

Full Length Research Paper

Norcantharidin (NCTD) induces mitochondria mediated apoptosis in human HepG2 cells

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Norcantharidin (NCTD), a demethylated form of cantharidin, is now in used as a routine anticancer drug. However, the detailed mechanisms underlying this process are generally unclear. The aims of this study were to evaluate the apoptotic effects and molecular mechanisms of NCTD. MTT assay was used to determine the cell growth inhibitory rate. Flow cytometry were used to detect the apoptosis and the loss of mitochondrial membrane potential ($\Delta\psi_m$) induced by NCTD. Caspase detection kit were used to detect the activity of caspase-3 -9. Western-blot was used to detect the expression of Bcl-2, Bax and cytochrome C (cyt C). Our results indicated that, treatment of NCTD resulted in significant decrease in cell viability in a dose-and time-dependent manner. A dose-dependent apoptosis was also observed by flow cytometry analysis. Molecular mechanistic studies of apoptosis revealed that, NCTD treatment resulted in a significant loss of $\Delta\psi_m$, release of cyt C, enhanced expression of pro-apoptotic protein Bax and suppression of anti-apoptotic protein Bcl-2. These were followed by activation of caspases-9 and -3, subsequently leading to cell apoptosis. These results indicate that, NCTD induced cytotoxicity in HepG2 cells by apoptosis, which is mediated through mitochondrial pathway.

Key words: Norcantharidin, apoptosis, caspase, Bax/Bcl-2, cyto C, HepG2 cells.

INTRODUCTION

Hepatoma is one of the most common malignancies worldwide with an overall 5-year survival of less than 14%. Jemal et al. (2010) estimated 17,430 new cases for men and 6,690 new cases for women in 2010 with 3.3% of all cancer deaths for both sexes being attributable to hepatoma. However, the available treatment and surgical options have proven to be inadequate in controlling the mortality and morbidity associated with this disease. It is therefore, necessary to intensify our efforts to develop new therapeutic agents for its prevention and treatment.

Many lines of evidence have shown that the Chinese medicine contains many chemical compounds with anticancer effects (Li et al., 2010; Mack et al., 1996). Thus, we tested whether the active ingredients of specific Chinese medicines have a therapeutic effect on human liver cancer. Norcantharidin (NCTD) is a demethylated

analog of cantharidin purified from the dried body of the Chinese blister beetle, *Mylabris*, which is a traditional medicine long been used for treating malignant tumors (Wang, 1989). In comparison with cantharidin, the major bioactive compound in *Mylabris*, NCTD has the advantage of easy synthesis and reduced intrinsic toxicity, while retaining its anti-cancer activity (Kok et al., 2005). The anticancer mechanisms of NCTD have been illuminated by the evidence showing its anti-proliferation, pro-apoptotic and anti-migratory effects on many cancer cells (Peng et al., 2002; Chen et al., 2002; Sun et al., 2000; Hong et al., 2000). However, the signaling pathways governing apoptosis in human HepG2 cells remains unclear.

This study was conducted to investigate NCTD induced toxicity in human HepG2 cells and the possible role of mitochondrial pathway in the toxic effects. Under experimental conditions, NCTD inhibited proliferation and induced apoptosis of HepG2 cells in a concentration-dependent manner. The results suggest that, NCTD -induced apoptosis in HepG2 cells is associated with increased expression of Bax protein and decreased Bcl-2 protein. Furthermore, the data show that, NCTD

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Abbreviation: $\Delta\psi_m$; Mitochondria membrane potential Chinese

treatment of HepG2 cells causes loss of mitochondrial membrane potential ($\Delta\psi_m$), releases of Cyt C from the mitochondrial intermembrane space toward the cytosol, and proteolytic activation of caspases 9 and 3.

MATERIALS AND METHODS

Chemicals and reagents

NCTD of analytical grade purity was obtained from Sigma Chemical Co. (St Louis, USA); a stock solution (5 mg/ml) in RPMI1640 (HyClone, USA) was prepared and stored in the dark at 4°C. Final concentrations of NCTD used for different experiments were prepared by diluting the stock with RPMI-1640. D-Hanks' solution, penicillin, streptomycin, fetal bovine serum and EDTA, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), propidium iodide in this study were purchased from Sigma Chemical Co. (St Louis, USA). Anti-rabbit Bcl-2, Bax, cyt C and β -actin antibodies and HRP-conjugated goat anti-rabbit Ig were from R and D Systems Inc (Minneapolis, USA). Caspase 3 and caspase 9 were obtained from Beyotime Institute of Biotechnology (Nantong, China). JC-1 kit were purchased from keygen Biotechnology Co., LTD (Nanjing, China). Annexin V-FITC kit was obtained from Beijing Biosea Biotechnology Co., LTD (Beijing, China).

Cell line and cell culture

The human hepatoma cell line, HepG2 was obtained from the department of oncology, Zhongnan Hospital of Wuhan University (Wuhan, China). The cells were cultured in RPMI-1640 medium (HyClone, USA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, USA), 100 U/ml penicillin and 100 μ g/ml streptomycin. Cultures were maintained in a humidified atmosphere of 5% CO₂ at 37°C.

Cell growth inhibition studies

The effect of NCTD on cell proliferation was measured by MTT assay (Liu et al., 2010). *In vitro* growth inhibition effect of NCTD on HepG2 cells was determined by measuring MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye absorbance of living cells. Briefly, cells (2×10^4 cells per well) were seeded in 96-well plates. After exposure to NCTD (2.5, 5, 10, 20 and 40 μ g/ml) for 24, 36 and 48 h, 20 μ l MTT solution (5 mg/ml in phosphate buffered saline (PBS)) was added to each well and the plates were incubated for an additional 4h at 37°C. The MTT solution in medium was aspirated off. To achieve solubilisation of the formazan crystal formed in viable cells, 200 μ l DMSO was added to each well. The absorbance rate of each well optical density (OD value) was measured at 570 nm by a spectrophotometer. The cell proliferation inhibition rate was calculated as 1-(average OD value of wells with administered drug/average OD value of control wells) \times 100%.

Annexin V/PI staining assay

The extent of apoptosis was evaluated by annexin V-FITC and flow cytometry (Bai et al., 2010; Xu et al., 2010). Briefly, 1×10^6 cells/ml cells grown to about 60% confluence were treated with 10, 20 or 40 μ g/ml of NCTD for 24 h. For annexin V/PI binding assay, cells were collected and washed with serum-containing media. Cells collected were washed with serum-containing media before being resuspended in 500 μ l of 1 \times binding buffer, followed by the addition

of 5 μ l of annexin V-FITC and 10 μ l of PI to cell suspension. Cells used as controls (0 μ l NCTD) were incubated with the vehicle (RPMI1640) alone. The mixture was then, incubated for 15 min at room temperature in the dark and immediately analyzed with the flow cytometer and cell quest software analysis.

Measurement of mitochondrial membrane potential ($\Delta\psi_m$)

$\Delta\psi_m$ was measured with the lipophilic cation JC-1 probes, which is able to selectively enter mitochondria. JC-1 exists in a monomeric form emitting at 525 nm after excitation at 490 nm. Depending on $\Delta\psi_m$, JC-1 is able to form J-aggregates that are associated with a large shift in emission (575 nm). A shift in fluorescence from red (JC-1 aggregates, FL2) to green (JC-1 monomers, FL1) is detected as a reduction of

$\Delta\psi_m$, which implies JC-1 dissociation from mitochondria into the cytosol. Briefly, (Cossarizza et al., 2010), cells (1×10^6 cells/ml) in 10 cm culture dishes were treated without or with NCTD (10, 20 and 40 μ g/ml) for 24 h. Cells were trypsinized, washed in ice-cold PBS and incubated with 10 mM JC-1 at 37°C for 20 min in darkness. Subsequently, cells were washed twice with PBS and analyzed by flow cytometry. Excitation wave was set at 490 nm and the emitted green fluorescence of annexin V-FITC (FL1) and red fluorescence of PI (FL2) were collected using 525 and 575 nm band pass filters, respectively.

Detection of cyt C release from the mitochondria to the cytosol

Cyt C determination in cytosolic and mitochondrial fractions was done by western blotting. The cells were harvested after the respective treatments and washed once with ice-cold PBS. For isolation of mitochondria and cytosol, the cells were sonicated in buffer containing 10mM Tris-HCl pH 7.5, 10 mM NaCl, 175 mM sucrose and 12.5 mM EDTA and the cell extract centrifuged at 1000 g for 10 min to pellet nuclei. The supernatant thus, obtained was centrifuged at 18000 g for 30 min to pellet the mitochondria and purified as previously described. The resulting supernatant was termed the cytosolic fraction. The pellet was lysed and protein content estimated in both fractions by Bradford's method. Equal amounts of protein were separated on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) then were electrotransferred to PVDF membrane. The membrane was then incubated in 5% non-fat milk in TBST (TBS: Tris-buffered-saline, 10 mM Tris, 150 mM NaCl, pH 7.6 with 0.1% Tween 20) for 2 h followed by overnight incubation with the primary antibody separately. The incubated membranes were extensively washed with TBST before incubation for 2 h with the secondary anti-body. After extensive washing with TBST, the immune complexes were detected by enhanced chemiluminescence detection kit.

Western blot analysis

HepG2 cells were treated with various concentrations of NCTD (10, 20 and 40 μ g/ml) for 24 h. Both adherent and floating cells were collected. Then, cells were lysed in ice-cold RIPA buffer (20 mmol/l Tris pH 7.5, 150 mmol/l NaCl, 1% NP40, 0.5% sodium deoxycholate, 1 mmol/l EDTA and 0.1% SDS) by pipetting up and down. Thereafter, cell lysates were placed on ice for 30 min and cleared by centrifugation at 12 000 \times g for 20 min at 4°C. The supernatants were collected. The protein concentration was determined by the Bradford protein assay kit. Equivalent amounts of protein lysates were separated by electrophoresis in 12% SDS polyacrylamide gel and blotted onto nitrocellulose membrane (Amersham Biosciences, UK). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween

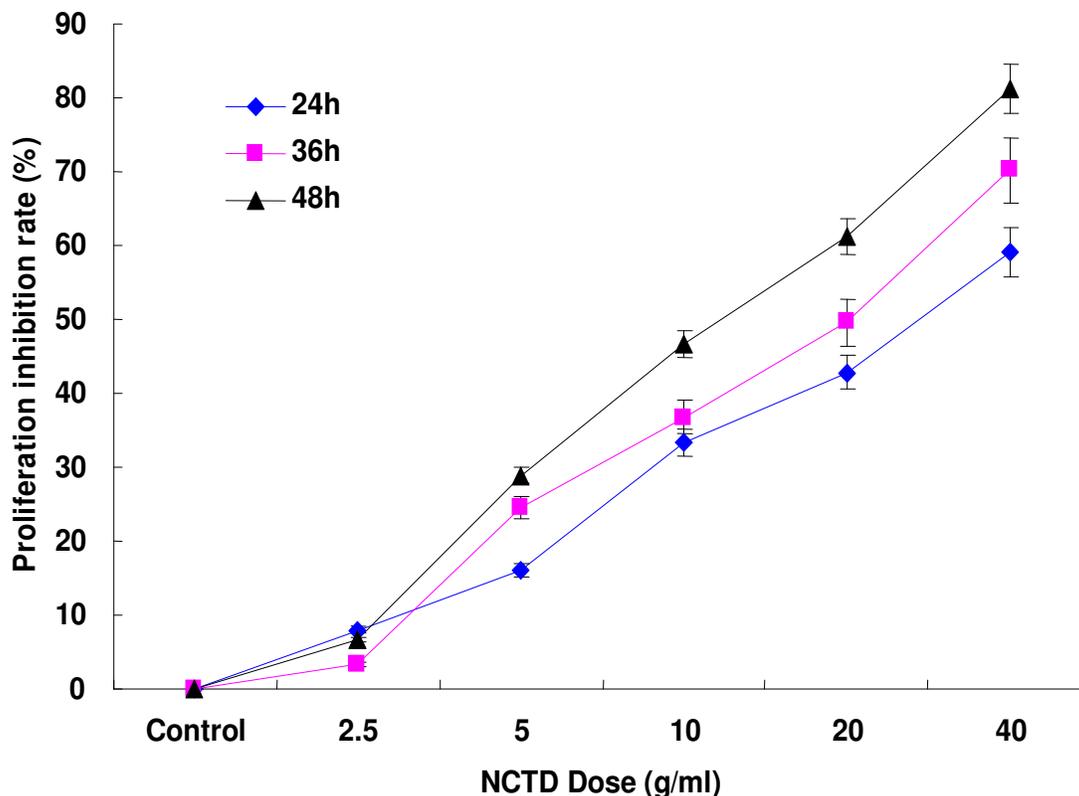


Figure 1. Proliferation inhibitory effect of NCTD.

20(TBS-T) and then probed with the primary antibody. The primary antibodies were diluted in 1% nonfat dry milk in TBS-T as indicated and used for immunoblotting. Protein expression was detected using a primary polyclonal antibody against Bax, Bcl-2, β -actin and a secondary polyclonal antibody conjugated with peroxidase. After washing in TBS-T, membranes were incubated with their appropriate horseradish peroxidase-conjugated secondary antibodies and developed using an enhanced chemiluminescence detection system according to the instructions of the manufacturer and were exposed to x-ray film.

Caspase activity assay

Analysis of caspase-3 and caspase-9 activities was performed using caspase apoptosis detection kit according to the manufacturer's instruction. In brief, cells (1×10^6) were pelleted by centrifugation, washed with PBS two times and incubated in 500 μ l lysis buffer on ice for 10 min, then 1 \times reaction buffer and 10 μ l caspase-3 (DEVD-AFC), caspase-9 (IEVD-AFC) substrates was added to lysis buffer. The reaction mixtures were incubated at 37°C for 60 min. Activities of caspase-3 and -9 were measured by spectrofluorometry.

Statistical analysis

The data were expressed as mean \pm standard deviation (SD) and analyzed by the SPSS 13.0 software to evaluate the statistical difference. Statistical analysis was done using the ANOVA and Bonferroni test. Values between different treatment groups were compared. Results were considered significant at a *P* value of <0.05. All experiments were repeated thrice.

RESULTS

Proliferation inhibitory effect of NCTD

To determine the inhibitory effects of NCTD on HepG2 cells, cytotoxicity assays were carried out as described earlier. Figure 1 shows the apoptotic curves of HepG2 cells treated with various concentrations of NCTD for different exposure times. As shown in the figure, the inhibitory effect of NCTD at low concentrations (2.5 μ g/ml) was not evident, with 8.01 ± 0.44 at 24 h; as the concentration increased, the growth of HepG2 cells was markedly inhibited in a dose-dependent manner, with $59.09\% \pm 3.25$ at 24 h ($p < 0.05$). These data indicate that, NCTD exerts a significant cytotoxic effect upon HepG2 cells in a dose- and time-dependent manner.

Flow cytometric estimation of NCTD induced apoptosis and necrosis

We characterized NCTD-induced apoptosis by annexin V/PI dual staining. Apoptotic cells lose asymmetry of membrane phospholipid, which leaves phosphatidylserine (PS) on the outer leaflet of the plasma membrane. One of the tools for studying apoptosis is to quantify the PS externalization by binding of annexin V. PI is a non-specific DNA intercalating agent, which is excluded by

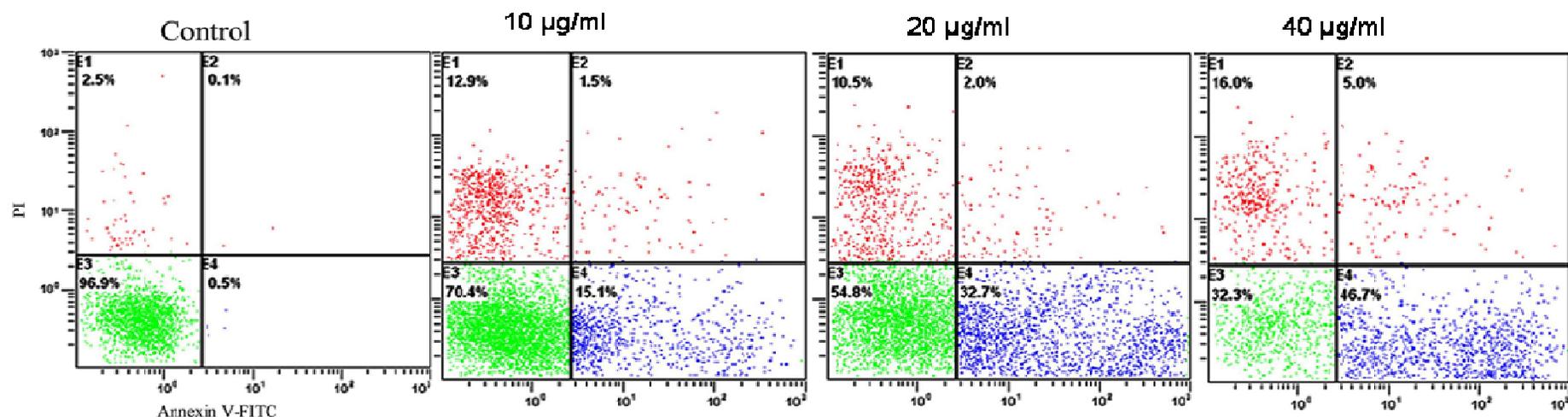


Figure 2. Apoptosis Induced by NCTD. HepG2 cells were treated without or with NCTD (10, 20 and 40 µg/ml) for 24 h then processed for annexin V/PI staining and analyzed by flow cytometry.

the plasma membrane of living cells and thus, can be used to distinguish necrotic cells from apoptotic and living cells by supravital staining without prior permeabilization. This assay divides apoptotic cells into two stages: early (annexin V⁺/PI⁻) and late apoptotic/necrotic (V⁺/PI⁺). As shown in Figure 2, treatment with various concentrations of NCTD (10, 20 and 40 µg/ml) for 24 h resulted in a concentration-dependent increase in both early and late apoptotic/necrotic cells. Furthermore, the percentage of early apoptotic cells was greater than that of late apoptotic/necrotic cells.

Mitochondria membrane potential ($\Delta\psi_m$) determination

There is general agreement that mitochondria are required for apoptosis. Change in mitochondria membrane potential ($\Delta\psi_m$) is one of the early events leading to functional alterations and

compromise of integrity of mitochondria. To examine whether the $\Delta\psi_m$ pathway is involved in NCTD-induced apoptosis, $\Delta\psi_m$ detection was performed using dye JC-1, which was used to assess mitochondrial membrane depolarization. As shown in Figure 3, exposure to NCTD for 24 h resulted in a significant decrease in the ratio between red and green fluorescence by approximately 34.1, 42.6 and 55.8% at 10, 20 and 40 µg/ml, respectively. This suggests that, treatment with various concentrations of NCTD (10, 20 and 40 µg/ml) for 24 h resulted in significant decreases of $\Delta\psi_m$. The results imply that, NCTD induces $\Delta\psi_m$ dissipation in a concentration-dependent manner.

Cyt C release from mitochondria to cytosol

Cyt C release from mitochondria is a critical step in the apoptotic cascade since this activates downstream caspases. To investigate the release

of cyt C in NCTD-treated HepG2 cells, we conducted western blot in both the cytosolic and mitochondrial fractions. The results demonstrate a concentration-dependent increase in the cytosolic cyt C after treatment with NCTD. Simultaneously, there was a decrease in cyt C in the mitochondria fraction (Figure 4).

Expression of Bax and Bcl-2 protein

The collapse of the mitochondria membrane potential occurs through the formation of pores on the mitochondria by dimerized Bax or activated Bid, Bak or Bad proteins. Bax and Bcl-2 are proteins that belong to the Bcl-2 family and they serve as pro- and anti-apoptotic effectors, respectively, to govern mitochondria outer membrane permeabilization (MOMP). The imbalance of anti- and pro-apoptotic protein expression is one of the major mechanisms underlying the ultimate fate of

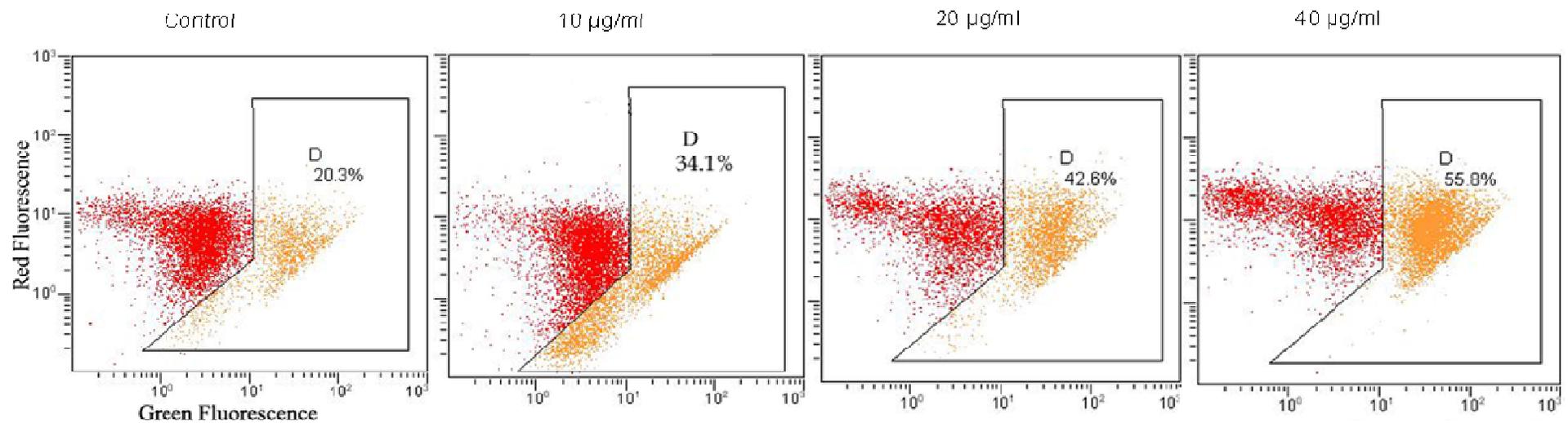


Figure 3. NCTD-Induced $\Delta\psi_m$ depolarization in HepG2 cells. Cells were treated without or with NCTD at the concentrations indicated. Change in $\Delta\psi_m$ was determined by flow cytometric analysis with JC-1.

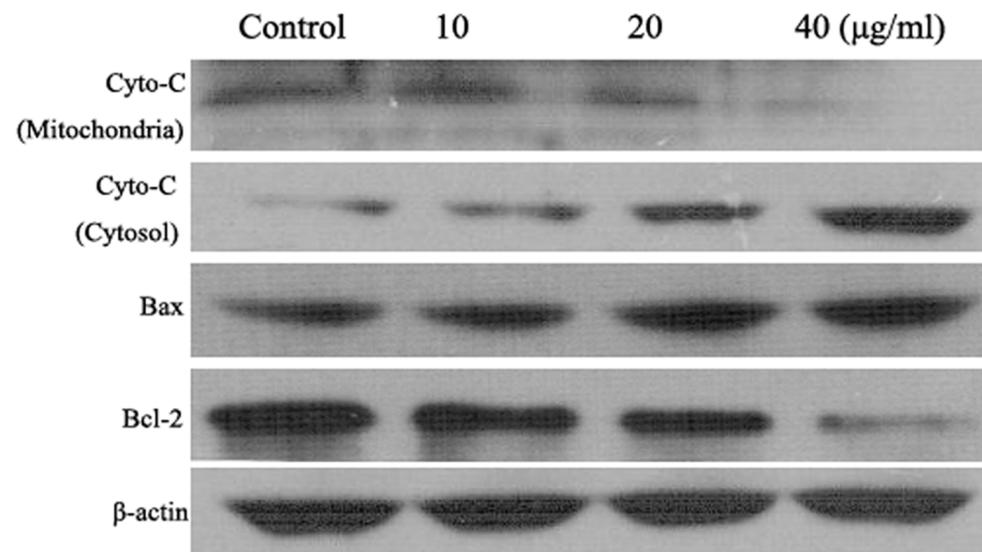


Figure 4. Effect of NCTD on expression of cyto C Bax and Bcl-2 proteins in HepG2 cell.

Table 1. Effects of NCTD on the activation of caspase-3, -9.

Concentration	Caspase 3	Caspase 9
Control	10.07±1.13	36.32±4.39
10 (µg/ml)	18.76±1.22*	48.87±1.72*
20 (µg/ml)	35.71±2.83**	53.89±2.54**
40 (µg/ml)	37.32±1.28**	55.92±3.16**

*P < 0.05 versus control; **P < 0.01 versus control.

cells during the apoptotic process. The results show that, Bax protein levels increased in a dose-dependent manner in NCTD-treated cells, whereas Bcl-2 levels decreased slightly (Figure 4).

NCTD activation of caspase 9 and caspase 3

It is well-known that, proteins of the Bcl-2 family play a pivotal role in apoptosis by affecting caspase activation. Caspase-9, an aspartic-acid-specific protease, is an initiator caspase linked to the mitochondria death pathway and it is activated dearily in the apoptotic cascade by cyt C. Activated caspase-9 stimulates the proteolytic activity of other downstream caspases, including caspase-3. Activated caspase-3, then, leads to the cleavage of numerous proteins. In this study, we demonstrated that, NCTD increased caspase 9 and caspase 3 activities in a concentration-dependent manner (Table 1).

DISCUSSION

Hepatoma is the leading cause of cancer-related death in the world. Treatment of this disease has largely been unsuccessful, mean survival after diagnosis being limited to a few months. Thus, there is an urgent need to identify new therapeutic agents for the treatment of hepatoma *in vivo*. Natural product extracts are a rich source of small molecules that display antitumor activity (Dholwani et al., 2008). Cantharidin, the form of the dried body of the Chinese blister beetles, displays antitumor activity and induces apoptosis in many types of tumor cells (Huang et al., 2011; Kuo et al., 2010; Zhang et al., 2010). However, the highly toxicity for the gastrointestinal tract and kidney limits its usage. A series of bioactive analogues have been synthesized in an attempt to increase the utility and to reduce the toxicity of cantharidin. NCTD, the demethylated form of cantharidin, appeared to cause the least nephrotoxic and inflammatory side effects. The Chinese herb cantharidin has been used in traditional Chinese medicine for more than two thousand years. The first recorded use of cantharidin as an anti-cancer agent was in 1264 (Wang, 1989). Currently, multiple studies *in vitro* and *in vivo* have shown that, NCTD was cytotoxic to various types of tumor cells. The significant apoptotic effects was also observed in tumor cells treated by NCTD. However, the exact mechanism responsible for the apop-

totic effect is not thoroughly elucidated.

A large family of related protein products, which are able to balance mitogenesis and cell death stimuli, regulate apoptosis. This well-defined class of genes is the Bcl-2 family. The Bcl-2 family consists of intracellular, membrane-associated proteins and there is evidence that they may act by controlling mitochondrial permeability (Xiao et al., 2009; Leibowitz et al., 2010). Bcl-2 can inhibit apoptosis caused by a variety of physiological and pathological stimuli. However, its exact mechanism of action is mostly unknown. Several proteins have an amino acid sequence homology with Bcl-2, but some of them function as promoters of cell death rather than suppressors of apoptosis. One of these is Bax, a protein with an extensive amino acid homology with Bcl-2 and it functions to counter the effects of Bcl-2 on cell survival. Moreover, Bax has been shown to bind the C-terminal domain of Bcl-2, forming heterodimers with it. It has been proposed that the ratio of Bcl-2 to Bax expression determines whether a cell is protected or sent to programmed cell death. The Bax gene is ubiquitously expressed and represents a protective factor whose expression is activated by different stimuli such as drug. In addition, Bax is a well known upstream mediator of caspases-9-3, which subsequently, lead to DNA fragmentation and cell apoptosis. In this study, cells treated with NCTD increased the level of the pro-apoptotic protein Bax and decreased the level of the anti-apoptotic protein Bcl-2, suggesting that, NCTD induced apoptosis in HepG2 cells by modulating Bcl-2 family proteins.

Early cellular apoptosis is accompanied by disruption of the mitochondrial membrane, resulting in a rapid collapse in the electrochemical gradient (Jeong et al., 2008). Previous studies have shown that, activation of the mitochondrial pathway by toxicants frequently results in the release of cyt C from the mitochondria into the cytosol. The significance of cyt C to the apoptotic process was revealed by the finding that, mitochondrially released cyt C combines with apoptosis protease activating factor-1, procaspase-9 and dATP in the cytosol, producing active caspase-9 (Oberst et al., 2008; Scorrano et al., 2009; Brooks et al., 2009). The activation of this initiator caspase then leads to the proteolytic activation of caspase-3, the primary effector caspase of the cell. In this study, a remarkable attenuation of $\Delta\psi_m$ occurred in cells exposed to NCTD in a concentration-dependent manner, but not in the untreated cells. Moreover, a concentration-dependent decrease in the mitochondrial cyt C level with a concomitant increase in the corresponding cytosolic fraction, was clearly observed when cells were incubated with NCTD. In addition, in this study, we also observed that, NCTD increased caspase 9 and caspase 3 activities.

Conclusions

In this study, our data indicated that NCTD induced apoptosis in HepG2 cells via mitochondrial pathway and

these findings may provide new insights for understanding the toxic effects of NCTD.

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