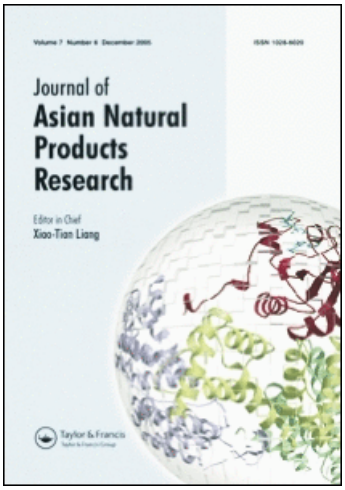

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

Anti-apoptotic effect and the mechanism of orientin on ischaemic/reperfused myocardium

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To cite this Article Fu, X. -C. , Wang, M. -W. , Li, S. -P. and Wang, H. -L.(2006) 'Anti-apoptotic effect and the mechanism of orientin on ischaemic/reperfused myocardium', Journal of Asian Natural Products Research, 8: 3, 265 — 272
To link to this Article: DOI: 10.1080/10286020500207347
URL: http://dx.doi.org/10.1080/10286020500207347

Journal of Asian Natural Products Research, Vol. 8, No. 3, April–May 2006, 265–272

Anti-apoptotic effect and the mechanism of orientin on ischaemic/reperfused myocardium

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(Received 14 September 2004; in final form 29 March 2005)

We investigated the anti-apoptotic effect of orientin, from bamboo leaves (*Phyllostachys nigra*), on rat heart after treatment with ischemia/reperfusion (I/R), and on rat cardiomyocytes injured by hypoxia/reoxygenation (H/R). I/R injury was induced by occluding the left anterior descending coronary artery for 45 min and restoring perfusion for 240 min. Orientin (0.5, 1.0 and 2.0 mg kg^{-1}) or its vehicle was injected i.v. 10 min prior to ischemia. Cultured cardiomyocytes were subjected to hypoxia for 120 min, then reoxygenated for 60 min to induce H/R. Vehicle or orientin (3, 10, 30 μmol l^{-1}) was added 10 min before hypoxia and reoxygenated. TUNEL assay and DNA fragmentation assay demonstrated that myocardium apoptosis was attenuated by pretreatment with orientin (0.5, 1.0 and 2.0 mg kg^{-1}). Flow cytometric analysis also showed that apoptosis of cardiomyocytes was reduced by pretreatment with orientin (3, 10 and 30 μmol l^{-1}). In addition, results of immunohistochemistry and Western blot analysis showed that orientin increased the expression of bcl-2 and reduced Bax expression, resulting in up-regulation of the bcl-2/Bax ratio. Cytochrome *c* (Cyt-*c*) and caspase-3 expression was also reduced in myocardium and cardiomyocytes injured by I/R and H/R. These observations indicate that orientin exerts a potent cardioprotective effect on I/R- and H/R-treated myocardium and cardiomyocytes, and inhibits apoptosis by preventing activation of the mitochondrial apoptotic pathway (cytochrome *c*–caspase-3).

Keywords: Orientin; Ischemia/reperfusion; Hypoxia/reoxygenation; Apoptosis; Mitochondria; Myocardium

1. Introduction

Bamboo leaves, *Phyllostachys nigra* (Lodd.ex.lind-1 Munro.L), have been a staple of Chinese medicine for thousands of years. Bamboo leaves have anti-free-radical activity and are comparable to the leaves of *Ginkgo biloba*, which are a potential source of natural antioxidants and free radical scavengers [1]. Our previous investigation revealed that bamboo leave extracts (BLEs) have significant protective effects on myocardial ischemia in the rat and restrain blood platelet aggregation in the rabbit. Four BLE flavonoid C-glycosides, orientin (luteolin-8-C-glucoside), isoorientin (luteolin-6-C-glucoside), isovitexin (apigenin-6-C-glucoside) and vitexin (apigenin-8-C-glucoside), were isolated for the

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Journal of Asian Natural Products Research
ISSN 1028-6020 print/ISSN 1477-2213 online © 2006 Taylor & Francis
http://www.tandf.co.uk/journals
DOI: 10.1080/10286020500207347

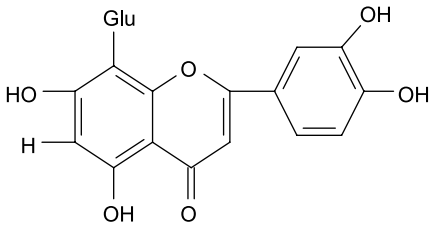

Figure 1. Chemical structure of orientin.

first time and have since been found to be distributed widely in other plants [2]. The most potent protective effect of orientin (figure 1) on myocardium was found through screening and its anti-apoptotic effect on I/R- or H/R-treated myocardium was investigated. Developing a therapy for I/R-injured myocardium is of major interest in the field of cardiology and heart surgery [3]. Since reperfusion after myocardial ischemia results in exacerbation of the injury and apoptosis (programmed cell death) [4], it is hypothesized that this type of injury can be attenuated if a portion of the injured myocardial cells could be rescued from apoptotic cell death. Also, identifying the intracellular signaling pathways that inhibit apoptosis in myocardium could lay the foundation for novel therapeutic strategies. Bcl-2 is one of the pivotal anti-apoptotic gene products, and Bax, Cyt-*c* and caspase-3 are pro-apoptotic gene products [5]. Therefore, signaling pathways that modulate cardiomyocyte apoptosis were identified *in vivo* and *in vitro*, and the function of orientin in governing these pathways was examined.

2. Results and discussion

2.1 Effects of orientin on myocardial apoptotic index after I/R injury

Recent experiments and clinical evidence suggest that coronary occlusion followed by reperfusion leads to reversible myocardial dysfunction [6], whereas myocardial death during I/R is partially mediated by apoptosis [7]. Apoptosis is a critical cellular event involved in the pathogenesis of myocardial ischemia–reperfusion (I/R) injury. In the present study, myocardial tissue during I/R *in vivo* was obtained to determine the significance of apoptosis in myocardium. A TUNEL assay was used to identify apoptotic cell number per thousand myocardial cells. Ten slices per group were prepared, 10 different regions were observed in each slice, and 100 cells were counted in each region. The incidence of TUNEL-positive myocardial cells caused by I/R was significantly reduced in orientin-pretreated hearts compared with that in I/R hearts (table 1). I/R-induced apoptosis in myocardial tissue was attenuated by orientin (0.5, 1.0 and 2.0 mg kg^{-1}) pretreatment in a dose-dependent manner.

2.2 Effects of orientin on DNA fragmentation of myocardium

Cell apoptosis in myocardium was determined by fragmentation of DNA in multiples of 180–200 bp, as shown by DNA gel electrophoresis. Spontaneous DNA fragmentation was observed in I/R myocardial cells, evidencing the characteristic 'DNA ladder' profile that is considered the hallmark of apoptosis, and DNA fragmentation was prevented in

Table 1. Effect of orientin on the number of apoptotic cells in I/R-injured rat myocardium detected by TUNEL.

Group	*n*	*Dose (mg kg^{-1})*	*Number of apoptotic cells (‰)*
Sham	10	–	7.8 ± 4.3**
I/R	10	–	434 ± 50$^{\#}$
Orientin	10	0.5	390 ± 30*
	10	1.0	331 ± 31**
	10	2.0	268 ± 28**
Ver	10	2.0	301 ± 26**

$n = 10$, $\bar{x} \pm$ SD. Sham, sham control; I/R, ischemia/reperfusion; Ver, verapamil control. $*P < 0.05$, $**P < 0.01$ vs. I/R group; $^{\#}P < 0.01$ vs. sham group.

orientin-pretreated hearts (0.5, 1.0 and 2.0 mg kg^{-1}) (figure 2). These results further indicate that orientin inhibits apoptosis of myocardial cells induced by I/R.

2.3 Effects of orientin on bcl-2, Bax, Cyt-c and Caspase-3 expression of myocardium injured by I/R

It has been reported that overexpression of the anti-apoptotic gene *Bcl-2* is protective [8], pro-apoptotic caspase-3 has been shown to be detrimental [9] to the heart with I/R injury, and the activation of downstream caspase-3 is essential for the induction of apoptosis by various stimuli [10]. One of the major mechanisms of caspase activation has been shown to involve the release of cytochrome *c* from the mitochondria to the cytosol. It was shown that Bax activated caspase-3 by stimulating Cyt-*c* release from mitochondria [11]. Overexpression of Bcl-2 blocked Cyt-*c* release and caspase-3 activation in myocardial cells. In view of the roles of the Bcl-2 families in the apoptotic pathway, immunohistochemistry was performed to observe the changes in bcl-2, Bax, caspase-3 and Cyt-*c* protein expression during I/R. The results showed that Bcl-2, Bax, Cyt-*c* and caspase-3 expression in the operation control was higher (207 ± 42, 300 ± 65, 256 ± 22 and 340 ± 58, respectively) than in the sham control (154 ± 40, 37 ± 9, 99 ± 10 and 122 ± 9, respectively). Orientin (2.0 mg kg^{-1}) significantly inhibited Bax, Cyt-*c*

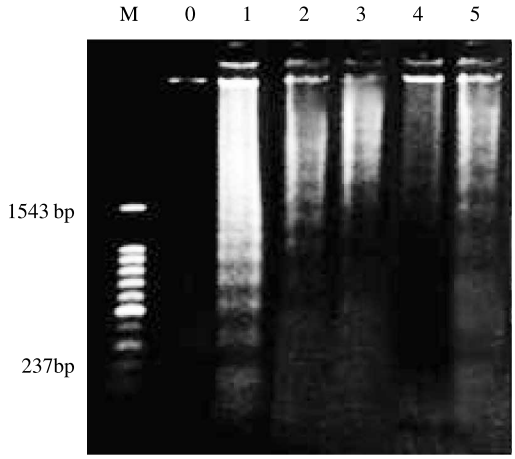

Figure 2. I/R-induced DNA fragmentation of myocardium. The normal myocardium (non-I/R, Lane 1); I/R myocardium (Lane 2); I/R myocardium pretreated with orientin, 0.5 mg kg^{-1} (Lane 3); I/R myocardium pretreated with orientin, 1.0 mg kg^{-1} (Lane 4); pretreated with orientin, 2.0 mg kg^{-1} (Lane 5); pretreated with Ver, 2.0 mg kg^{-1} (Lane 6).

Table 2. Effect of orientin on Bax, bcl-2, Cyt-*c* and caspase-3 expression in I/R myocardium of rats.

	PEI (%)				
	Bcl-2	*Bax*	*Cyt-c*	*Caspase-3*	*Bcl-2/Bax*
Sham	154 ± 40**	37 ± 9**	99 ± 10**	122 ± 9**	4.26 ± 1.41**
I/R	207 ± 42$^{\#}$	300 ± 65$^{\#}$	256 ± 22$^{\#}$	340 ± 58$^{\#}$	0.70 ± 0.13$^{\#}$
Orientin (mg kg^{-1})					
0.5	233 ± 44	225 ± 40*	202 ± 19*	273 ± 24*	1.05 ± 0.20*
1.0	254 ± 43	155 ± 24*	172 ± 16*	223 ± 31*	1.67 ± 0.35**
2.0	281 ± 44	99 ± 22**	130 ± 13**	174 ± 21**	3.00 ± 0.93**
Ver (mg kg^{-1})					
2.0	247 ± 38	191 ± 39*	168 ± 19*	223 ± 22*	1.34 ± 0.35**

$n = 10$, $\bar{x} \pm$ SD. PEI, positive expressive index; Sham, sham control; I/R, ischemia/reperfusion; Ver, verapamil control. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. I/R group; $^{\#}P < 0.01$ vs sham group.

and Caspase-3 expression (99 ± 22, 130 ± 13, 174 ± 21, respectively), but did not inhibit bcl-2 expression (281 ± 44), and significantly increased the Bcl-2/Bax ratio (3.0 ± 0.93) (table 2). Ver, a potent Ca^{2+} channel inhibitor, has a protective effect on myocardium induced by I/R, and, in this study, Ver (2.0 mg kg^{-1}) had almost the same effect as orientin (1.0 mg kg^{-1}). These observations indicate that orientin exerts a potent cardioprotective effect on myocardium treated with I/R, and inhibits apoptosis by preventing activation of the mitochondrial apoptotic pathway (Cyt-*c*–caspase-3).

2.4 Effects of orientin on the apoptosis of cardiomyocytes injured by H/R

Loss of DNA may occur as a result of the shedding of apoptotic bodies containing fragments of nuclear chromatin, and apoptotic cells often show a deficit in DNA content. These can be recognized in DNA content histograms on staining with a DNA-specific fluorescein [12]. In the H/R-injured cardiomyocytes, the percentage of apoptotic cells with fragmented DNA content was 83.6%. The ratio of apoptotic cells was significantly reduced to 13.8, 5.43 and 2.02%, respectively, by orientin (3, 10, 30 μmol l^{-1}) pretreatment, and Ver (5 μmol l^{-1}) pretreatment also reduced the apoptotic ratio (3.17%) (figure 3). These results suggest that orientin has an anti-apoptotic effect on H/R-injured neonatal mouse cardiomyocytes.

2.5 Effects of orientin on bcl-2, Bax, Cyt-c and Caspase-3 expression of cardiomyocyte injured by H/R

The change in expression of apoptosis-related proteins during the course of H/R injury *in vitro* was analysed by Western blot analysis. As shown in figure 4, orientin (3, 10, 30 μmol l^{-1}) increased the expression of bcl-2 and reduced Bax expression, and Cyt-*c* and caspase-3 expression were also reduced by pretreatment with orientin (3, 10, 30 μmol l^{-1}) in cardiomyocytes injured by H/R. The present study, on the cellular level, has demonstrated that orientin (3, 10, 30 μmol l^{-1}) attenuates cardiomyocyte apoptosis after H/R injury. Ver (5 μmol l^{-1}) has almost the same effect as orientin (10 μmol l^{-1}). These data further indicate that orientin inhibits H/R-induced apoptosis by preventing activation of the mitochondrial apoptotic pathway. These results confirm the data obtained from immunohistochemistry.

It is clear that apoptosis plays an important role in a variety of physiological and pathological states. However, in the cardiovascular system, we have only begun to clarify the role of apoptosis and the therapeutic potential associated with its inhibition. Reoxygenation

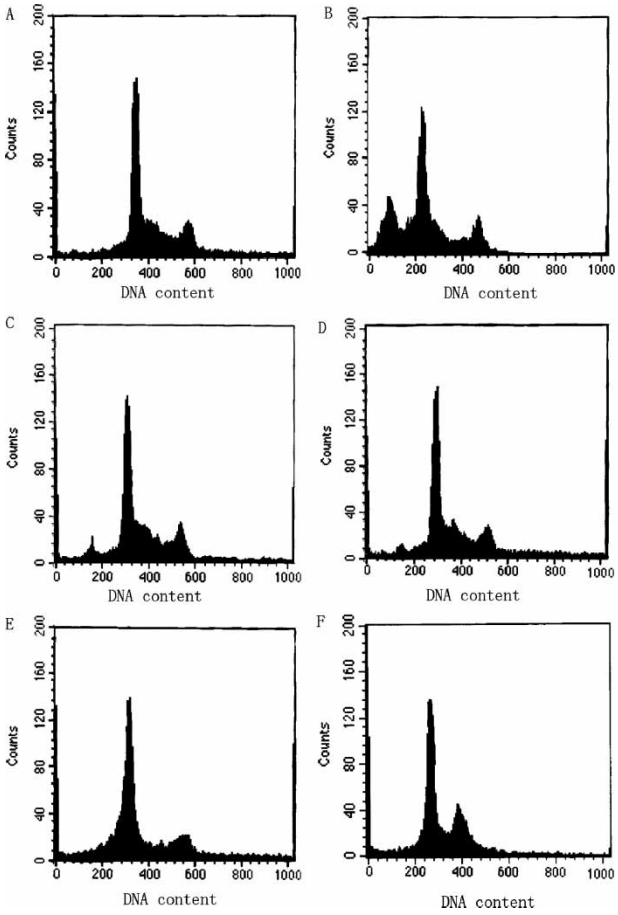

Figure 3. Cardiomyocytic apoptosis rate obtained by flow cytometry. (A) Non-H/R myocardium (apoptosis 0.63%). (B) H/R myocardium (apoptosis 83.6%). (C) H/R cardiomyocyte pretreated with orientin, 3 μmoll^{-1} (apoptosis 13.8%). (D) H/R cardiomyocyte pretreated with orientin, 10 μmoll^{-1} (apoptosis 5.43%). (E) H/R cardiomyocyte pretreated with orientin, 30 μmoll^{-1} (apoptosis 2.02%). (F) H/R cardiomyocyte pretreated with Ver, 5 μmoll^{-1} (apoptosis 3.17%).

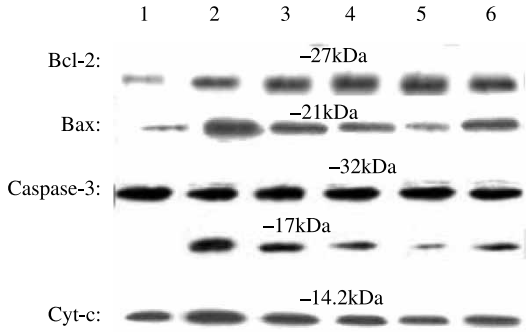

Figure 4. Expression of Bcl-2, Bax and cytochrome *c*, caspase-3 on cardiomyocytes injured by H/R. Cell lysates were separated by 12% SDS-PAGE electrophoresis, and the protein bands were detected by Western blot analysis. Lane 1, non-H/R; Lane 2, H/R cardiomyocyte; Lane 3, H/R cardiomyocyte pretreated with orientin, 3 μmoll^{-1}; Lane 4, H/R cardiomyocyte pretreated with orientin, 10 μmoll^{-1}; Lane 5, H/R cardiomyocyte pretreated with orientin, 30 μmoll^{-1}; Lane 6, H/R cardiomyocyte pretreated with Ver, 5 μmoll^{-1}.

or reperfusion may emerge as a clinically important area in which inhibition of apoptosis may prove to be of clinical benefit. However, more work is necessary to clarify the significance of apoptosis and the molecular mechanisms involved in ischemic heart disease.

3. Experimental

3.1 Drug and reagents

Orientin (purity, 99.8%; wt, 464; character, yellow powder) was purchased from Extrasynthese (Genay, France). It was dissolved in DMSO to make stock solutions, then diluted in 0.9% NS (I/R) or cell culture medium to different concentrations. In all assays, the final concentrations of DMSO in the culture medium were below 0.05%. Antibodies against rat Bcl-2, Bax, Cyt-*c* and caspase-3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The cell apoptosis checking kit and the SABC (strepy–sidin–botin–enzyme complex) immunohistochemistry checking kit were obtained from Wuhan Boster Biological Technology (Wuhan, China). Fetal bovine serum (FBS), trypsin, DMEM, agarose gel, sodium dodecyl sulfate (SDS), propidium iodide (PI) and ethidium bromide were purchased from Sigma (St Louis, MO, USA). Verapamil was from Lianyungan Pharmacy Factory (Lianyungan, China).

3.2 I/R of the rat heart in vivo

Two-month-old male Wistar rats were anesthetized with sodium pentobarbital (60 mg kg^{-1}, i.p.), and the trachea was intubated using a stainless-steel tube and a breathing machine. Electrodes connected to the cardiogram machine were placed under the skin of a limb. After left thoracotomy and exposure of the heart, the left anterior descending coronary artery (LAD) was ligated with a 4–0 silk just proximal to its main branching point, and the suture was tied over a 1 mm polyethylene tube that was left in place during the planned period of ischemia (45 min). Ischemia was confirmed by electrocardiographic evidence of injury. Blood flow was then re-established by removal of the tube for 240 min [13]. For sham I/R, thoracotomy was performed without LAD ligation. Three subgroups of animals received orientin (0.5, 1.0 and 2.0 mg kg^{-1}) before ischemia for 10 min.

3.3 TUNEL assay

The TUNEL assay was used for detection of DNA strand breaks in the rat heart induced by I/R [14]. Detection was carried out according to the instructions of the supplier. Briefly, the left ventricular myocardium was collected and fixed in 10% formalin. Each fixed myocardium was embedded in paraffin. Paraffin-fixed sections were pretreated with 3% H_2O_2, and end-labeling was performed for 1 h with TdT (0.06 $\mu\mu l^{-1}$) in TdT buffer containing 40 μmol dig-11-dUTP. Nuclei exhibiting DNA fragmentation were visualized by incubation in 3′,3-diaminobenzidine (DAB), 0.5 g l^{-1}, for 10 min. Finally, the sections were counterstained with hematoxylin and observed under light microscopy. The nuclei of apoptotic cells, which contained DNA fragments, were stained dark brown. TUNEL-positive myocytes were determined by randomly counting 1000 cells (× 200).

3.4 Immunohistochemistry

Tissue sections were deparaffinized and hydrated through graded alcohol to water. Endogenous peroxidase activities were blocked by incubation in 3% H_2O_2 for 10 min. After three washes with TBS (Tris–HCl, 0.05 mol l^{-1}, 0.9% NaCl, pH 7.8) the sections were blocked with 1:50 normal horse serum at 37°C for 30 min to suppress non-specific background staining. The primary antibodies, rabbit anti-mouse Bax monoclonal antibody, rabbit anti-mouse Bcl-2 monoclonal antibody, rabbit anti-mouse Cyt-*c* monoclonal antibody and rabbit anti-mouse caspase-3 polyclonal antibodies, were then applied. For control sections, TBS was used in place of primary antibody. After incubation at 4°C for 20 h, the sections were incubated with biotinlyated goat anti-rabbit IgG (1:50, 37°C, 30 min), followed by 1:100 diluted avidin–biotin–peroxidase complex at 37°C for 30 min. The sections were subsequently incubated with 3′,3-diaminobenzidine (DAB), 0.5 g l^{-1}, for 10 min, counter-stained with hematoxylin and observed under light microscopy [15]. Brown staining in the cytoplasm was evaluated as positive expression, and positive expression indexes (bcl-2 and Bax, caspase-3 and cytochrome *c*) were determined by randomly counting 1000 cells.

3.5 DNA gel electrophoresis

Frozen tissue samples were homogenized in equal volumes of homogenization buffer (10 mmol l^{-1} Tris–HCl, 25 mmol l^{-1} EDTA, 100 mmol l^{-1} NaCl, pH 8.0). To assess DNA fragmentation, the homogenate (100 μl) was mixed with 1.25 ml of lysis buffer (10 mmol l^{-1} Tris–HCl, 25 mmol l^{-1} EDTA, 100 mmol l^{-1} NaCl, 0.5% SDS). The suspension was centrifuged at 13 000 × *g* for 15 min, and the supernatant was treated with 100 μg ml^{-1} proteinase K at 50°C for 30 min. The DNA was precipitated by adding ethanol and NaCl, dissolved in TE buffer, and extracted with phenol/chloroform. DNA extract was dissolved in 50 μl TE buffer and treated with RNase (100 μg ml^{-1}) at 37°C for 30 min. Electrophoresis was performed at 50 V with 1.5% agarose gel. DNA was visualized with ethidium bromide.

3.6 Preparation of cardiomyocytes

The hearts were cut into small pieces and digested in cell suspension by 0.25% trypsin [16]. The cardiomyocytes were obtained by centrifugation, resuspended in DMEM containing 20% FBS, and assessed by immunohistochemical staining with α-actin monoclonal antibody. The cell density was adjusted to 5 × 10^8 cells/l and incubated in 5% CO_2 in a humidified atmosphere for 4 days. Cultured cardiomyocytes were randomly divided into six groups, including the normal group, the H/R group, the H/R + orientin (3, 10 and 30 μmol l^{-1}) group, and the H/R + Ver group. For H/R, the cells were cultured in 100 ml glass culture bottles saturated with 99.9% N_2 for 2 h (the medium was changed to serum-free DMEM saturated with 99.9% N_2 in advance), followed by normal incubation for 1 h to form the cell model of hypoxia–reoxygenation injury. Orientin (3, 10, 30 μmol l^{-1}) or Ver (5 μmol l^{-1}) was added 10 min before H/R.

3.7 Flow cytometry

Following H/R, cardiomyocytes were harvested for flow cytometry [17]. Briefly, cells were fixed in 70% ethanol at 4°C overnight. They were then treated with Tris–HCl buffer (pH 7.4)

containing 1% RNase A and stained with PI, 5 mg l^{-1}.The distribution of cells with different DNA contents was determined by flow cytometry (Facscalibur, Becton Dickinson, USA) and the data were analysed by multicycle DNA content and cell analysis software (Modfit LT 2.0).

3.8 Western blot analysis for apoptosis-related proteins

Both adherent and non-adherent cells were collected and frozen at -80°C. Western blot analysis was performed as previously described [18] with some modification. Briefly, the cell pellets were resuspended in lysis buffer (mmol l^{-1}: HEPES-KOH 20, pH 7.5, KCl 10, $MgCl_2$ 1.5, sodium EDTA 0.5, sodium EGTA 0.5, dithiothreitol 0.5, phenylmethylsulfonyl fluoride 0.1, and sucrose 250), supplemented with protease inhibitors (in μg ml^{-1}: pepstatin A 10, leupeptin 10, and aprotinin 10). The protein content of the supernatant was determined using Bio-Rad protein assay (Bio-Rad, USA), then lysed on ice for 60 min. After centrifugation at 13 000 $\times$ g for 10 min, the protein lysates were separated by electrophoresis in 12% SDS polyacrylamide gel and blotted onto a nitrocellulose membrane. Antibodies against rat Bcl-2, Bax, Cyt-*c* and caspase-3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bcl-2, Bax, Cyt-*c* and caspase-3 were visualized using anti-rabbit IgG conjugated with peroxidase (HRP) and DAB as the HRP substrate.

3.9 Statistical analysis of the data

All values are expressed as mean $\pm$ SD. The differences in the data between two groups were determined by a Student's t test. A value of $P < 0.05$ was considered statistically significant.

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