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Protective role of Coptidis Rhizoma alkaloids against peroxynitrite-induced damage to renal tubular epithelial cells

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Abstract

A study was conducted to elucidate and compare the protective activity of alkaloids from Coptidis Rhizoma (berberine, coptisine, palmatine, epiberberine, jatrorhizine, groenlandicine and magnoflorine) using an LLC-PK_1 cell under peroxynitrite ($ONOO^-$) generation model. Treatment with 3-morpholinosydnonimine (SIN-1) led to an increase in cellular $ONOO^-$ generation in comparison with non-treated cells. However, Coptidis Rhizoma extract and its alkaloids, except for berberine, reduced the cellular $ONOO^-$ level. In addition, DNA fragmentation induced by SIN-1 was significantly decreased by the extract, and also by coptisine, epiberberine, jatrorhizine, groenlandicine and magnoflorine. Moreover, treatment with berberine, coptisine, palmatine and epiberberine exerted a protective effect against G_0/G_1 phase arrest of cell cycle induced by SIN-1. The increase in cellular $ONOO^-$ generation, DNA damage and disturbance of the cell cycle by SIN-1 resulted in a decrease in cell viability. However, Coptidis Rhizoma extract, epiberberine, jatrorhizine, groenlandicine and magnoflorine significantly increased cell viability even at a concentration as low as $10\,\mu g\,mL^{-1}$. These findings demonstrate that Coptidis Rhizoma extract and its alkaloids can ameliorate the cell damage associated with $ONOO^-$ generation in renal tubular LLC-PK_1 cells, and that the various alkaloids have distinctive mechanisms of action, such as $ONOO^-$ scavenging, protection from DNA damage and control of the cell cycle. Furthermore, the data suggest that among the Coptidis Rhizoma alkaloids, coptisine is the most effective for protection against SIN-1-induced cellular injury in terms of its potency and content.

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Introduction

Peroxynitrite ($ONOO^-$), formed in-vivo from the superoxide anion (O_2^-) and nitric oxide (NO), has been suggested to be an important causative agent of pathogenic cellular damage and organ dysfunction (Radi et al 1991; Douki et al 1996). $ONOO^-$ exhibits toxic oxidative and nitrosative reactivity with biological molecules, leading to impaired function, toxicity and alterations in signalling pathways that are implicated in diverse forms of free radical-induced damage (Traystman et al 1991; Fukuyama et al 1997; Ischiropoulos 1998; Nakazawa et al 2000; Mallozzi et al 2001). Such evidence suggests that protection against $ONOO^-$ is an important defence against various pathological diseases. Therefore, antioxidant therapy to reduce the toxicity of $ONOO^-$ and its metabolites is considered to be a new avenue of therapeutic intervention that may have beneficial effects by ameliorating the damage and lesions characteristic of various pathological conditions.

The development of novel classes of safe and effective therapeutic compounds with protective activity against $ONOO^-$ is attracting much attention. In particular, traditional crude drugs and their active components have been highlighted because of their excellent pharmacological properties without side effects or toxicity. In previous studies, we have found that Coptidis Rhizoma, widely used as a traditional medicine for treating diarrhoea and gastrointestinal disorders, ameliorated renal oxidative injury under both in-vivo and in-vitro experimental conditions as an antioxidant (Yokozawa et al 1999, 2004). Coptidis Rhizoma extract effectively scavenged the NO radical in-vitro, and in an

animal model of $ONOO^-$ generation, the extract attenuated the renal damage induced by $ONOO^-$ by scavenging $ONOO^-$ and strengthening the antioxidative defence system. The protective role of Coptidis Rhizoma against $ONOO^-$ was attributable mainly to its component alkaloids – berberine, palmatine and coptisine. However, the protective potency of Coptidis Rhizoma alkaloids and their mechanisms of action against $ONOO^-$ remain unclear. Therefore, this study was conducted to elucidate and compare the protective activity of alkaloids from Coptidis Rhizoma against $ONOO^-$ using a cellular $ONOO^-$ generation model involving LLC-PK_1 cells, which are proximal tubule cells known to be susceptible to free radicals.

Materials and Methods

Reagents

Ethylenediamine tetraacetic acid (EDTA), calcium- and magnesium-free phosphate-buffered saline (PBS), Tris-HCl buffer, Triton X-100, trichloroacetic acid (TCA), acetic acid, diphenylamine, sulfuric acid, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F-12) and fetal bovine serum (FBS) were purchased from Life Technologies Inc. (Grand Island, NY). 3-Morpholinosydnonimine (SIN-1) and dihydrorhodamine-123 (DHR-123) were obtained from Sigma Chemical Co. (St Louis, MO). Propidium iodide (PI), trypsin solution and ribonuclease A (RNase) were purchased from Molecular Probes (Eugene, OR), Nakarai (Kyoto, Japan) and Funakoshi (Tokyo, Japan), respectively.

Apparatus

Melting points were measured on a Yanaco micro melting point apparatus and were uncorrected. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. NMR spectra were recorded in CD_3OD on a JEOL ECA-500 or A-400 spectrometer. Chemical shifts are reported as δ (ppm) with tetramethylsilane (TMS) as the internal standard. Column chromatography was performed with MCI-gel CHP 20P (75–150 μm; Mitsubishi Chemical Industries Ltd), Dia-ion HP-20 (Mitsubishi Chemical Industries Ltd), YMC-gel ODS-A120 (230–400 mesh; YMC Co. Ltd) and Toyopearl HW40F (45 μm; TOSOH Corporation). TLC was performed on pre-coated Kieselgel 60 F_{254} plates (0.2 mm, Merck).

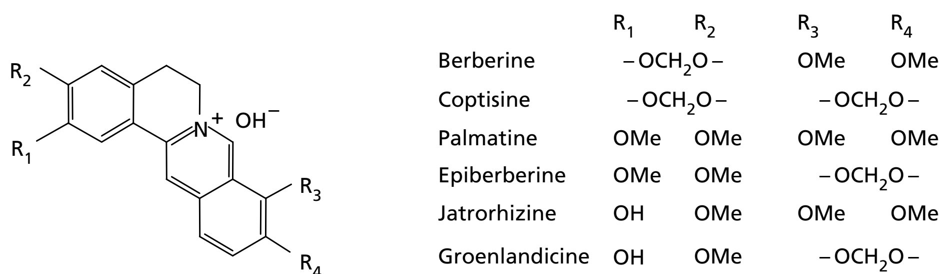
Preparation of Coptidis Rhizoma extract

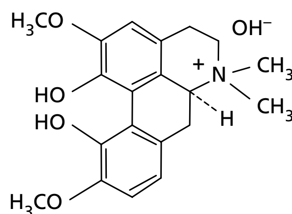
To prepare the extract, Coptidis Rhizoma (*Coptis chinensis* Franch) (100 g), purchased from Uchida Wakan-yaku Co. Ltd (Tokyo, Japan), was cut into small pieces and boiled gently in 1 L of water for 1 h. After filtration, the solution was evaporated under reduced pressure to give an extract at a yield of 20.3%.

Isolation of alkaloids

Coptidis Rhizoma (1.5 kg) was cut into small pieces and extracted with methanol (MeOH, 2.2 L × 10) at room temperature. The combined extract was concentrated under reduced pressure to give 390 g of residue. The extract residue was suspended in distilled water (2 L), and the solution was shaken three times with ethyl acetate (EtOAc, 1 L). The aqueous layer was subsequently applied to a Dia-ion HP 20P column. Elution with water yielded a fraction containing sugars. Further elution with 50% MeOH, MeOH and 2% acetic acid (AcOH)–MeOH successively gave three fractions (fractions I (148 g), II (103 g) and III (2.7 g)). Fraction II was subsequently subjected to MCI-gel CHP 20P chromatography with water containing increasing amounts of MeOH (1:0→0:1) to yield three fractions (II-1 to II-3). Fraction II-2 was further fractionated on MCI-gel CHP 20P (2% AcOH–MeOH (1:0→0:1)) into three fractions (II-2.1 to II-2.3). Fractions II-2.1 (8.3 g) and II-2.2 (4.6 g) were individually chromatographed repeatedly on YMC-gel ODS (2% AcOH–MeOH (1:0→0:1)) to yield magnoflorine (920 mg) and groenlandicine (560 mg). Fraction II-2.3 (22.8 g) was subsequently chromatographed on Toyopearl HW40F (2% AcOH–MeOH (1:0→0:1)) to give a further three fractions (II-2.3.1 to II-2.3.3). Repeated chromatography of II-2.3.3 (8.2 g) on MCI-gel CHP 20P (MeOH–H_2O–AcOH (70:30:2)), followed by crystallization with MeOH, afforded coptisine (290 mg). Fraction II-2.3.3 (8.2 g) was repeatedly chromatographed on MCI-gel CHP 20P (MeOH–H_2O–AcOH (60:40:2)) and YMC-gel ODS (0.2% trifluoroacetic acid (TFA)–MeOH (1:0→0:1)), and then crystallized to yield epiberberine (420 mg, from acetonitrile), jatrorhizine (1.3 g, from ethanol (EtOH)), palmatine (510 mg, from EtOH) and berberine (2.3 g, from MeOH). All the compounds (Figure 1) were identified using authentic samples or by comparison of their physical and spectral data with those described in the literature (Hussain et al 1989; Mizuno et al 1992).

Selected spectroscopic data for alkaloids isolated from Coptidis Rhizome are as follows. Groenlandicine: red powder (from EtOH); mp 186–190°C (decomp); ^{1}H NMR (400 MHz, CD_3OD) δ: 3.19 (1H, t, $J = 6$ Hz, H-5), 4.01 (3H, s, 3-OCH_3), 4.87 (1H, t, $J = 6$ Hz, H-6), 6.45 (2H, s, -O-CH_2-O-), 6.84 (1H, s, H-4), 7.61 (1H, s, H-1), 7.85 (2H, s, H-11 and -12), 8.76 (1H, s, H-13); ^{13}C NMR (100 MHz, CD_3OD) δ: 28.5 (C-5), 57.9 (OCH_3), 58.3 (C-6), 107.0 (-O-CH_2-O-), 110.9 (C-1), 114.5 (C-8a), 116.9 (C-4), 120.2 (C-13b), 122.7 (C-13), 123.1 (C-11), 123.8 (C-12), 131.1 (C-4a), 135.6 (C-12a), 140.5 (C-13a), 145.9 (C-8), 146.5 (C-9), 149.8 (C-10), 150.6 (C-2), 152.9 (C-3). Coptisine: yellow needles (from MeOH); mp > 300°C; ^{1}H NMR (500 MHz, CD_3OD) δ: 3.27 (1H, t, $J = 6.5$ Hz, H-5), 4.89 (1H, t, $J = 6.5$ Hz, H-6), 6.10, 6.46 (each 2H, s, -O-CH_2-O-), 6.95 (1H, s, H-4), 7.64(1H, s, H-1), 7.87 (2H, s, H-11 and -12), 8.72 (1H, s, H-13); ^{13}C NMR (125 MHz, CD_3OD) δ: 29.1 (C-5), 58.2 (C-6), 104.6, 107.1 (-O-CH_2-O-), 107.4 (C-1), 110.3 (C-4), 114.7 (C-13b), 122.9 (C-8a), 123.3 (C-12), 123.5 (C-13), 124.1 (C-11), 132.7 (C-4a), 135.4 (C-12a), 140 (C-13a), 146.2 (C-8), 146.7 (C-9), 150.3 (C-2), 151 (C-10), 153.2 (C-3).





Magnoflorine

Figure 1 Chemical structures of alkaloids extracted from Coptidis Rhizoma.

Cell culture

The porcine kidney cell line LLC-PK_1 was maintained on culture plates containing 5% FBS-supplemented DMEM/F-12 medium (pH 7.2) at 37°C in a humidified atmosphere of 5% CO_2 in air. All subsequent procedures were carried out under these conditions. The cells were sub-cultured weekly with 0.05% trypsin-EDTA in PBS. To measure $ONOO^-$ formation and the DNA fragmentation rate, LLC-PK_1 cells were seeded at a density of 1×10^5 cells per well in 24-well culture plates and incubated for 48 h. To determine cell viability, the same number of cells were seeded in 96-well plates and incubated for 2 h to enable them to adhere, whereas 5×10^5 cells per well were seeded in 6-well culture plates and incubated for 48 h to observe the cell cycle distribution. Subsequently, the cells were treated with 10 or 25 $\mu g\,mL^{-1}$ of sample together with 0.8 mM SIN-1 for 24 h. The concentration of samples was determined on the basis of a preliminary investigation on their cytotoxicity.

Cellular $ONOO^-$ formation

$ONOO^-$-dependent oxidation of DHR-123 to rhodamine-123 was measured on the basis of the method described by Haddad et al (1994). DHR-123 was added to each 24-well plate (to produce a concentration of 1.25 μM in each well) and incubated at 37°C, after which the absorbance at 500 nm of rhodamine-123 in the medium was measured.

DNA fragmentation assay

According to the method of Sellins & Cohen (1987), the cells were lysed in an ice-cold hypotonic lysis buffer (10 mM Tris-HCl buffer, 1 mM EDTA, pH 7.5) containing 0.2% Triton X-100 and centrifuged for 10 min at 13 000 *g*. After that, 500 μL of 12.5% TCA was added and each mixture was incubated for 24 h at 4°C and then centrifuged at 13 000 *g* for 5 min. The resulting pellet was dissolved in 80 μL of 5% TCA, heated at 90°C for 10 min, then 100 μL diphenylamine solution (a mixture of AcOH, diphenylamine and sulfuric acid, 1:15:0.15 v/v) was added and the mixture was kept at 25–30°C overnight in the dark. The amount of DNA was determined colorimetrically at 600 nm by the method of Burton (1956). The DNA fragmentation rate, expressed as a percentage, was calculated as the ratio of the amount of DNA in the supernatant to the total amount of DNA in the pellet and supernatant.

Flow-cytometric cell cycle analysis

Cultured LLC-PK_1 cells in 6-well plates were collected by centrifugation, fixed for at least 30 min at 4°C in 3 mL of 70% ice-cold EtOH, washed twice with PBS, incubated with RNase solutions (1 $mg\,mL^{-1}$ in PBS) for 30 min at 37°C and then treated with PI solution (1 $mg\,mL^{-1}$ in PBS) at 4°C for 30 min. The cells were transferred to analysis tubes on ice and then analysed on a FACSCalibur flow cytometer (Becton Dickinson, CA) with laser excitation at 488 nm using a 639 nm band pass filter to collect the red PI fluorescence. The percentages of cells at various phases of the cell cycle, namely G_0/G_1, S and G_2/M, were assessed using ModFit LT software (Verity Software House, Topsham, ME) from the analysis data (Block et al 1987; Ho et al 2000).

Cell viability

Cell viability was assessed using the MTT colorimetric assay (Mosmann 1983). MTT solution (50 μL, 1 $mg\,mL^{-1}$) was

added to each 96-well culture plate and incubated for 4 h at 37°C and then the medium containing MTT was removed. The incorporated formazan crystals in the viable cells were solubilized with 100 μL DMSO and the absorbance at 540 nm of each well was read using a microplate reader (Model 3550-UV; Bio-Rad, Tokyo, Japan).

Statistics

The results for each group are expressed as mean values ± s.e. (n = 5/group). The effect on each parameter was examined using the one-way analysis of variance. Differences among groups were evaluated by Dunnett's test and those at $P < 0.05$ were considered to be statistically significant.

Results

Cellular $ONOO^-$ formation

Table 1 shows the effect of Coptidis Rhizoma extract and its alkaloids on $ONOO^-$ formation in renal tubular LLC-PK_1 cells. Treatment of LLC-PK_1 cells with 0.8 mM SIN-1 for 24 h led to an increase in $ONOO^-$ formation compared with non-treated normal cells. However, treatment with Coptidis Rhizoma extract and its alkaloids, except for berberine, significantly decreased the cellular $ONOO^-$ formation. Coptisine and groenlandicine exerted stronger inhibitory effects on cellular $ONOO^-$ formation than any of the other alkaloids or the extract. On the other hand, berberine-treated LLC-PK_1 cells did not show any significant decrease in cellular $ONOO^-$ formation.

DNA fragmentation

DNA fragmentation in 0.8 mM SIN-1-treated LLC-PK_1 cells was significantly increased compared with that in non-treated normal cells (Table 2). However, cells treated with Coptidis Rhizoma extract, coptisine, jatrorhizine, groenlandicine or magnoflorine showed a significant decrease in DNA fragmentation compared with SIN-1-treated control cells. Among the alkaloids, jatrorhizine, groenlandicine and magnoflorine showed the strongest protective effect against DNA damage, exceeding that of the Coptidis Rhizoma crude extract.

Table 1 $ONOO^-$ in media treated with Coptidis Rhizoma extract and its alkaloids together with SIN-1

Material	$ONOO^-$ formation (% compared with SIN-1-treatment values)
None	10.0 ± 2.3
SIN-1 (0.8 mM)	100.0 ± 13.4#
Extract (10 μg mL^{-1}) plus SIN-1 (0.8 mM)	68.4 ± 6.5#,***
Extract (25 μg mL^{-1}) plus SIN-1 (0.8 mM)	50.4 ± 6.3#,***
Berberine (10 μg mL^{-1}) plus SIN-1 (0.8 mM)	123.2 ± 6.5#,**
Berberine (25 μg mL^{-1}) plus SIN-1 (0.8 mM)	113.9 ± 7.3#
Coptisine (10 μg mL^{-1}) plus SIN-1 (0.8 mM)	48.5 ± 7.3#,***
Coptisine (25 μg mL^{-1}) plus SIN-1 (0.8 mM)	25.6 ± 4.0#,***
Palmatine (10 μg mL^{-1}) plus SIN-1 (0.8 mM)	85.3 ± 7.2#
Palmatine (25 μg mL^{-1}) plus SIN-1 (0.8 mM)	66.9 ± 8.0#,***
Epiberberine (10 μg mL^{-1}) plus SIN-1 (0.8 mM)	87.5 ± 10.1#
Epiberberine (25 μg mL^{-1}) plus SIN-1 (0.8 mM)	77.8 ± 9.2#,**
Jatrorhizine (10 μg mL^{-1}) plus SIN-1 (0.8 mM)	96.4 ± 8.3#
Jatrorhizine (25 μg mL^{-1}) plus SIN-1 (0.8 mM)	63.9 ± 9.4#,***
Groenlandicine (10 μg mL^{-1}) plus SIN-1 (0.8 mM)	30.6 ± 1.7#,***
Groenlandicine (25 μg mL^{-1}) plus SIN-1 (0.8 mM)	34.5 ± 2.6#,***
Magnoflorine (10 μg mL^{-1}) plus SIN-1 (0.8 mM)	83.3 ± 4.4#,*
Magnoflorine (25 μg mL^{-1}) plus SIN-1 (0.8 mM)	45.3 ± 4.4#,***

Data are means ± s.e., n = 5. #$P < 0.001$ compared with non-treatment values; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with SIN-1-treatment values.

Table 2 DNA fragmentation in LLC-PK_1 cells treated with Coptidis Rhizoma extract and its alkaloids together with SIN-1

Material	DNA fragmentation (% compared with SIN-1-treatment values)
None	55.4 ± 3.8
SIN-1 (0.8 mM)	100.0 ± 17.1###
Extract (10 μg mL^{-1}) plus SIN-1 (0.8 mM)	67.9 ± 4.0***
Extract (25 μg mL^{-1}) plus SIN-1 (0.8 mM)	65.1 ± 4.6***
Berberine (10 μg mL^{-1}) plus SIN-1 (0.8 mM)	97.6 ± 1.6###
Berberine (25 μg mL^{-1}) plus SIN-1 (0.8 mM)	82.0 ± 6.5##
Coptisine (10 μg mL^{-1}) plus SIN-1 (0.8 mM)	63.9 ± 3.6***
Coptisine (25 μg mL^{-1}) plus SIN-1 (0.8 mM)	68.3 ± 1.3***
Palmatine (10 μg mL^{-1}) plus SIN-1 (0.8 mM)	82.3 ± 0.4###,*
Palmatine (25 μg mL^{-1}) plus SIN-1 (0.8 mM)	85.5 ± 1.6###
Epiberberine (10 μg mL^{-1}) plus SIN-1 (0.8 mM)	72.8 ± 8.4#,**
Epiberberine (25 μg mL^{-1}) plus SIN-1 (0.8 mM)	70.5 ± 3.7***
Jatrorhizine (10 μg mL^{-1}) plus SIN-1 (0.8 mM)	43.3 ± 8.7***
Jatrorhizine (25 μg mL^{-1}) plus SIN-1 (0.8 mM)	38.8 ± 5.1***
Groenlandicine (10 μg mL^{-1}) plus SIN-1 (0.8 mM)	59.1 ± 0.4***
Groenlandicine (25 μg mL^{-1}) plus SIN-1 (0.8 mM)	60.2 ± 3.3***
Magnoflorine (10 μg mL^{-1}) plus SIN-1 (0.8 mM)	57.2 ± 4.4***
Magnoflorine (25 μg mL^{-1}) plus SIN-1 (0.8 mM)	52.1 ± 4.1***

Data are means ± s.e., n = 5. #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ compared with non-treatment values; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with SIN-1-treatment values.

Cell cycle

Table 3 shows the effects of Coptidis Rhizoma extract and its alkaloids on the cell cycle disturbance induced by SIN-1. After treatment with SIN-1, the proportion of LLC-PK_1 cells in G_0/G_1 phase increased significantly from 52.9% to 62.8% and that of cells in S phase decreased significantly from 36.2% to 24.2% compared with non-treated normal cells. Treatment with Coptidis Rhizoma extract decreased the proportion of cells in G_0/G_1 phase from 62.8% to 49.8% and increased that of cells in S phase from 24.2% to 35.1%. In addition, berberine, coptisine, palmatine and epiberberine showed protective effects against G_0/G_1 phase arrest by SIN-1. On the other hand, treatment with groenlandicine and magnoflorine resulted in arrest in G_2/M phase. However, cells treated with jatrorhizine showed no difference in cell cycle distribution from SIN-1-treated control cells.

Cell viability

The viability of renal tubular LLC-PK_1 cells was decreased significantly to 25% of the normal value by treatment with 0.8 mM SIN-1 for 24 h (Figure 2). However, LLC-PK_1 cells treated with 10 and 25 $\mu g\,mL^{-1}$ Coptidis Rhizoma extract together with SIN-1 showed significantly increased viability. In addition, cells treated with epiberberine, jatrorhizine, groenlandicine and magnoflorine at a low concentration of 10 $\mu g\,mL^{-1}$ showed increased viability, and coptisine and palmatine increased cell viability significantly at the higher concentration of 25 $\mu g\,mL^{-1}$. Jatrorhizine and groenlandicine showed the highest potency in protecting against SIN-1-induced cell death.

Discussion

Coptidis Rhizoma has long been considered to have antiphlogistic, sedative, antidotal, haemostatic, stomachic and anti-tumour properties, and it has been used as a therapeutic agent for gastrointestinal disorders, infectious or inflammatory diseases, and as an anti-tumour agent. Our previous study also indicated that Coptidis Rhizoma had a protective effect against renal ischaemia–reperfusion injury, reducing the tissue damage caused by oxidative stress, and thus ameliorating renal functional impairment (Cho et al 2004). In addition, Coptidis Rhizoma extract was shown to provide protection against $ONOO^-$-induced oxidative damage in an in-vivo system, exhibiting NO, O_2^- and $ONOO^-$ scavenging activity (Yokozawa et al 2004). The alkaloid-rich fraction from Coptidis Rhizoma extract showed the strongest protective effect against $ONOO^-$-induced renal disorder caused by free-radical generation. It was suggested that the protective activity of Coptidis Rhizoma against $ONOO^-$ is mainly due to its alkaloid components. Although it remains unclear which of the Coptidis Rhizoma components exhibits pharmacological activity, its alkaloids berberine, coptisine, palmatine and jatrorhizine have been suggested to be active (Otsuka et al 1974; Peng et al 1997; Schmeller et al 1997). Therefore, this study was carried out to verify which of the alkaloids are active against $ONOO^-$-induced oxidative damage.

Several studies have provided evidence that renal tubule obstruction plays an important role in acute ischaemic renal injury, and it is well known that renal tubular cells, including LLC-PK_1 renal proximal tubule cells, are susceptible to free radicals (Gobe et al 1999; Schena et al 2001). Therefore, an in-vitro model of oxidative damage, in which LLC-PK_1 cells are exposed to free radicals, would appear useful for searching for agents that can provide effective protection. To study in detail the alkaloid components of Coptidis Rhizoma extract that are effective against renal disorders, we investigated the effect of Coptidis Rhizoma extract and its components on the damage to renal tubular LLC-PK_1 cells induced by SIN-1.

SIN-1 generates both NO and O_2^-, which then react rapidly to form $ONOO^-$. SIN-1 has been reported to significantly and consistently decrease the adhesion of

Table 3 LLC-PK_1 cell cycle treated with Coptidis Rhizoma extract and its alkaloids together with SIN-1

Material	Percentage of cells in each phase of cell cycle (%)		
	G_0/G_1	S	G_2/M
None	52.9 ± 2.1	36.2 ± 1.5	14.2 ± 0.6
SIN-1 (0.8 mM)	62.8 ± 2.1##	24.2 ± 2.8##	15.1 ± 2.3
Extract (25 $\mu g\,mL^{-1}$) plus SIN-1 (0.8 mM)	49.8 ± 3.5**	35.1 ± 3.6**	17.1 ± 2.7
Berberine (25 $\mu g\,mL^{-1}$) plus SIN-1 (0.8 mM)	53.1 ± 1.6**	31.8 ± 1.6**	15.2 ± 1.9
Coptisine (25 $\mu g\,mL^{-1}$) plus SIN-1 (0.8 mM)	52.3 ± 0.8**	37.0 ± 0.7**	12.8 ± 0.2
Palmatine (25 $\mu g\,mL^{-1}$) plus SIN-1 (0.8 mM)	48.8 ± 1.8**	38.1 ± 1.0**	15.1 ± 1.6
Magnoflorine (25 $\mu g\,mL^{-1}$) plus SIN-1 (0.8 mM)	58.7 ± 4.4##	25.3 ± 6.4#	21.3 ± 0.2##,**
Epiberberine (25 $\mu g\,mL^{-1}$) plus SIN-1 (0.8 mM)	56.4 ± 2.0*	20.2 ± 5.6##	25.4 ± 3.9##,**
Groenlandicine (25 $\mu g\,mL^{-1}$) plus SIN-1 (0.8 mM)	61.0 ± 0.8##	18.3 ± 1.2##	22.7 ± 1.1##,**
Jatrorhizine (25 $\mu g\,mL^{-1}$) plus SIN-1 (0.8 mM)	59.7 ± 1.1##	27.6 ± 3.9#	18.0 ± 2.1

Data are means ± s.e., n = 5. #$P < 0.01$, ##$P < 0.001$ compared with non-treatment values; *$P < 0.01$, **$P < 0.001$ compared with SIN-1-treatment values.

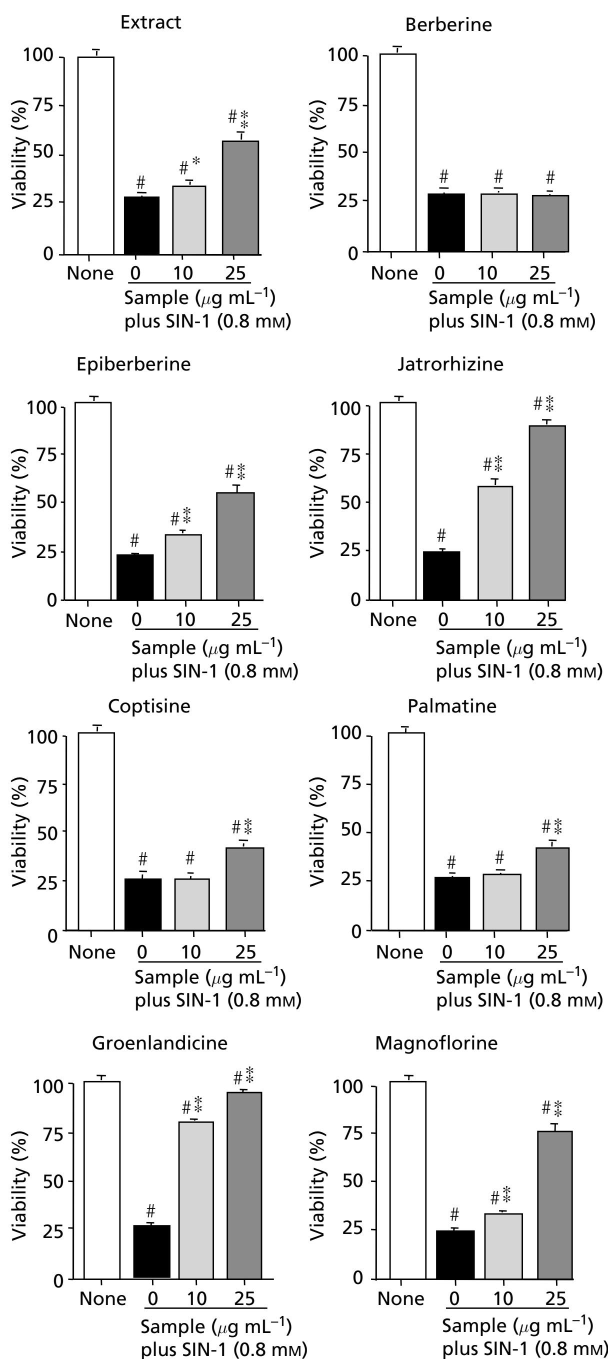


Figure 2 Viability of $LLC\text{-}PK_1$ cells treated with Coptidis Rhizoma extract and its alkaloids (10 and 25 $\mu g\,mL^{-1}$) together with 0.8 mM SIN-1. Data are means ± s.e., n = 5. #$P < 0.001$ compared with non-treatment values; *$P < 0.01$, **$P < 0.001$ compared with SIN-1-treatment values.

the renal tubular epithelial cell line $LLC\text{-}PK_1$ (Kelm et al 1997; Wangsiripaisan et al 1999). These results also confirmed that treatment of cells with SIN-1 led to generation of $ONOO^-$, induction of DNA damage, disturbance of the cell cycle and down-regulation of cell proliferation. Because measurement of $ONOO^-$ formation is difficult, the yield of rhodamine-123 in response to extracellular addition of SIN-1 is generally measured (Haddad et al 1994). Our study showed that SIN-1 induced markedly higher formation of $ONOO^-$ in $LLC\text{-}PK_1$ cells. However, treatment with Coptidis Rhizoma extract and alkaloids, except for berberine, inhibited the increase in $ONOO^-$ formation significantly, suggesting that these agents would ameliorate the cellular damage caused by excessive $ONOO^-$ formation. Among the alkaloids, coptisine and groenlandicine scavenged $ONOO^-$ most efficiently.

Several mechanisms of $ONOO^-$ toxicity in the pathogenesis of tissue injury have been suggested. $ONOO^-$ is thought to play a role in induction of DNA damage, inhibition of DNA repair and induction of cell death via apoptosis or necrosis (Lin et al 1995; Sandoval et al 1997; Patel et al 1999; Doulias et al 2001). In addition, it has been demonstrated that DNA damage and tubule cell death are caused by reactive oxygen species or reactive nitrogen species under pathological conditions of renal injury. Therefore, protection against DNA damage is essential for preventing renal injury. Our study showed that DNA fragmentation in $LLC\text{-}PK_1$ cells was increased by SIN-1 treatment, whereas DNA fragmentation was inhibited in cells treated with Coptidis Rhizoma extract. Among the component alkaloids, coptisine, jatrorhizine, groenlandicine and magnoflorine showed especially strong inhibition of $ONOO^-$ generation, and inhibited DNA fragmentation efficiently. These results suggest that Coptidis Rhizoma extract and its alkaloids would prevent DNA damage induced by SIN-1.

G_1 arrest and G_2 checkpoints in the cell cycle are crucial points for the detection of DNA damage, DNA repair and induction of apoptosis (Bonfoco et al 1995; Lin et al 1995; Sandoval et al 1997; Li et al 1998; Patel et al 1999; Doulias et al 2001). Consistent with other reports that cells damaged by free radicals show G_0/G_1 or G_2/M arrest, or both, our study demonstrated G_0/G_1 arrest in $LLC\text{-}PK_1$ cells after treatment with SIN-1. However, cells treated with Coptidis Rhizoma extract, berberine, coptisine, palmatine and epiberberine showed a near-normal cell cycle distribution through inhibition of G_0/G_1 arrest. Regulation of the cell cycle is of obvious importance for the proper structure and function of a biological system. These results support the protective effect of Coptidis Rhizoma extract and its alkaloids on cell cycle regulation. On the other hand, the cells treated with groenlandicine revealed a similar distribution of cell cycle to SIN-1 treated control cells, although they showed the strong protective effect from $ONOO^-$. Mechanisms of protection against biological damage also include apoptosis and DNA repair pathways. Therefore, the protection by groenlandicine from $ONOO^-$ is probably related to cellular protective mechanisms other than cell cycle control.

Excessive generation of $ONOO^-$, and the resulting damage, finally results in cell loss (Poot et al 1988, Poot 1991). Our investigation also showed that the viability of $LLC\text{-}PK_1$ cells was reduced by the oxidative damage induced by SIN-1. However, treatment with Coptidis Rhizoma extract improved cell viability by protecting against SIN-1-induced oxidative damage. The protective

effect was attributed to decreases in $ONOO^-$ formation and DNA fragmentation, and control of the cell cycle through inhibition of G_0/G_1 arrest. In addition, jatrorhizine, groenlandicine and magnoflorine also increased cell viability by decreasing the extent of $ONOO^-$ formation and DNA damage.

This study also suggests a relationship between the structure of the alkaloids and the protective activity from $ONOO^-$. Coptisine and groenlandicine contain methylene-dioxy group at the C-9 and C-10 positions, suggesting that the presence of the methylene-dioxy group at the C-9 and C-10 positions play an important role for exhibiting $ONOO^-$ scavenging activity. In contrast, berberine and epiberberine were shown to be completely inactive and very weak compounds, respectively, though they also possess a methylene-dioxy group. Since berberine and epiberberine contain two methoxy groups, together with a methylene-dioxy group, it was suggested that the presence of both of these groups reduces the activity. In addition, moderate $ONOO^-$ scavenging activity was found in magnoflorine and jatrorhizine, which have phenolic hydroxyl group(s). Groenlandicine also possesses a phenolic hydroxyl group at the C-2 position, and was considered to demonstrate the most potent activity. Therefore, it was implied that the phenolic hydroxyl group also contributes to the $ONOO^-$ scavenging potency.

The major alkaloids present in Coptidis Rhizoma, such as berberine, coptisine and palmatine, have been reported to have various biological actions, including antimicrobial, antimalarial and antifungal activity (Thakur & Srivastava 1982; Iwasa et al 1996). In addition, these alkaloids exhibit a wide variety of pharmacological effects via their inhibition of enzymes and their interaction with proteins (Lee & Kim 1996; Schmeller et al 1997; Kong et al 2001). Among these alkaloids, berberine and coptisine have been reported to exert antinephritic effects through their antiplatelet action and improve renal haemodynamics by mediating prostanoid synthesis (Hattori et al 1992). In particular, of its several types of biological activity, interest has been focused on the antioxidative potential of berberine (Chun et al 1979; Rabbani et al 1987; Marin-Neto et al 1988; Jin et al 2000; Choi et al 2001). In our study, although treatment with berberine inhibited the G_0/G_1 phase arrest induced by SIN-1, it failed to exert the protective effect against excessive $ONOO^-$ generation and its related cellular loss. On the other hand, we previously reported that berberine exerted protective activity against $ONOO^-$-induced renal failure in an in-vivo model (Yokozawa et al 2004). We consider that the inconsistency in results between cellular and in-vivo systems may be related to the metabolism of berberine, although the specific mechanisms are unclear and need to be elucidated.

This study has demonstrated that Coptidis Rhizoma extract ameliorates the cellular damage associated with $ONOO^-$ formation in renal tubular LLC-PK_1 cells, and that the protective activity is attributed to alkaloid compounds, such as coptisine, epiberberine, jatrorhizine, groenlandicine and magnoflorine. Furthermore, the respective alkaloids showed distinctive mechanisms of protection against $ONOO^-$-induced cellular damage, such as $ONOO^-$ scavenging, protection against DNA damage and control of the cell cycle. Coptidis Rhizoma extract is composed of 20.8% berberine, 6.1% coptisine and 5.2% palmatine (data not shown). As coptisine appears to be the second most abundant component of Coptidis Rhizoma after berberine, our findings suggest that coptisine is the major compound responsible for protection against SIN-1-induced injury to renal epithelial cells. These data indicate that Coptidis Rhizoma alkaloids might be useful for development as pharmacological agents for the treatment of disorders related to oxidative stress.

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