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Protection by the Chinese prescription Wen-Pi-Tang against renal tubular LLC-PK$_1$ cell damage induced by 3-morpholinosydnonimine

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Abstract

We investigated the effects of Wen-Pi-Tang extract on the protective mechanisms of renal tubular LLC-PK$_1$ cells, as renal tubular cells are the most vulnerable renal tissue to oxidative stress. Exposure to 800 μM 3-morpholinosydnonimine (SIN-1) resulted in a marked increase in cellular peroxynitrite ($ONOO^-$), which converted nonfluorescent dihydrorhodamine 123 to fluorescent rhodamine 123, a detectable probe for the long-lived $ONOO^-$. In addition, it resulted in apoptotic cell death, assessed by a DNA fragmentation assay. However, treatment with Wen-Pi-Tang extract, at concentrations of 50 and 100 μg mL^{-1} together with SIN-1 protected renal tubular cells against $ONOO^-$ through scavenging $ONOO^-$ and inhibiting apoptotic cell death in a dose-dependent manner. Moreover, treatment with Wen-Pi-Tang extract both before and after exposure to SIN-1 was also protective: it reduced cellular $ONOO^-$ levels, increased cell viability and decreased the DNA fragmentation rate. These results suggest that Wen-Pi-Tang would have protective activity against $ONOO^-$-induced renal tubular injury through the inhibition of $ONOO^-$ production and apoptotic cell death by both preventing and treating renal injury. Furthermore, morphological characteristics of apoptosis were observed in SIN-1 treated tubular cells, while the addition of Wen-Pi-Tang extract with SIN-1 attenuated these morphological changes. $ONOO^-$ generated by SIN-1 also disturbed the cell cycle by decreasing the cellular G_2/M phase ratio, while Wen-Pi-Tang extract regulated the cell cycle by G_2/M phase arrest.

Introduction

Progressive acute and chronic renal diseases are characterized by impairment of glomerular and tubular function, and cytotoxic free radicals have been identified as important pathological mediators in impaired renal function (Remuzzi et al 1997; Jörres 2002). In particular, the actual site of generation of reactive oxygen and/or nitrogen species that have been implicated in the initiation and development of renal diseases is known to be the tubular cells and such generation results in the eventual injury and death of renal cells (Lash & Tokarz 1990; Yaqoob et al 1993, 1994). Since the functional changes of tubular cells mediated by free radicals are essentially related to renal injury, protection of renal tubular cells against free radical damage may ameliorate renal injury and dysfunction.

Peroxynitrite ($ONOO^-$), a strong oxidant formed via the reaction of nitric oxide (NO) and superoxide (O_2^-), has been suggested to be cytotoxic itself and to decompose into other toxic species, such as the hydroxyl radical (·OH) (Beckman & Koppenol 1996). Among the reactive oxygen and/or nitrogen species that are generated in response to pathological situations, the overproduction or uncontrolled formation of the potent toxic radical $ONOO^-$ was identified, indicating that it was an important factor in tissue damage, including the kidney. Specifically in the kidney, $ONOO^-$ generation has been implicated in the pathophysiology of renal ischemia-reperfusion. Noiri et al (2001) demonstrated that injury of the kidney by ischemia is attributable to increased lipid peroxidation and DNA damage caused by $ONOO^-$. Thus, strategies to protect against the toxic effects of $ONOO^-$ have been devised in an attempt to

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attenuate renal damage. An effective modulator of the effects of $ONOO^-$ that can eliminate the toxicity of $ONOO^-$ and/or its metabolites or precursor radicals involved in tubular damage is highly desirable.

We have reported some evidence that the Chinese prescription Wen-Pi-Tang is able to improve impaired kidney function under conditions of oxidative stress (Oura et al 1984; Yokozawa et al 1986, 1989, 1994, 2001). In particular, in an animal model of $ONOO^-$ generation, Wen-Pi-Tang extract attenuated the renal damage induced by $ONOO^-$ through scavenging $ONOO^-$ and boosting the antioxidative defence system (Rhyu et al 2002). The aim of our current study was to use an in-vitro cell system to investigate how Wen-Pi-Tang extract affects cellular protective mechanisms, especially those in renal tubular cells, which are the most vulnerable kidney cells to oxidative stress. We also wondered whether Wen-Pi-Tang extract prevents or treats renal injury caused by $ONOO^-$. Thus, we not only evaluated the effects of Wen-Pi-Tang extract on scavenging $ONOO^-$ and apoptotic cell death under different experimental conditions to elucidate whether it can prevent and/or treat renal injury, but also investigated its effects on the cellular morphological changes and regulation of the cell cycle resulting from 3-morpholinosydnonimine (SIN-1)-induced renal LLC-PK_1 cell damage.

Materials and Methods

Medium and reagents

Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F-12) and fetal calf serum (FCS) were purchased from Life Technologies Inc. (Grand Island, NY) and Cell Culture Laboratories (Cleveland, OH), respectively. SIN-1, dihydrorhodamine 123 (DHR 123) and Hoechst 33342 were obtained from Sigma Chemical Co. (St Louis, MO). Propidium iodide (PI) was obtained from Molecular Probes (Eugene, Oregon). 3-(4,5-Dimethyl-2-thiazoyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) and diphenylamine were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Preparation of Wen-Pi-Tang extract

The Wen-Pi-Tang prescription was prepared with 15 g Rhei Rhizoma (*Rheum officinale* Baillon), 3 g Ginseng Radix (*Panax ginseng* C. A. Meyer), 9 g Aconiti Tuber (*Aconitum japonicum* Thunberg), 3 g Zingiberis Rhizoma (*Zingiber officinale* Roscoe) and 5 g Glycyrrhizae Radix (*Glycyrrhiza glabra* Linn. var. *glandulifera* Regel et Herder). Aconiti Tuber was obtained from Japan, Ginseng Radix was produced in Korea and the other elements were from China. As previously described (Oura et al 1984), an extract was manufactured by boiling the above crude drugs gently in 1000 mL of water for 60 min and the mixture was concentrated under reduced pressure, resulting in a yield of about 30% by weight of the original preparation.

Cell culture

The porcine kidney cell line LLC-PK_1 was maintained in culture plates containing 5% FCS-supplemented DMEM/F-12 medium (pH 7.2) at 37 °C in a humidified atmosphere of 5% CO_2 in air. All the subsequent procedures were carried out under these conditions. The cells were subcultured weekly with 0.05% trypsin-EDTA in calcium- and magnesium-free phosphate buffered saline (PBS). In order 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. In addition, 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 morphological changes and cell cycle distribution. Subsequently, the cells were treated with 50 or 100 $\mu g\,mL^{-1}$ Wen-Pi-Tang extract together with 800 μM SIN-l for 24 h. The concentration of Wen-Pi-Tang extract was determined on the basis of the preliminary investigation on its cytotoxicity. The doses of 50 and 100 $\mu g\,mL^{-1}$ did not affect the experimental conditions without toxicity. In order to investigate whether the protective activity of Wen-Pi-Tang extract is attributable to prevention or treatment of renal injury, two different incubation conditions, pre- and post-Wen-Pi-Tang extract treatment with SIN-1, respectively, were employed as follows: LLC-PK_1 cells were treated with Wen-Pi-Tang extract for 24 h prior to and after exposure to SIN-1 for 4 h. The normal group without treatment of SIN-1 and Wen-Pi-Tang extract was also included in each individual experiment.

Cellular $ONOO^-$ formation

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

Cell viability

Cell viability was assessed using the MTT colorimetric assay (Mosmann 1983). A 50-μL aliquot of MTT solution (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 formazan crystals incorporated by the viable cells were solubilized with 100 μL dimethyl sulfoxide and the absorbance at 540 nm of each well was read using a Microplate Reader (Model 3550-UV, Bio-Rad, Tokyo, Japan).

DNA fragmentation assay

According to the method of Sellins and Cohen (1987), the cells were lysed in an ice-cold hypotonic lysis buffer

(10 mM Tris, 1 mM EDTA, pH 7.5) containing 0.2% Triton X-100 and centrifuged for 10 min at 13 000 *g*, 500 μL 12.5% trichloroacetic acid (TCA) was added, 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 with 800 μL 5% TCA, heated at 90 °C for 10 min, 100 μL diphenylamine solution (mixture of acetic acid, diphenylamine and sulfic acid, 1:15:0.15 v/v) was added and the mixture was kept at 25–30 °C in the dark overnight. 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.

Morphological changes

To observe the SIN-1-induced morphological changes in unfixed cells, the dye Hoechst 33342 (10 μg mL^{-1}) and PI (10 μg mL^{-1}) were added to unfixed monolayer cells, which were kept on ice (Stapper et al 1995). Other monolayer cells were fixed with PBS containing 2% paraformaldehyde for 30 min, washed twice with PBS and then reacted with Hoechst 33342 (10 μg mL^{-1}) and PI (10 μg mL^{-1}) in PBS at 4 °C for 30 min (Fabisiak et al 1997). The fixed and unfixed cell nuclei were viewed under a fluorescence microscope.

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 70% ice-cold EtOH (Block et al 1987; Ho et al 2000), washed twice with PBS, incubated with RNase solution for 30 min at 37 °C and then treated with PI at 4 °C for 30 min. The cells were 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 in the G_0/G_1, S and G_2/M phases of the cell cycle were assessed using ModFit LT software (Verity Software House, Topsham, ME) in the analysis data.

Statistics

Results are presented as means ± s.e. of five replicates for each of the independent experimental conditions. The effect of Wen-Pi-Tang extract on each parameter was examined using a one-way analysis of variance. Individual differences between groups were evaluated using Dunnett's test and those at $P < 0.05$ were considered to be statistically significant.

Results

Cellular ONOO$^-$ formation, cell viability and DNA fragmentation

Figure 1 shows the effects of Wen-Pi-Tang extract on cellular ONOO$^-$ formation, cell viability and DNA fragmentation in renal tubular LLC-PK$_1$ cells treated with Wen-Pi-Tang extract together with SIN-1. The exposure of LLC-PK$_1$ cells to 800 μM SIN-1 led to marked cellular formation of ONOO$^-$, loss of cell viability and increased DNA fragmentation. However, Wen-Pi-Tang extract protected against the cellular damage induced by SIN-1 in a concentration-dependent manner. Although ONOO$^-$ was not detected in the absence of SIN-1, ONOO$^-$ was produced dramatically in response to exposure to 800 μM SIN-1 and its mean concentration was 912 pmol mL^{-1}. In

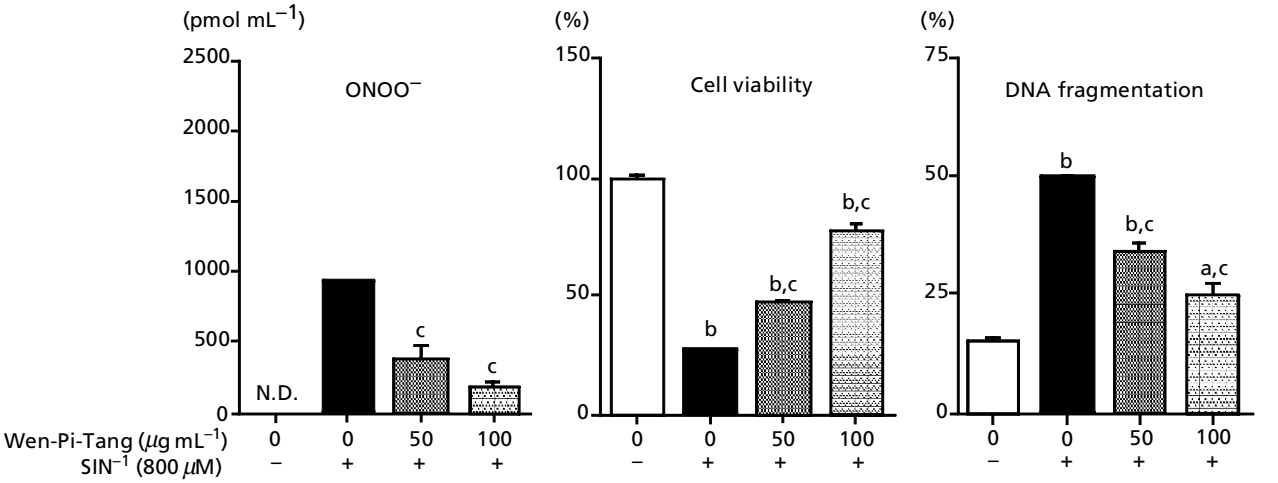

Figure 1 Effects of Wen-Pi-Tang extract on cellular ONOO$^-$ formation, cell viability and DNA fragmentation in renal tubular LLC-PK$_1$ cells treated with Wen-Pi-Tang extract together with SIN-1. N.D., not detectable; [a] $P < 0.01$, [b] $P < 0.001$ vs no treatment values, [c] $P < 0.001$ vs SIN-1 treatment values.

contrast, the cellular $ONOO^-$ levels of the Wen-Pi-Tang extract plus SIN-1-treated groups were significantly lower than those of the only SIN-1-treated group. Wen-Pi-Tang extract, at concentrations of 50 and 100 $\mu g\,mL^{-1}$, reduced cellular $ONOO^-$ production from 912 $pmol\,mL^{-1}$ to 350 and 162 $pmol\,mL^{-1}$ (62 and 82% decreases), respectively. Moreover, while the viability of LLC-PK_1 cells decreased significantly to 27% of the normal value after exposure to SIN-1, Wen-Pi-Tang extract, at concentrations of 50 and 100 $\mu g\,mL^{-1}$, reversed it to 47 and 77%, respectively. Furthermore, SIN-1 treatment increased the DNA fragmentation rate in LLC-PK_1 cells from the control value of 15% to 50%, whereas in the presence of Wen-Pi-Tang extract, 50 and 100 $\mu g\,mL^{-1}$, DNA fragmentation was prevented significantly and the rates were 35 and 24%, respectively. The results of treatment with Wen-Pi-Tang extract prior to and after SIN-1 exposure, in order to clarify whether the extract prevents or treats the renal cellular damage, are shown in Figures 2 and 3. Wen-Pi-Tang extract exerted a protective effect against SIN-1-induced renal tubular cell damage under both experimental conditions. Treatment with Wen-Pi-Tang extract, 50 and 100 $\mu g\,mL^{-1}$, prior to SIN-1 exposure reduced $ONOO^-$ production from 802 $pmol\,mL^{-1}$ to 563 and 385 $pmol\,mL^{-1}$ (30 and 52% decreases), respectively, and reduced the DNA fragmentation rate from 29% to 24 and 25%, respectively

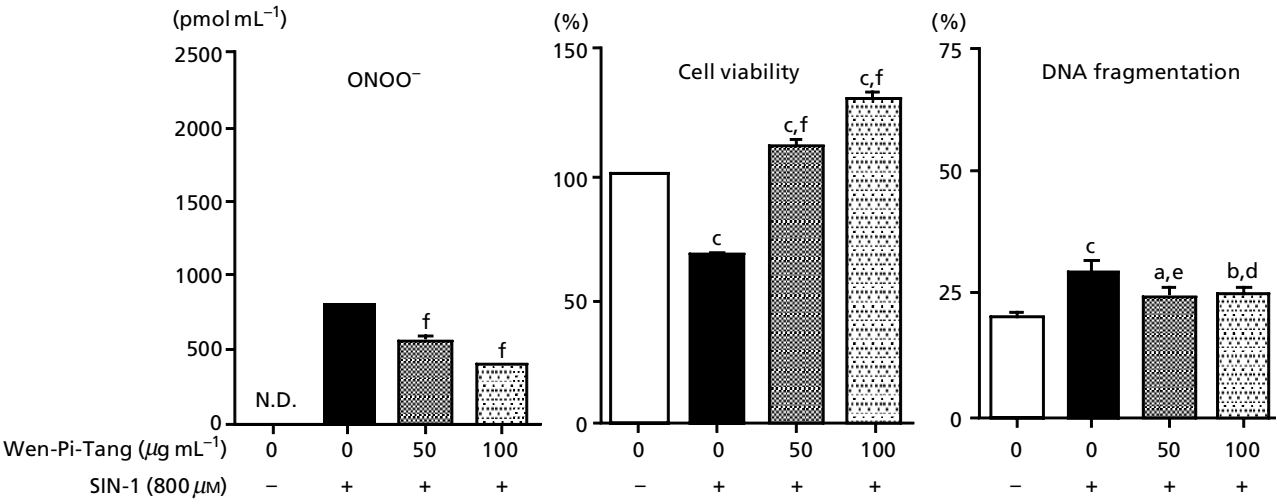

Figure 2 Effects of Wen-Pi-Tang extract on cellular $ONOO^-$ formation, cell viability and DNA fragmentation in renal tubular LLC-PK_1 cells treated with Wen-Pi-Tang extract prior to SIN-1 exposure. N.D., not detectable; $^aP < 0.05$, $^bP < 0.01$, $^cP < 0.001$ vs no treatment values, $^dP < 0.05$, $^eP < 0.01$, $^fP < 0.001$ vs SIN-1 treatment values.

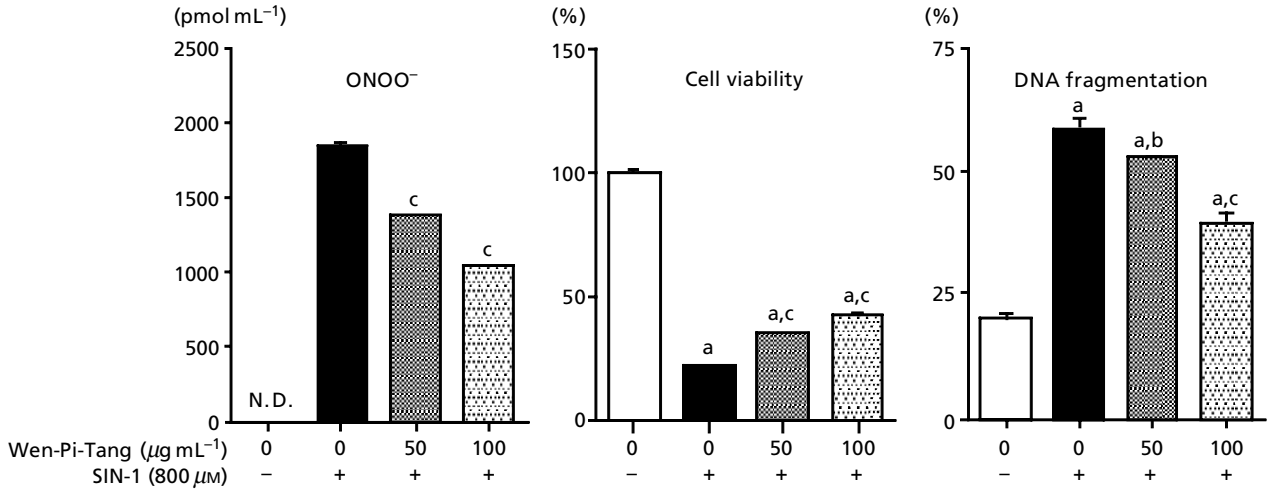

Figure 3 Effects of Wen-Pi-Tang extract on cellular $ONOO^-$ formation, cell viability and DNA fragmentation in renal tubular LLC-PK_1 cells treated with Wen-Pi-Tang extract after SIN-1 exposure. N.D., not detectable; $^aP < 0.001$ vs no treatment values, $^bP < 0.01$, $^cP < 0.001$ vs SIN-1 treatment values.

(Figure 2). In addition, the loss of cell viability was reversed from 67% to 111 and 113%, respectively. Furthermore, treatment with Wen-Pi-Tang extract at 100 $\mu g\,mL^{-1}$ after SIN-1 exposure also resulted in decreased $ONOO^-$ production from 1845 to 1031 $pmol\,mL^{-1}$ (44% decrease) and reduced the DNA fragmentation rate from 59 to 40%, but it led to an increase in the cell viability from 27 to 42% (Figure 3).

Morphological changes

SIN-1 induced morphological changes in fixed and unfixed LLC-PK_1 cells are shown in Figure 4. The cells treated with SIN-1 show nuclear fragmentation and slight membrane changes representing morphological characteristics of apoptosis (B), whereas normal cells in the absence of SIN-1 do not show these morphological changes (A) (Figure 4, upper panel). Damage to unfixed cells (Figure 4, lower panel) was observed as a red color in the nuclei with SIN-1-induced infiltration by the PI staining probe (B). However, the addition of Wen-Pi-Tang extract, at 100 $\mu g\,mL^{-1}$, with SIN-1 attenuated the apoptotic morphological changes induced by SIN-1 in both fixed and unfixed cells (C).

Cell cycle

Table 1 shows the effect of Wen-Pi-Tang extract on the cell cycle disturbance caused by SIN-1. After treatment with 800 μM SIN-1, the proportion of LLC-PK_1 cells in the G_2/M phase decreased significantly from 3.2 to 0.5%,

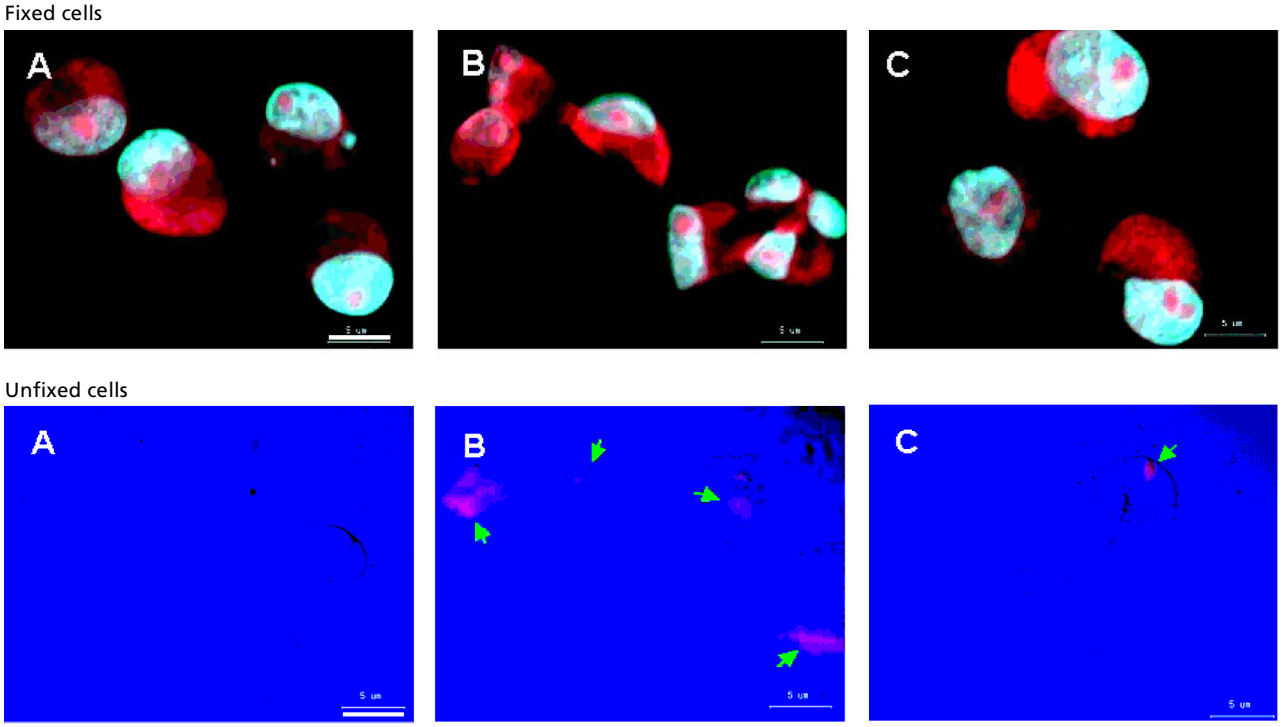

Figure 4 Morphological changes of fixed (upper panel) and unfixed (lower panel) cells treated with Wen-Pi-Tang extract together with SIN-1. (A) No treatment; (B) SIN-1 (800 μM) treatment; (C) SIN-1 (800 μM) and Wen-Pi-Tang extract (100 $\mu g\,mL^{-1}$). Arrows indicate apoptotic cells. Magnification, × 2400. Bar represents 5 μm.

Table 1 Effect of Wen-Pi-Tang extract on the cell cycle.

Treatment	Percentage of cells in each phase of cell cycle		
	G_0/G_1	S	G_2/M
None	67.4 ± 1.1	29.4 ± 1.2	3.2 ± 0.2
SIN-1 (800 μM)	72.6 ± 1.0^{c}	26.9 ± 1.1^{a}	0.5 ± 0.0^{c}
SIN-1 (800 μM) and Wen-Pi-Tang extract (100 $\mu g\,mL^{-1}$)	$70.4 \pm 0.1^{b,d}$	26.7 ± 0.5^{a}	2.9 ± 0.4^{e}

$^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ vs no treatment values; $^{d}P < 0.05$, $^{e}P < 0.001$ vs SIN-1 treatment values.

but concomitant treatment with Wen-Pi-Tang extract, at 100 $\mu g\,mL^{-1}$, increased this proportion from 0.5 to 2.9%.

Discussion

In our recent report, we suggested that the Chinese prescription Wen-Pi-Tang had therapeutic potential for $ONOO^-$-mediated renal dysfunction and injury in an animal model subjected to lipopolysaccharide (LPS) plus ischemia-reperfusion (Rhyu et al 2002). Its protective mechanism was attributed to the direct elimination of $ONOO^-$ formation, rather than inhibition of NO or O_2^- generation, and to elevation of the activities of the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase. In addition, the administration of Wen-Pi-Tang extract reduced the hydroxylation of *m*- and *p*-tyrosine, indicating that it modulated generation of ·OH, a secondary reactive end-product of $ONOO^-$ stimulated by the LPS plus ischemia-reperfusion process. Moreover, Wen-Pi-Tang extract reduced the plasma urea nitrogen and creatinine levels, which are parameters of renal function, implying that it ameliorated renal impairment. These previous results of ours provided the first evidence that Wen-Pi-Tang extract contributes to the regulation of $ONOO^-$ formation and plays a beneficial role against $ONOO^-$-induced oxidative injury and renal dysfunction in vivo. Although the previous study indicated the protective activity of Wen-Pi-Tang extract from $ONOO^-$-induced renal damages, which of the Wen-Pi-Tang components contribute to the activity has not been elucidated. The three-dimensional HPLC profile of Wen-Pi-Tang extract (data not shown) and our previous serial studies suggested that the activity of Wen-Pi-Tang extract is mainly attributed to (−)-epicatechin 3-*O*-gallate (Rhyu et al 2002; Yokozawa et al 2003). However, the mechanism responsible for the protective effect of Wen-Pi-Tang extract against $ONOO^-$-mediated tubular injury remains unclear, although it is well known that renal tubular cells are susceptible to free radicals, which have been related to renal injury and dysfunction. This study was therefore designed to investigate the effects of Wen-Pi-Tang extract on renal tubular LLC-PK_1 cellular damage induced by SIN-1.

SIN-1 releases NO and O_2^- and these reactive species generate $ONOO^-$ under physiological conditions (Huie & Padmaja 1993). As the direct determination of $ONOO^-$ levels in a cellular system is difficult, the yield of rhodamine 123 generated from DHR 123 in response to the extracellular addition of SIN-1, one of the most efficient $ONOO^-$-mediated oxidations, is generally measured (Kooy et al 1994). With respect to $ONOO^-$ formation in renal cells, Bian et al (1999) and Zhang et al (2000) reported that nitrotyrosine, an indirect marker of $ONOO^-$ in vivo, is generally distributed in the proximal regions of LPS-treated rat kidneys and that nitrotyrosine distributed in kidney participates in renal injury. In addition, to measure cell viability, MTT assay was employed. The measurement of cell growth by MTT reduction correlates well with the indicator of viable cell number (Mosmann 1983; Carmichael et al 1987). The MTT assay offers an excellent opportunity for the rapid testing of large numbers of samples with good reproducibility, particularly in adherent cell lines. Our present study also demonstrates that the exposure of renal tubular LLC-PK_1 cells to SIN-1 results in marked $ONOO^-$ formation, loss of cell viability and increased apoptosis demonstrated by the DNA fragmentation assay (Figure 1). However, the concomitant addition of Wen-Pi-Tang extract with SIN-1 dramatically suppressed SIN-1-induced $ONOO^-$ production and apoptotic cell death. These findings imply that Wen-Pi-Tang would protect against SIN-1-induced renal tubular damage through scavenging $ONOO^-$ and inhibiting cell death resulting from $ONOO^-$-induced apoptosis. Thus, our results suggest that the potential protective activity of Wen-Pi-Tang extract against tubular injury is closely involved with $ONOO^-$ formation.

Our previous study also showed that Wen-Pi-Tang extract did not scavenge NO or O_2^- radicals (Rhyu et al 2002). Taken together, this evidence and our current findings indicate that the protective effect of Wen-Pi-Tang against $ONOO^-$ is attributable to direct scavenging of $ONOO^-$, but not of its precursors NO and O_2^-. However, we had not shown whether the effect of Wen-Pi-Tang on $ONOO^-$ under the conditions of treatment with Wen-Pi-Tang extract and SIN-1 together is due to direct activity against $ONOO^-$ or an interaction between SIN-1, or the products generated by SIN-1, and Wen-Pi-Tang extract, and whether the protective effect is attributable to prevention or treatment of renal injury caused by $ONOO^-$. We therefore also determined the effects of Wen-Pi-Tang extract treatment prior to and after the addition of SIN-1 (Figures 2 and 3). This study showed the difference in cellular $ONOO^-$ formation between pre- and post-treatment of SIN-1. The addition of fresh medium or Wen-Pi-Tang extract after SIN-1 exposure led to much more cellular $ONOO^-$ formation than for those additions before SIN-1 treatment. This suggests that exposure to SIN-1 at once resulted in continuous $ONOO^-$ generation even after removal of SIN-1 and the treatment of fresh medium after exposure to SIN-1 did not effectively scavenge the cellular $ONOO^-$. Under these conditions, Wen-Pi-Tang extract also showed protective activity against SIN-1, resulting in reduction of $ONOO^-$ production and inhibition of apoptotic cell death. Accordingly, we would expect Wen-Pi-Tang to exert direct protective activity against $ONOO^-$, and consider that its activity is not due to an interaction between Wen-Pi-Tang extract and SIN-1 or the products generated by SIN-1. In addition, it contributes to the prevention as well as treatment of renal injury induced by $ONOO^-$.

Several mechanisms of $ONOO^-$ toxicity in the pathogenesis of tissue injury have been suggested. The contribution of $ONOO^-$ has been ascribed to induction of DNA damage, inhibition of DNA repair and cell death due to either apoptotic or necrotic responses (Lin et al 1995; Szabo et al 1996; Doulias et al 2001). In particular, cellular damage and apoptotic or necrotic death during the development of acute and chronic renal failure are well known to be important pathogenic mechanisms. In addition,

several studies have demonstrated DNA damage and tubular cell death caused by reactive oxygen or nitrogen metabolites under pathological conditions of renal injury (Hagar et al 1996; Lieberthal & Levine 1996; Chatterjee et al 2000). The inhibition of apoptotic cell death and DNA damage in renal tubular cells is therefore essential for protection against renal damage and much effort has been devoted to the amelioration of cellular and DNA damage induced by oxidative stress. We therefore investigated the protective effects of Wen-Pi-Tang extract on $ONOO^-$-mediated DNA damage and apoptotic cell death. We observed apoptotic morphological changes in tubular cells that resulted from toxic $ONOO^-$ generated in response to SIN-1 exposure, whereas the characteristic morphological markers of apoptosis and nuclear fragmentation recovered after treatment with Wen-Pi-Tang extract (Figure 4). These findings suggest that Wen-Pi-Tang would ameliorate cellular and DNA damage induced by $ONOO^-$.

The division of cells must be carefully regulated and coordinated with both cell growth and DNA replication in order to ensure the formation of progeny cells containing intact genomes. The cellular injury resulting from oxidative stress triggers a variety of responses, including DNA damage, decreased DNA repair activity, cell cycle disturbance and cellular proliferation or death due to either apoptosis or necrosis (Tang et al 1996). Thus, the regulation of the cell cycle in many types of cells is an obvious check-point for the proper structure and function of cells in a biological system. In our present study, the decrease in the proportion of cells in the G_2/M phase caused by $ONOO^-$ formation as a result of SIN-1 treatment was reversed significantly by the concomitant addition of Wen-Pi-Tang extract, which indicates that Wen-Pi-Tang extract regulates the cell cycle by G_2/M phase arrest (Table 1).

The present results with renal tubular LLC-PK_1 cells demonstrate that the Chinese prescription Wen-Pi-Tang ameliorates the cellular damage associated with $ONOO^-$ formation, suggesting that it has a role in the prevention and treatment of $ONOO^-$-induced renal injury.

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