Research Article

Norcantharidin Enhances High Concentrations of Fetal Bovine Serum-Induced Apoptosis in Human Mesangial Cells by Regulating the Mitogen-Activated Protein Kinase Signaling Pathway

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Keywords

Norcantharidin · Human mesangial cells · Apoptosis · Mitogen-activated protein kinase pathway

Abstract

Aim: This study aimed to investigate the effect of norcantharidin (NCTD) on human mesangial cells (HMCs) apoptosis in vitro and further examine its molecular mechanism. **Methods:** HMCs were divided into 5 groups: control group, 25% fetal bovine serum (FBS)-treated group, and NCTD groups (NCTD [2.5, 5 and 10 µg/mL] + 25% FBS, respectively). Cell proliferation was determined by MTT assay, while apoptosis was evaluated by Hoechst 33258 staining, the level of cytochrome c, immunohistochemistry, and apoptotic-related proteins/gene expression. **Results:** Cell viability was inhibited in NCTD-treated HMCs in a dose-dependent manner. The number of apoptotic cells and the content of cytochrome c were significantly increased by NCTD treatment but that of mitochondrial membrane was decreased. Moreover, the expression of bcl-2 and caspase-3 was prompted by NCTD, but the expression of bax, MMP-2, and MMP-9 in 25% FBS-treated HMCs was inhibited. In addition, NCTD markedly unregulated the expression of apoptosis-related gene/protein, including p-Erk1/2, phosphorylated-Jun N-terminal kinase (JNK), p-p38, and p53. **Conclusion:** NCTD enhances 25% FBS-treated HMC apoptosis in vitro, and this effect may be attributed to the modulation of the ERK, JNK, and p38 mitogen-activated protein kinase signaling pathways.

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Introduction

Glomerulonephritis is an important cause of morbidity and mortality in patients of all ages worldwide [1, 2]. Mesangial proliferative glomerulonephritis (MsPGN) is the common pathological type of glomerulonephritis, which may contribute to poor patient outcome and high risk of end-stage renal failure [3]. To date, there is no optimal treatment for MsPGN [4]. Hence, it is urgent to find novel therapies to prevent this disease. The imbalance between mesangial cell proliferation and apoptosis is a critical pathological cause for MsPGN. Accumulating evidence has shown that mitogen-activated protein kinase (MAPK) signaling pathways, which consist of at least 3 distinct subfamilies (extracellular regulated protein kinases [ERK], Jun N-terminal kinase [JNK], and p38 MAPK pathways), are involved in the initiation and progression of MsPGN [5–7]. JNK signaling inhibits cell proliferation and promotes apoptosis [8, 9], while P38 signaling inhibits cell proliferation but promotes cell migration [10]. Phosphorylated JNK (p-JNK) makes cell cycle arrest and apoptosis by activating p53, caspase-9, and caspase-3 [11]. As downstreams of p38 MAPK signaling, MMP-2, and MMP-9 are key enzymes involved in cell proliferation and migration [12]. ERK is primarily associated with cell proliferation, migration, and senescence [13, 14]. Thus, the cell proliferation could be inhibited by inducing the cell apoptosis [15].

Norcantharidin (NCTD) is a synthetic demethylated analog of cantharidin, which is a traditional Chinese herb. NCTD has been used as an anticancer drug, such as liver cancer [16, 17], gastric cancer [18], renal cancer [19], colon cancer [20], prostate cancer [21], and so on. It has been reported that NCTD can enhance oxidative stress, promote apoptosis, and modulate the cell cycle [22–24]. NCTD exhibits a protective effect on renal tubule interstitial fibrosis by modulating the TGF- β 1-Smad signal cascade, inhibiting NF- κ B, and decreasing PP2Ac expression, which is a profibrotic factor [25–27]. Interestingly, it has been revealed that NCTD possesses antiangiogenesis effects on human umbilical endothelial cells via downregulating MAPK signaling [28]. Recently, it was found that NCTD was effective in inhibiting human mesangial cell (HMC) proliferation and apoptosis [29]. In addition, NCTD appeared to induce little side effects and lesser metabolic distribution in vitro [30].

In the present study, the effect of NCTD on MsPGN proliferation model, which was induced by high concentration fetal bovine serum (FBS), was investigated, and the apoptosis level and underlying MAPK signaling pathway were explored. The present study expands our knowledge of therapeutic potentials of NCTD for the treatment of glomerulonephritis, especially MsPGN.

Materials and Methods

Materials

NCTD was purchased from Sichuan Sunnyhope Pharmaceutical Co. Ltd. (Sichuan, China). FBS and RPMI-1640 were purchased from Gibco (Grand Island, NY, USA). Dimethyl sulfoxide and MTT were purchased from Beijing Solarbio Science and Technology Co. Ltd. (Beijing, China). The Hoechst 33258 fluorescent dye kit and mitochondrial membrane potential assay kit with JC-1 were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Human Cytochrome C enzyme-linked immunosorbent assay Kit was purchased from Cuasbio Biotech Co. Ltd. (Wuhan, China).

Cell Culture

HMCs were provided by Professor Wen-ge Li of China-Japan Friendship Hospital. These HMCs were cultured in RPMI-1640 supplemented with 10% FBS, 100 U/mL of penicillin, and 100 mg/mL of streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂.

Proliferative Assay and Half Maximal Inhibitory Concentration Determination

The proliferation inhibition potential of NCTD was determined in HMCs using the MTT method. Briefly, cells were seeded in a 96-well plate at a density of 5×10^3 cells/well and were incubated with 25% FBS for 48 h. Then, these were treated with NCTD at final concentrations of 2.5, 5.0, 10.0, or 20.0 $\mu\text{g/mL}$ for 12 h. Untreated cells served as the control group. The viability of cells was assessed using the MTT kit (Amresco, USA), according to manufacturer's protocol. The absorbance of the mixture was measured at 490 nm using a GF-M3000 microplate reader (Shandong Caihong, China). The results were calculated as previously described [31]. The inhibitory concentration values were calculated using nonlinear regression analysis.

Nuclear Morphology Observation by Hoechst 33258 Staining Assay

In order to investigate the cell apoptosis, nuclear morphological analysis was performed by Hoechst 33258 staining. HMCs of 1×10^6 cells per well were plated in a 6-well plate. After the fresh medium was changed with 25% FBS after 48 h, different concentrations of NCTD (2.5, 5, and 10 $\mu\text{g/mL}$) were added into these HMCs. Untreated cells served as the control group. About 12 h later, each well was fixed in 4% paraformaldehyde for 15 min at room temperature. Subsequently, these cells were gently washed 3 times with PBS, followed by incubation with 1 mL (5 $\mu\text{g/mL}$) of Hoechst 33258 solution per well at 37 °C for 10 min in the dark. At the end of the incubation, cells were washed with PBS and mounted by antifade mounting medium. Then, the images were captured and observed using a fluorescence microscope (Olympus IX71, Tokyo, Japan) with an excitation wavelength of 330–380 nm. Strong fluorescence was observed in the nuclei of apoptotic cells, while weak fluorescence was observed in nonapoptotic cells. The quantification of apoptotic cells was performed *via* taking the images in random fields and counting at least 200 cells in 4 random fields in each well.

Determination of Mitochondria Membrane Potential ($\Delta\psi_m$)

HMCs at 1×10^6 cells were cultured in 6-well plates in 2.5 mL of RPMI-1640 medium for 24 h. The medium was changed with fresh medium with RPMI-1640 plus 25% FBS after 48 h. When the cell fusion rate reached approximately 80%, the supernatant was discarded, 2 mL of FBS-free RPMI-1640 medium was added to each well, and these cells were cultured for 24 h. Next, cells were further treated with NCTD (2.5, 5.0, and 10.0 $\mu\text{g/mL}$) for 12 h. Then these cells were stained with mitochondrial membrane potential dye. Cells were incubated with 1 mL of working solution of JC-1 fluorescent dye for 20 min in the dark at 37 °C. Then, cells were gently washed twice with JC-1 dyeing buffer. The mitochondrial membrane potential ($\Delta\psi_m$) was imaged using a fluorescence microscope at 550 nm excitation and 570 nm emission for JC-1.

Determination of Cytochrome c Release in the Cytoplasm

HMCs were seeded in a white-walled 96-well plate and treated with different concentrations (2.5, 5.0, and 10.0 $\mu\text{g/mL}$) of NCTD for 12 h. Then, cytochrome c activity was determined using the enzyme-linked immunosorbent assay kit, according to manufacturer's protocol. Briefly, the treated cells were added with biotin-labeled antibody and incubated at 37 °C for 1 h. After washing working fluid was washed 3 washes, the wells were incubated with horseradish peroxidase-conjugated avidin at 37 °C for 1 h. Then, the samples were measured using a SpectraMax Plus384 microplate reader (Molecular Devices, USA). The progress of the reaction was monitored by measuring absorbance at 450 nm. The results were expressed in ng/mL.

Immunohistochemical Examination of Bax, bcl-2, Caspase-3, MMP-2, and MMP-9

HMCs were seeded in a 6-well plate and treated with different concentrations (2.5, 5.0, and 10.0 µg/mL) of NCTD for 12 h. According to the immunohistochemistry staining method previously described [32], cells were fixed, permeabilized, and stained with the corresponding primary antibody: bax (Abcam, 1:1,000), bcl-2 (Abcam, 1:1,000), caspase-3 (Abcam, 1:1,000), MMP-2 (Cell Signaling, 1:1,000), and MMP-9 (Cell Signaling, 1:1,000). Images were taken using a Leica DM6000 microscope with a magnification of ×200 (Olympus, Germany), and these were analyzed using ImagePro-Plus software. A cytoplasm stained brown was considered a standard for positive cells.

Detection of Erk1/2, Phospho (p)-Erk1/2, JNK, p-JNK, p38, p-p38, and p53 Protein Expression by Western Blot

HMCs were treated with various concentrations (2.5, 5.0, and 10.0 µg/mL) of NCTD for 12 h. Protein extraction and western blot were performed, as previously described [33]. Briefly, the proteins were detected by incubating with primary antibodies (Erk1/2, p-Erk1/2, JNK, p-JNK, p38, p-p38, and p53 [all were purchased from Cell Signaling Technology, USA]) overnight at 4 °C. After hybridization with horseradish peroxidase-conjugated secondary antibody, the protein bands were detected using Odyssey V 3.0 software. GAPDH was the protein loading control.

Analysis of p53 mRNA by Real-Time Polymerase Chain Reaction

HMCs were seeded in a 6-well plate at a density of 30,000 cells/well and treated with various concentrations (2.5, 5.0, and 10.0 µg/mL) of NCTD for 12 h. The total RNA was extracted from cells using TRIzol reagent (Ambion, USA) and quantified by a spectrophotometer (Thermo Scientific, USA). Reverse transcription was carried out in 20 µL of reaction mixture containing 1 µg of total RNA. A reverse transcriptase (Takara) kit was used to synthesize the cDNA by incubating at 25 °C for 10 min, and subsequently at 50 °C for 60 min. Then, DNA amplification was performed using the cDNA template and primers (Invitrogen, USA) with the 7500 real-time polymerase chain reaction system (Applied Biosystems, USA). The sequences of the primers used in the present study were as follows: P53 (forward primer: 5'-GAACAGCTTTGAGGTGCGTG-3', reverse primer: 5'-CTTCTTTGGCTGGGAGAGG-3') and GAPDH (forward primer: 5'-TTCACCACCATGGAGAAGGC-3', reverse primer: 5'-AGAGGGGGCAGAGATGATGA-3'). The final results were described with the relative values ($2^{-\Delta\Delta C_t}$) [34].

Statistical Analysis

Statistical analysis was performed by SPSS version 17.0, and all data were presented as mean ± SD. Differences between 2 groups were compared using Student *t* test when these had a normal distribution. One-way analysis of variance was used to compare data among groups when these had normal distribution and homogeneous variances. A *p* value of <0.05 was considered statistically significant.

Results

NCTD Inhibits the Viability of HMCs

MTT assay was used to evaluate the viability of HMCs. Following NCTD treatment on HMCs, it was found that cells grew slowly, distorted, round in, and detached from the bottom of the plate. Furthermore, the number of detached cells increased as the increasing drug concentration. As shown in Figure 1, cell viability was significantly higher in cells only treated

Fig. 1. Cell viability. **A** The cytotoxicity of NCTD on 25% FBS-induced HMCs. **B** NCTD decreased cell viability in 25% FBS-induced HMCs. Values are presented as the mean $\pm$ SD ($n = 3$). ^a $p < 0.05$, compared with control group, ^b $p < 0.05$, compared with 25% FBS group. NCTD, norcantharidin; FBS, fetal bovine serum.

with 25% FBS, which was inhibited by NCTD (10 and 20 $\mu\text{g/mL}$; $p < 0.05$) in a dose-dependent manner. The inhibitory concentration value of NCTD in HMCs was 19.75 $\mu\text{g/mL}$, which was calculated by the SPSS software.

NCTD Causes Nuclear Condensation and Cell Apoptosis

The bodies of apoptotic cells shrank in volume and became round. The concentration of the cell nucleus was observed, and the cell nucleus became white after being stained by Hoechst 33258 under a fluorescent microscope. Untreated HMCs, which displayed normal chromatin patterns and cell size, were stained with Light blue fluorescence (Fig. 2). After 12 h of treatment with NCTD, these cells exhibited chromatin condensation, cellular shrinkage, apoptotic bodies, and cytoplasmic condensation and were stained with higher blue fluorescence. The number of apoptotic cells underwent similar change in cells subjected to 25% FBS, when compared to the normal control group (Fig. 2; $p > 0.05$). However, compared with the model group, the number of apoptotic cells increased after NCTD treatment (2.5, 5.0, and 10.0 $\mu\text{g/mL}$; $p < 0.05$).

Fig. 2. Multiplex morphological analysis of the cytotoxicity of NCTD on 25% FBS-induced HMCs. **A** Representative cell images in Hoechst 33258 staining and JC-1 determination of mitochondrial membrane potential. Te arrows point to apoptotic cells. **B** Changes in apoptotic cells. Values are presented as the mean $\pm$ SD ($n = 3$). ^a $p < 0.05$, compared with control group, ^b $p < 0.05$, compared with 25% FBS group. FBS, fetal bovine serum; NCTD, norcantharidin.

Fig. 3. NCTD-enhanced cytochrome c release on 25% FBS-induced HMCs. Values are presented as the mean $\pm$ SD ($n = 3$). ^a $p < 0.05$, compared with control group, ^b $p < 0.05$, compared with 25% FBS group. FBS, fetal bovine serum; NCTD, norcantharidin.

NCTD Causes the Disruption of $\Delta\psi_m$ in HMCs

The representative microphotographs of the fluorescence stain were presented in Figure 2. Following the treatment with NCTD (2.5, 5.0, and 10.0 µg/mL), the red light was significantly decreased, which was accompanied by increasing green light in HMCs.

Cytochrome c Release

As shown in Figure 3, NCTD treatment could trigger cytochrome c release from the mitochondria after 12 h of intervention. The cytochrome c level in HMCs decreased in the model group, when compared to the control group ($p < 0.05$). Interestingly, these alternations reversed following NCTD treatment (2.5, 5.0, and 10.0 µg/mL; $p < 0.05$).

NCTD Induced the Apoptosis of HMCs

The immunohistochemical changes of HMCs were presented in Figure 4. A decline in the number of bcl-2, MMP-2, and MMP-9 positive cells and an increase in the number of bax and caspase-3 positive cells were observed in the 25% FBS group, when compared to the control group. The treatment with NCTD (5.0 and 10.0 µg/mL) produced a significant reverse on these changes.

Effect of NCTD on Apoptotic-Related Protein Expression

As shown in Figure 5, in the model group, the phosphorylation level of Erk1/2, JNK and p38 significantly decreased by 20.0, 36.4, and 23.8%, respectively, when compared to the control group. By treatment with NCTD (2.5, 5.0, and 10.0 µg/mL), the expression of p-Erk1/2, p-JNK, and p-p38 increased, when compared with the model group. Meanwhile, the protein expression of p53 was significantly lower in the model group, when compared with the control group, and the effect of that was significantly ameliorated by NCTD treatment (2.5, 5.0, and 10.0 µg/mL).

Effect of NCTD on p53 mRNA Expression

In the present study, the effect of NCTD on p53 mRNA level in HMCs was determined by real-time-polymerase chain reaction. As shown in Figure 6, compared to the control group, the p53 mRNA level in the model group was significantly reduced, but markedly increased after NCTD treatment at various concentrations (2.5, 5.0, and 10.0 µg/mL).

Fig. 4. Immunohistochemical changes. HMCs were stained with DAB and antibody against bax, bcl-2, caspase-3, MMP-2, and MMP-9. **A** Representative images displaying the number of positive cells. **B** Calculation of the number of positive cells (cells/mm²). Each column represents the mean $\pm$ SD ($n = 3$). Scale bar = 100 μm . ^a $p < 0.05$, compared with control group, ^b $p < 0.05$, compared with 25% FBS group. FBS, fetal bovine serum; NCTD, norcantharidin.

Fig. 5. Effect of NCTD on apoptotic-related protein expression. The bands are from a representative blot. Values are presented as the mean ± SD ($n = 3$). ^a $p < 0.05$, compared with control group, ^b $p < 0.05$, compared with 25% FBS group. p-JNK, Jun N-terminal kinase; FBS, fetal bovine serum; NCTD, norcantharidin.

Fig. 6. Effect of NCTD on p53 mRNA expression. Values are presented as the mean $\pm$ SD ($n = 3$). ^a $p < 0.05$, compared with control group, ^b $p < 0.05$, compared with 25% FBS group. FBS, fetal bovine serum; NCTD, norcantharidin.

Discussion

HMC proliferation is the common feature of glomerulosclerosis, which can be induced by high concentration of FBS [35, 36]. The previous study demonstrated that the growth of HMCs could be significantly inhibited by NCTD [29], and this may be mediated by inducing cell apoptosis and modulating the cell cycle. In the present study, a successful HMC proliferation model was established by incubating with 25% FBS, which was consistent with previous reports [35, 37]. The present results revealed that the administration of NCTD inhibited HMC proliferation in a concentration-dependent manner. Moreover, NCTD improved HMC apoptosis, which was evidenced by the decrease in $\Delta\psi_m$, the increase in apoptosis rate, the release of cytochrome c, and the apoptotic-related gene/protein expression. Importantly, the underlying mechanism of NCTD proapoptosis may involve the regulation of MAPK signaling pathway. These results suggested that the NCTD had the potential to attenuate MsPGN.

Apoptosis and programmed cell death are crucial mechanisms of proliferation inhibition [15]. The present study demonstrated that NCTD reduced cell viability and increased apoptosis in HMCs treated with 25% FBS. In addition to nuclear condensation, the number of apoptotic cells increased after NCTD treatment, which was confirmed by Hoechst 33258 staining and higher blue fluorescence occurrence.

Apoptosis can be induced by the intrinsic or extrinsic pathway. It was found that NCTD-induced cell apoptosis was accompanied with cytochrome c release, which is the hallmark of apoptosis induction through the intrinsic pathway [38, 39]. Moreover, it was also found that NCTD regulated the expression of the bcl-2 family, which is mainly involved in the intrinsic pathway of apoptosis. In addition, NCTD enhanced the extrinsic pathway factor caspase-3, which improves cell apoptosis.

Mitochondria, as the major energy generator in cells, play a vital role in cell apoptosis induced by stimuli. It has been reported that agents decreased the levels of $\Delta\psi_m$, which may lead to apoptosis in cells through the mitochondria-dependent pathway [40]. Thus, $\Delta\psi_m$ is further characterized, as indicated by the decrease in JC-1 dye. It is well-known that JC-1 red fluorescence in normal cells and the reduction of mitochondrial membrane potential leads to the replacement of red fluorescence by green JC-1 monomers, and the decreasing of ratio of red/green fluorescence indicates the loss of $\Delta\psi_m$ [41]. The present study revealed that mitochondrial depolarization became remarkable with NCTD treatments, as evidenced by NCTD, which caused decrease in JC-1 aggregates and dose-dependent increase in JC-1 monomers. The present study was in agreement with another study, in which NCTD-induced cytotoxicity

in tumor cells by apoptosis, and this was probably mediated through the mitochondrial-dependent pathway [18]. The present results suggested that the reason why NCTD could induce apoptosis of HMCs was that the induced release of cytochrome c into the cytoplasm disrupted the mitochondrial permeability.

In the present study, it was observed that NCTD increased the level of caspase-3, but inhibited that of MMP2 and MMP9. In order to further explore the mechanism underlying the NCTD-induced apoptosis, the MAPK signaling pathway was investigated. As a member of the MAPK family, JNK is mainly responsible for apoptosis induction. The activated p-p38 contributes to the induction of apoptosis and inhibition of cell proliferation [42, 43]. P53 is involved in coordinating apoptosis to preserve genomic stability and prevent cell excessive proliferation [11, 44]. Blocking the phosphorylation of MAPK generates the subsequent inhibition of apoptosis through the regulation of caspase-3 activity [45]. The present study demonstrated that NCTD induced the phosphorylation of JNK and p38 and increased the expression of p53, which suggested the involvement of the activation of JNK and p38, and the expression of p53 in the antiproliferation effects of NCTD.

The classical ERK family (p42/44 MAPK) is known to be an intracellular checkpoint for controlling cellular G₀/G₁ mitogenic signals and cell cycle progression [46, 47]. Traditionally, growth factors and several cytokine receptors activate the ERK pathway by the JAK cascade, eventually leading to cell proliferation, differentiation, and development [48]. Although the survival rate can be promoted by the activation and phosphorylation of Erk1/2 [49], contradictory evidence has revealed that the activation of Erk1/2 drives cells to death [50]. The present results supported the latter opinion and revealed that NCTD increased phosphorylation and induced apoptosis. However, the underlying mechanism remains to be determined through further studies.

Several limitations still exist in the present study. First, the sample size was relatively small. In addition, the up- and downstream signal factors in the ERK pathway were not measured in the present study, and animal models are needed to further evaluate the effect of NCTD on the inhibition of cell proliferation.

In conclusion, the present finding demonstrates for the first time that the administration of NCTD induces 25% FBS-treated HMC apoptosis. This effect may be mediated by the ERK, JNK, and p38 MAPK signaling pathways. The present findings suggest that NCTD may be a potential antiproliferation drug for the MsPGN. However, further studies *in vivo* are required to confirm these findings.

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Disclosure Statement

The authors declare that there are no conflicts of interest.

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