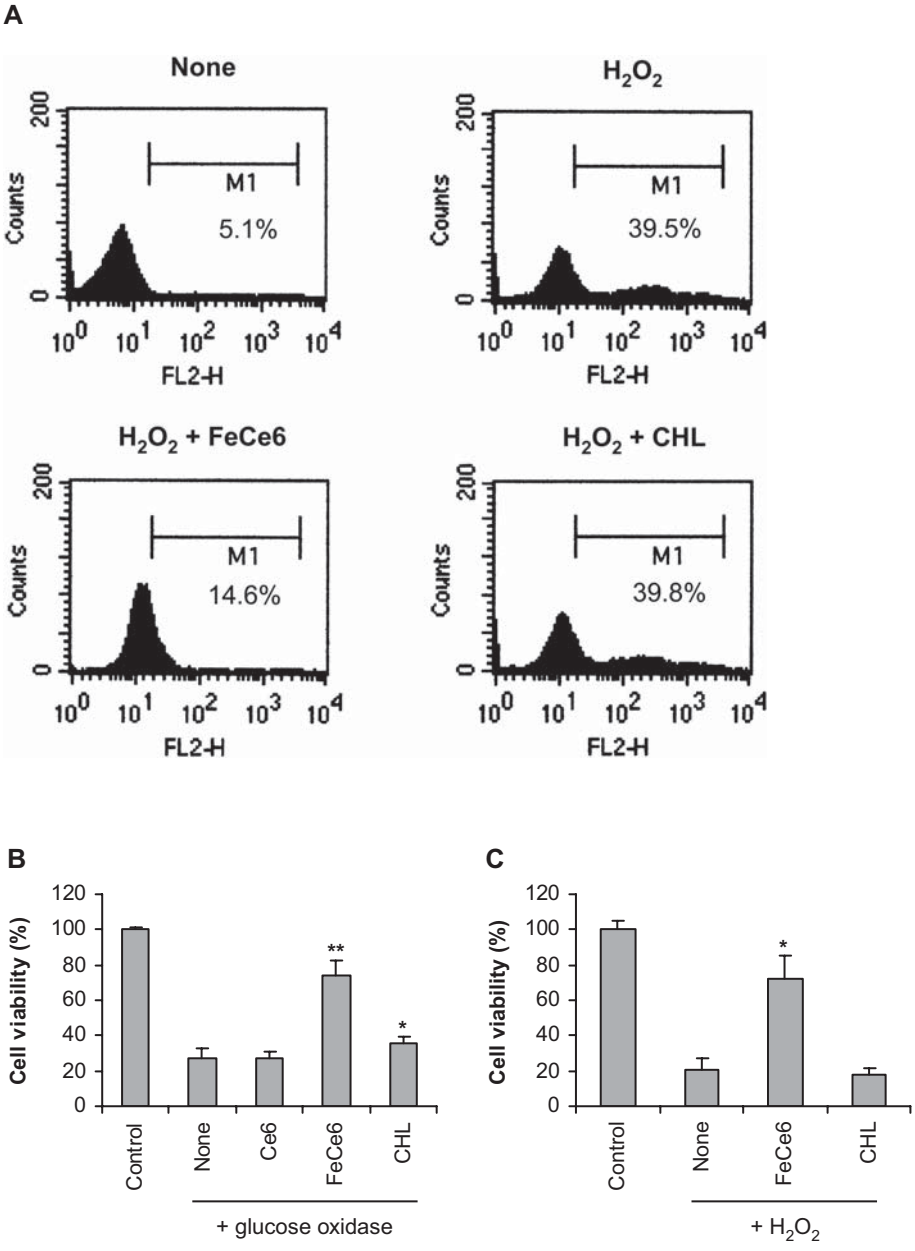


Figure 4. Protective effect of FeCe6 on $H_2O_2$-dependent cytotoxicity. (A) Jurkat T-cells were untreated (none) or treated with $H_2O_2$ (500 μM) for 6 h after pre-treatment with FeCe6 or CHL (100 μM) for 2 h. Cell death was determined by FACS analysis after staining with PI. (B) HUVEC cells, pre-treated with Ce6, FeCe6 or CHL (100 μM) for 6 h as indicated, were untreated (control) or treated with glucose oxidase (2.5 mU/mL) for 4 h. Cell viability was then measured by MTT assay and expressed as % of control. Asterisks indicate significant differences from the group treated with glucose oxidase only ($n = 6$; $^{*}p < 0.05$; $^{**}p < 0.0001$). (C) A549 cells, pre-treated with FeCe6 or CHL (100 μM) for 6 h as indicated, were untreated (control) or treated with 1 mM $H_2O_2$ for 6 h. Cell viability was determined by MTT assay. Asterisks indicate significant differences from the group treated with $H_2O_2$ only ($n = 12$; $^{*}p < 0.0001$).

Among the chlorin-related compounds tested, FeCe6 significantly prevented glucose oxidase-induced cell death of HUVEC, similar to the result in the direct treatment of $H_2O_2$ in Jurkat T-cells (Figure 4B), while the metal-lacking Ce6 showed no protective effect, indicating that the metal moiety is essential for cytoprotection of FeCe6 against $H_2O_2$. On the other hand, CHL exerted a slight protection against glucose oxidase-induced cytotoxicity. To verify the protective roles of these two chlorophyllins, human lung epithelial A549 cells were treated with 1 mM $H_2O_2$. As shown in Figure 4C, FeCe6 remarkably protected A549 cells against $H_2O_2$ toxicity, whereas CHL exhibited no cytoprotective effect as observed in Jurkat T-cells. Collectively, our results demonstrate that FeCe6, but not CHL, is a potent cytoprotective antioxidant to prevent $H_2O_2$-dependent cell death. Moreover, such observations raise the possibility that only FeCe6 is able to scavenge $H_2O_2$ or $H_2O_2$-mediated secondary free radicals such as hydroxyl radical, making FeCe6 a more efficient cytoprotective antioxidant.

### *Extracellular inhibitory action of FeCe6 on $H_2O_2$-dependent cytotoxicity*

$H_2O_2$ is known to exert its cytotoxic action by activating intracellular signalling pathways leading to cell death or by damaging cellular components [34]. To explore the possibility that the above protective effect of FeCe6 against $H_2O_2$ is due to its extracellular presence, possibly because of incomplete washing after pre-treatment, FeCe6 was treated into HUVEC in two different ways, i.e. together with $H_2O_2$ or glucose oxidase and pre-treated. As shown in Figure 5A, co-treatment of FeCe6 with $H_2O_2$ exhibited a remarkable protection of HUVEC cell death even in the low dose of FeCe6 as 10 μM, while the pre-treatment showed a dose-dependent protective effect as we observed earlier. This result indicates that extracellular FeCe6 could inhibit the diffusion of extracellular $H_2O_2$ into intracellular space through scavenging of $H_2O_2$ or $H_2O_2$-derived reactive species such as hydroxyl radical. To confirm the extracellular effect of FeCe6 against $H_2O_2$, glucose oxidase was added into HUVEC together with FeCe6 (Figure 5B). Similarly, the co-treatment of FeCe6 protected glucose oxidase-induced cell death of HUVEC much stronger than the pre-treatment in the low concentration of FeCe6. However, there were no significant differences in cell protection against $H_2O_2$ or glucose oxidase between co-treatment and pre-treatment of FeCe6 in the high dose as 100 μM, indicating that FeCe6 could act as an effective intracellular antioxidant when its concentration is enough to distribute through the cells.

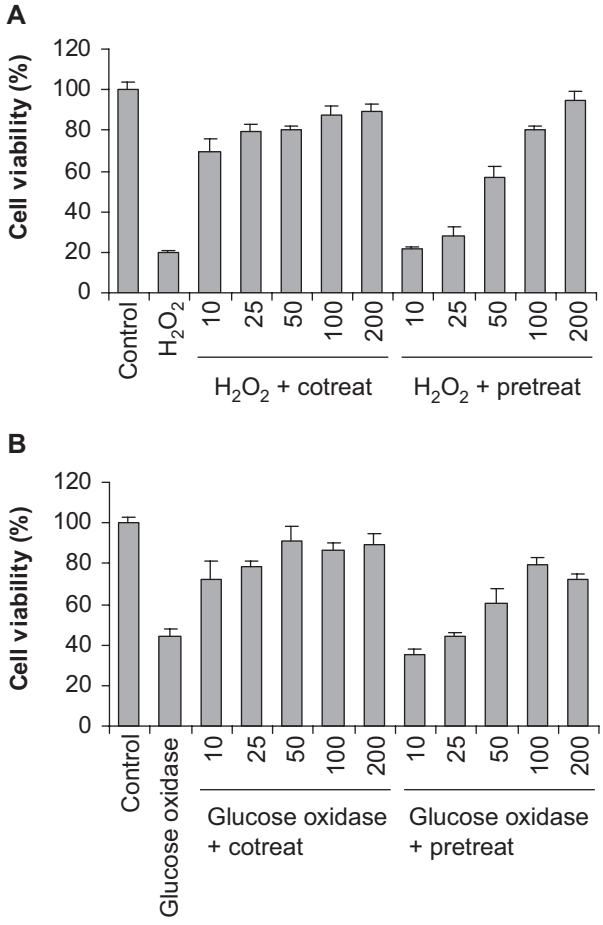


Figure 5. Effect of differential treatment of FeCe6 on $H_2O_2$-dependent cytotoxicity. (A) HUVEC cells were treated with $H_2O_2$ (0.5 mM) and the increasing amount of FeCe6 for 6 h (co-treat) or pre-treated with FeCe6 for 6 h, washed with PBS and treated with $H_2O_2$ for an additional 6 h (pre-treat). Cells were harvested and cell viability was measured by MTT assay ($n = 6$). (B) Cells were treated as in (A) except the treatment with glucose oxidase (2.5 mU/mL) instead of $H_2O_2$ ($n = 6$).

### *Inhibition of $H_2O_2$-induced apoptosis by chlorophyllins*

Given that low concentration of $H_2O_2$ is known to cause apoptotic cell death in various cell lines [35], we next examined the inhibitory role of two chlorophyllins on the low dose of $H_2O_2$-induced apoptosis. In a dose-dependent experiment, distinguished phosphatidylserine externalization, measured by the relative cells in Annexin $V^+/PI^-$ area, was observed by 6 h treatment with 50 μM and 100 μM of $H_2O_2$, but not with 250 μM and 500 μM of $H_2O_2$ in Jurkat T-cells (data not shown). As shown in Figure 6A, pre-treatment of FeCe6 significantly decreased $H_2O_2$-induced apoptotic cell death as measured by phosphatidylserine exposure (upper and lower panel). Standard antioxidant Trolox showed no effect on the $H_2O_2$-induced apoptosis, but significant inhibition was found by CHL, although to a much weaker extent, suggesting that CHL could exert an antioxidant effect against the low dose of $H_2O_2$.

To further verify the suppressive role of these two chlorophyllins, we examined their effects on $H_2O_2$-induced loss of mitochondrial membrane potential. ROS are known to cause a defect in mitochondrial function including the loss of membrane potential leading to release of apoptosis-inducing molecules such as cytochrome *c* [36]. $H_2O_2$-mediated depolarization of mitochondrial membrane was measured by using JC-1, as shown in Figure 6B. Consequently, 4 h treatment of $H_2O_2$ (100 μM) caused an obvious loss of membrane potential as red fluorescence (upper left area) shifted to green fluorescence (lower right area). The relative percentage of red fluorescence, which represents the intact mitochondrial membrane potential, was decreased by the treatment of $H_2O_2$ from 93.26% to 51.17%. Pre-treatment with FeCe6 markedly blocked the loss of membrane potential, as 90.27% of cells treated with $H_2O_2$ exhibited a red fluorescence. Interestingly, CHL also partially prevented the decrease of membrane potential by $H_2O_2$, even though it is unclear whether this inhibitory effect of CHL is dependent on its $H_2O_2$ scavenging.

### *Scavenging of $H_2O_2$ by FeCe6*

To understand this differential cytoprotective capability of CHL and FeCe6, we tested their direct $H_2O_2$

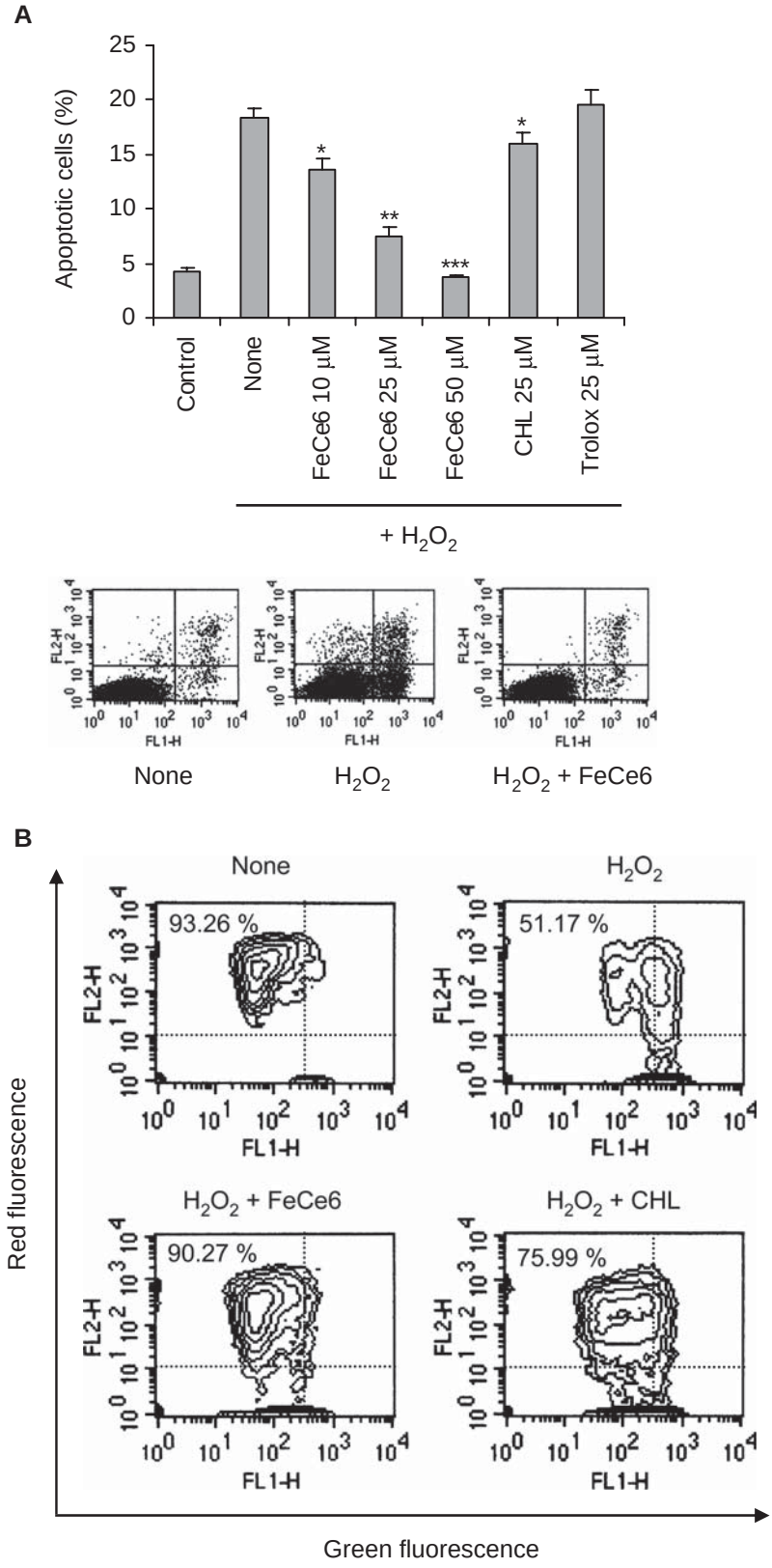


Figure 6. Inhibitory effect of FeCe6 on $H_2O_2$-induced apoptosis. (A) Jurkat T-cells were untreated (control) or treated with $H_2O_2$ (100 μM) for 6 h after pre-treatment with the indicated amount of compound (upper panel) or with FeCe6 (50 μM) for 2 h (lower right panel). Cells were then stained with Annexin V-FITC and PI and analysed by FACS flow cytometer. Apoptotic cells were presented as the relative cells in Annexin V-FITC$^+$/PI$^-$ area. Asterisks indicate significant differences from the group treated with $H_2O_2$ only ($n = 3$; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$). (B) Jurkat T-cells, pre-treated with FeCe6 or CHL (100 μM) for 2 h as indicated, were untreated (none) or treated with $H_2O_2$ (100 μM) for 4 h and mitochondrial membrane potential was measured by FACS flow cytometer after staining with JC-1. Values represent the relative cells in Red$^+$/Green$^-$ area (upper left area).

scavenging activity using two different assays. First, catalase-like activity was measured by monitoring the absorbance of $H_2O_2$ at 240 nm. As shown in Figure 7A, FeCe6 exhibited a dose-dependent $H_2O_2$ decomposing activity, while CHL did not show any effect on the decomposition of $H_2O_2$ within the range of concentrations used in our cytotoxic assay. This observation demonstrates that FeCe6, but not CHL, is capable of scavenging $H_2O_2$ directly. Specific catalase-like activity of 100 μM FeCe6 was estimated to 1.45 U.

Next, HRP-dependent phenol red oxidation assay was conducted to determine the rate constant for the reaction of chlorophyllins with $H_2O_2$. As shown in Figure 7B, both CHL and FeCe6 inhibited $H_2O_2$-mediated phenol red oxidation in a dose-dependent manner, demonstrating that the two chlorophyllins may compete with HRP for $H_2O_2$. The rate constant for FeCe6 with $H_2O_2$ was estimated to be $9.17 \times 10^5\ M^{-1}\ s^{-1}$, which was 7.3-times higher than that of CHL ($1.26 \times 10^5\ M^{-1}\ s^{-1}$). Considering that the rate constant of reaction between $Fe^{2+}$ and $H_2O_2$ to generate hydroxyl radical is $6.5 \times 10^2\ M^{-1}\ s^{-1}$ at neutral pH [37], two chlorophyllins, at least, could prevent hydroxyl radical formation from $H_2O_2$. Our results collectively demonstrated that FeCe6 is a prominent $H_2O_2$ scavenger and, notably, stronger $H_2O_2$ scavenging ability of FeCe6 may explain its potent cytoprotective effect against $H_2O_2$ toxicity.

### *Intracellular antioxidant action of FeCe6*

To verify the cytoprotective role of FeCe6 as an intracellular antioxidant, Jurkat T-cells were treated with redox-active menadione. Menadione, a synthetic quinone compound, is known to generate intracellular superoxide radical and hydrogen peroxide by redox cycling and to induce toxic damage to cellular components [38]. Menadione (50–500 μM) exhibited a marked cytotoxic effect to Jurkat T-cells after 3 h treatment (data not shown). When cells were treated with 250 μM menadione, the relative dead cell was up to 91% (Figure 8A, line b), as determined by PI uptake. Menadione-induced cell death was then effectively reduced by the pre-treatment with FeCe6 (Figure 8A, line c).

To confirm that the cytoprotection of FeCe6 resulted from its ROS scavenging activity, intracellular ROS level was measured by using a DCFH-DA as a probe. As shown in Figure 8B, menadione caused an increase in intracellular ROS (line b) and pre-treatment with FeCe6 significantly reduced intracellular oxidant level (line c). Intracellular ROS scavenging ability was also verified in a time-course experiment by fluorescent spectrophotometric assay. As shown in Figure 8C, DCF fluorescence, which represents intracellular ROS level, was constantly increased by the treatment with menadione. FeCe6 remarkably decreased menadione-induced ROS formation, indicating that cytoprotective effect of FeCe6 under oxidative stress condition is due to its scavenging of intracellular oxidant.

To provide more evidence of intracellular antioxidant action of FeCe6, TNF-α was added to Jurkat T-cells. TNF-α has been shown to induce intracellular ROS to activate transcriptional factor nuclear factor kappa B (NF-κB) signalling pathway and ROS-mediated necrotic cell death [39,40]. Treatment of TNF-α to Jurkat T-cells induced intracellular ROS formation as determined by DCF fluorescence (data not shown) and IκB phosphorylation, the essential step of NF-κB activation. We thus examined whether FeCe6 could inhibit the phosphorylation of IκB upon stimulation with TNF-α. As shown in Figure 8D,

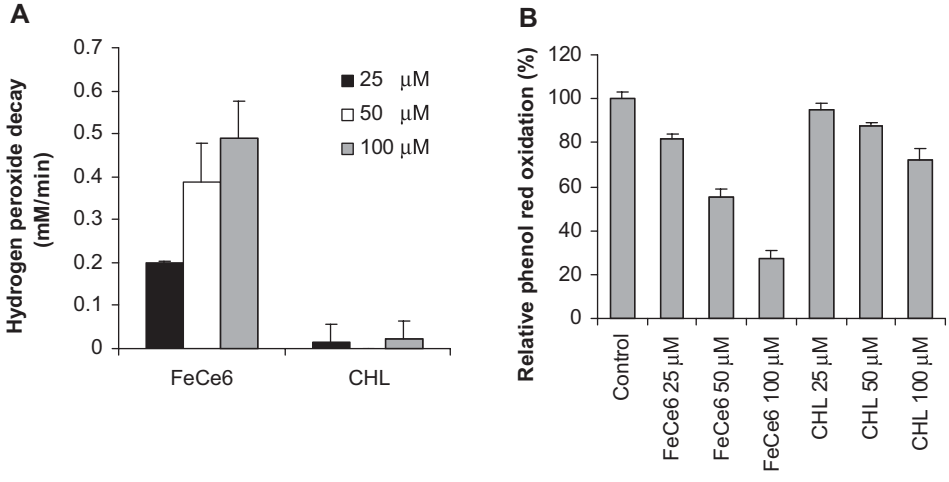


Figure 7. $H_2O_2$ scavenging activity of FeCe6. (A) Catalase-like activity of FeCe6. $H_2O_2$ was incubated with FeCe6 or CHL as indicated and the absorbance of $H_2O_2$ was monitored at 240 nm. Catalase-like activity was calculated as described in Materials and methods ($n = 3$). (B) $H_2O_2$-dependent phenol red oxidation. HRP (30 U/ml) was incubated with $H_2O_2$ (60 μM), phenol red (0.1 mg/ml) and FeCe6 or CHL for 5 min and the oxidation of phenol red was measured by the absorbance at 610 nm. Data were expressed as the relative phenol red oxidation as compared to control ($n = 3$).

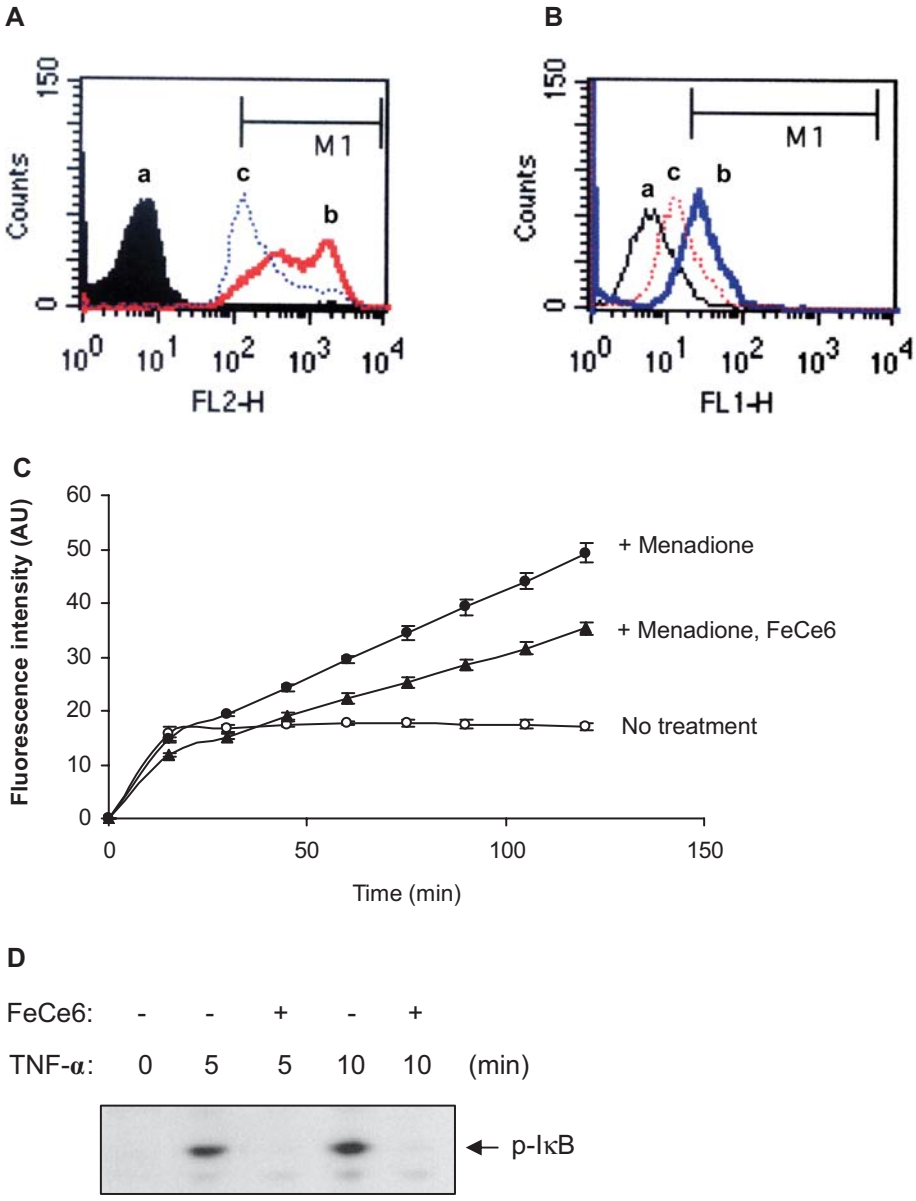


Figure 8. Intracellular antioxidant action of FeCe6. (A) Inhibition of menadione-induced cell death by FeCe6. Jurkat T-cells, pre-treated with FeCe6 (100 μM) for 1 h (line c) or buffer (line b) were untreated (line a) or treated with 0.25 mM menadione for 3 h (lines b and c) and the cell death was measured by FACS flow cytometer after staining with PI. (B) Inhibition of menadione-induced intracellular ROS by FeCe6. Jurkat T-cells, pre-treated with FeCe6 (100 μM, line c) or buffer (line b) for 1 h were loaded with DCFH-DA and treated with 0.25 mM menadione (lines b and c) for 1 h. Fluorescence of DCF was then measured by FACS flow cytometer. (C) Time-dependent formation of intracellular ROS. Jurkat T-cells were pre-treated with FeCe6 (100 μM) for 1 h and ROS level was measured in fluorescence spectrophotometer after treatment with 0.25 mM menadione ($n$ = 4). (D) Inhibition of TNF-α-induced IκB phosphorylation by FeCe6. Jurkat T-cells were pre-treated with FeCe6 (100 μM) for 1 h and treated with TNF-α (20 ng/mL) for the indicated times. Cell lysates were immunoblotted with anti-phospho-IκB antibody.

TNF-α induced a robust IκB phosphorylation after 5 min or 10 min treatment, and pre-treatment of FeCe6 clearly blocked the TNF-α-induced IκB phosphorylation, indicating that FeCe6 is capable of inhibiting TNF-α-mediated intracellular signalling pathways. Taken together, our results demonstrate that FeCe6 is able to function as a potent intracellular antioxidant to defend cellular homeostasis from inevitable oxidative stress threat.

## Discussion

ROS and oxidative stress have been implicated in the pathogenesis of many degenerative diseases [2]. As a result, there is increasing understanding for the therapeutic use of antioxidant to treat oxidative stress-mediated disease models [3,5,6]. In this regard, many putative antioxidants have been examined for their *in vitro* scavenging abilities on ROS and *in vivo* protective actions [5,7–10].

Biological functions of chlorophyll have been of particular interest because of its high content in green vegetables. As chlorophyll is water-insoluble and highly susceptible to metabolic modifications owing to its unstable structure, much effort has been paid to the stable chlorophyll derivatives [19]. Among the chlorophyll derivatives examined, chlorin-related compounds such as chlorophyllin, derived from dietary chlorophyll through metabolic modification, were found to possess antioxidant activity [11,20]. Two chlorophyllins, CHL and FeCe6, were previously proposed as superoxide and hydroxyl radical scavenger [14]. In the present study, we clearly demonstrated that CHL and FeCe6 execute multifunctional antioxidant functions to scavenge a broad range of ROS including singlet oxygen and peroxynitrite and to protect DNA breakage against peroxyl radical.

Recently, peroxynitrite has been extensively studied for its potent adverse effects on biological functions [23,29,41]. We here provide the first evidence that two chlorophyllins are able to scavenge peroxynitrite as efficient as FeTMPyP, a well-known peroxynitrite scavenger (Figure 2C). The chemistry of peroxynitrite scavenging by two chlorophyllins is still unknown. As for the manganese- or iron-porphyrins, decomposition of peroxynitrite by metalloporphyrins is involved in three different reactions, which are one electron reduction, two electron reduction and isomerization [29,31]. Like other iron porphyrins, FeCe6 may catalyse the decomposition of peroxynitrite by isomerization to produce nitrate or by one electron reduction to produce nitrogen dioxide, which potentially induce 3-nitrotyrosine formation [31]. As FeCe6 significantly reduced the formation of protein carbonyl by peroxynitrite (data not shown) and these two chlorophyllins attenuated peroxynitrite-mediated cell death of HUVEC (data not shown), we can exclude the possibility that the secondary reactive species produced during the scavenging of peroxynitrite by chlorophyllins may cause cellular damage. Although the detailed mechanism of the reaction of chlorophyllins with peroxynitrite remains to be determined, our result clearly demonstrates that they are putative antioxidant molecules against peroxynitrite. Further study will be needed to verify the cytoprotective functions of chlorophyllins as peroxynitrite scavengers.

With its high permeability to membrane, $H_2O_2$ is increasingly recognized as a key metabolite in ROS biology and a toxic intermediate in diverse pathologies in spite of its relatively low reactivity [35]. Excess $H_2O_2$ is able to modify or damage cellular components leading to the loss of cell function or cell death, while low or moderate dose of $H_2O_2$ may act as a signalling mediator to regulate diverse signalling pathways including apoptosis [34]. In this context, our results also demonstrated that $H_2O_2$ caused an evident cytotoxic effect and apoptosis in a dose-dependent fashion. Besides its potent ROS scavenging activity, FeCe6 exerted a marked protective function on $H_2O_2$-dependent cytotoxicity and apoptosis in our results. Neither standard antioxidant Trolox (data not shown) nor metal-lacking chlorophyllin, Ce6 (Figure 4B) prevented $H_2O_2$-induced cytotoxicity, indicating that FeCe6 is a very effective antioxidant to attenuate oxidative stress-mediated mammalian cell death and its cytoprotection may be due to its iron-catalysed ROS scavenging activity.

Previously, CHL has been reported to protect from $H_2O_2$- or hydroxyl radical-induced cell death in HUVEC or mouse macrophage RAW 264.7 cells [42,43]. On the contrary, our results showed that CHL exerted little cytoprotection and a very weak anti-apoptotic effect upon treatment with $H_2O_2$. In addition, CHL has been proposed to promote apoptosis in cancer cells by inhibiting extracellular signal-regulated kinases (ERKs) or activating death receptor-dependent pathway [44,45]. Several cell lines were thus treated with $H_2O_2$ to test the cell type-dependent cytoprotective role of CHL, but CHL did not confer a significant protection against $H_2O_2$-dependent cell death in our assay (Figure 4). Although intracellular actions of CHL are poorly understood, our results here demonstrate that CHL is not likely to prevent $H_2O_2$-dependent cell death.

In particular, $H_2O_2$ is known to generate highly reactive hydroxyl radical through the Fenton reaction with reduced metal such as $Fe^{2+}$ [2]. As FeCe6 exhibited excellent hydroxyl radical scavenging activity in our previous finding [14], we inferred that the protective effect of FeCe6 against $H_2O_2$ cytotoxicity may have partially resulted from its scavenging of hydroxyl radical. However, CHL failed to prevent $H_2O_2$-induced cell death in spite of its considerable hydroxyl radical scavenging ability, implying that $H_2O_2$ itself could damage cellular component or activate intracellular signalling pathways, leading to both necrotic and apoptotic cell death. In this regard, it was previously proposed that catalase-like activity of some metalloporphyrins might be responsible for their cytoprotective effects against $H_2O_2$-induced endothelial cell injury [46]. In this study, FeCe6, but not CHL, showed a dose-dependent $H_2O_2$ decomposing ability, suggesting that $H_2O_2$ scavenging capacity of FeCe6 may contribute to its potent cytoprotection against $H_2O_2$ toxicity. However, CHL also inhibited $H_2O_2$-mediated phenol red oxidation although with a much lower rate constant than FeCe6. This relatively weak $H_2O_2$ scavenging activity of CHL may explain its slight suppressive effect on the low dose of $H_2O_2$-induced apoptosis and the loss of mitochondrial membrane potential.

As the decay of metalloporphyrins in the presence of $H_2O_2$ is a critical determinant for catalytic scavenging of $H_2O_2$ [46], we examined the stability of FeCe6 by monitoring the decrease in the Soret band. After the reaction of FeCe6 with $H_2O_2$ for 5 min, the reduction

in the Soret band of FeCe6 (415 nm) was negligible compared to the decay of $H_2O_2$ (data not shown), indicating that FeCe6 is quite stable in the presence of $H_2O_2$, and could dismutate $H_2O_2$ catalytically.

At present, the molecular mechanism underlying the antioxidant function of FeCe6 against $H_2O_2$ is not fully understood. We observed a slight red shift of the Soret band of FeCe6 after reacting with $H_2O_2$ (data not shown), proposing an oxo- or hydroperoxo-ferric intermediate might be formed as previously reported in heme enzymes [47]. It is thus possible that FeCe6 may react with $H_2O_2$ to produce intermediate oxo-Fe(III)-chlorin radical and $H_2O$. To complete the catalytic cycle, oxo-Fe(II) radical may oxidize another $H_2O_2$ to form Fe(II), $O_2$ and $H_2O$. The chemical basis of $H_2O_2$ scavenging action of FeCe6 needs to be clarified in further study. The intermediate oxo-Fe(III)-chlorin may be highly reactive or could produce secondary reactive species. However, based on our earlier report, no DMPO adduct was detected after incubation of FeCe6 with $H_2O_2$ as assayed by ESR [14], while myoglobin, containing iron porphyrin prosthetic group, was shown to produce DMPO-thiyl radical after reaction with $H_2O_2$ [48]. Moreover, as FeCe6 significantly reduced the $H_2O_2$-dependent lipid peroxidation of Jurkat T-cell membrane (data not shown), we can exclude the possibility that the secondary species produced during the scavenging of $H_2O_2$ may possess adverse effects. We therefore infer that FeCe6 may catalytically dismutate $H_2O_2$ like catalase without producing any toxic substances. Future studies using electrochemical experiments including the determination of $O_2$ will be helpful to understand the peroxide scavenging action of FeCe6.

When the central $Fe^{2+}$ or $Cu^{2+}$ of chlorophyllins is released from the complex, both transition metals could generate highly reactive hydroxyl radicals through the Fenton reaction [2]. However, both Fe and Cu of chlorophyllins examined in this study were not released by metal-dechelating proteins or substances and horseradish peroxidase, while central Zn or Mg of chlorophyllin was easily dechelated in the previous report [49]. It was also shown that Fe-moiety of FeCe6 is tightly bound to chlorin ring and not released to generate hydroxyl radicals through Fenton reaction [20]. Besides its stability, FeCe6 was here demonstrated to function as a potent ROS scavenger and to exert a remarkable protective effect against $H_2O_2$-dependent cell death through its direct $H_2O_2$ dismutating ability. Furthermore, we verified the intracellular antioxidant action of FeCe6, as demonstrated by reducing menadione-induced cell death and TNF-α-mediated IκB phosphorylation. It is also possible that FeCe6 could affect the additional ROS-mediated intracellular signalling cascades. Therefore, it would be of interest to clarify the intracellular action of FeCe6 as a signalling modulator. Taken together, our results suggest that FeCe6 may be a useful therapeutic antioxidant in human diseases associated with oxidative stress based on its wide range of ROS scavenging capacity.

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