

## Original Article

# Effects of norcantharidin on histochemistry and ultra-microstructure of cholangiocarcinoma RBE cells

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**Abstract:** *Objective:* This study aimed to observe the effects of norcantharidin on the histochemistry and ultra-microstructure of human cholangiocarcinoma RBE cells, and explore its mechanism. *Methods:* Cholangiocarcinoma cell line RBE were cultured *in vitro*, and treated with different concentrations of norcantharidin (5-40 µg/ml). RBE cell apoptosis rate was calculated by AO/EB staining, and morphological changes were observed. Ultra-microstructure changes in the RBE cell surface were observed under a scanning electron microscope (SEM), and intra-cellular ultrastructural changes were observed under a transmission electron microscope. *Results:* acridine orange (AO)/ethidium bromide (EB) fluorescent staining results indicated that RBE cell apoptosis rate increased with the increase of norcantharidin dosage, and apoptotic cells accounted for  $83.1 \pm 15.6\%$  after norcantharidin treatment for 24 hours at a dose of 40 µg/ml. Typical morphological changes of apoptosis were observed under light microscope and a scanning electron microscope, and the formation of apoptotic bodies were observed. A crescent shaped change in the nucleus was observed under transmission electron microscope. *Conclusions:* Norcantharidin can lead to microscopic and ultrastructural changes in cholangiocarcinoma RBE cells. These changes may closely be related to variations in cell apoptosis and invasiveness.

**Keywords:** Cholangiocarcinoma, norcantharidin, morphology, electron microscopy

## Introduction

Cantharidin is extracted from traditional Chinese medicine cantharides, which has achieved certain curative effects in the treatment for a variety of malignant tumors [1]. On the base of cantharidin, two methyls at 1, 2 sites are absent in norcantharidin (NCTD), which makes its anti-tumor activity retained, while the toxicity and adverse reaction are significantly reduced [2]. In previous studies, NCTD showed significantly inhibitory effect on the proliferation of cholangiocarcinoma RBE cells, and could induce apoptosis through the mitochondrial pathway and the destruction of the tubulin cytoskeleton [3, 4]. In this study, we observed the microstructural and ultrastructural changes in the cholangiocarcinoma cell line RBE induced by NCTD, and explored the mechanism of cholangiocarcinoma cell apoptosis induced by NCTD.

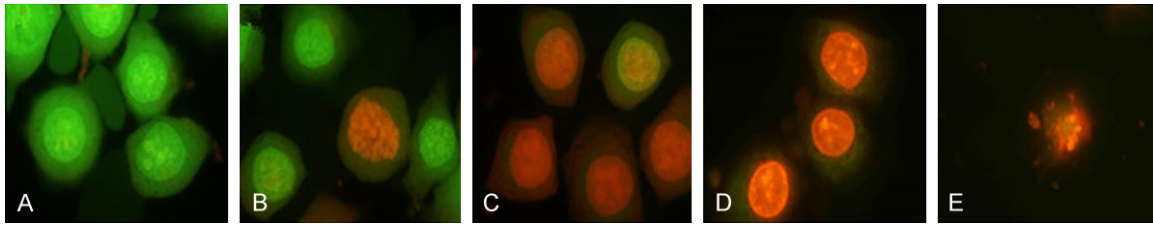
## Materials and methods

### Materials

Cholangiocarcinoma cell line RBE-TCHU179 was purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai), cultured in RPMI 1640 medium (GIBCO, USA) containing 10% fetal bovine serum (Hangzhou Sijiqing), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Solarbio), under the conditions of a saturated humidity environment with 5% CO<sub>2</sub> at 37°C.

NCTD injection was purchased from Sigma and diluted to a final concentration with serum-free RPMI 1640 medium prior to use. Acridine orange/ethidium bromide (AO/EB) staining reagents were purchased from Solarbio; fetal calf serum was purchased from Hangzhou Sijiqing; 100 U/ml penicillin and 0.1 mg/ml streptomycin were purchased from Solarbio;

## Effect of norcantharidin on human cholangiocarcinoma cells line RBE



**Figure 1.** Effect of norcantharidin on RBE cells, AO-EB fluorescence staining (1000 $\times$ ). A: Control group; B-E: Norcantharidin group; B: 5  $\mu\text{g/ml}$ ; C: 10  $\mu\text{g/ml}$ ; D: 20  $\mu\text{g/ml}$ ; E: 40  $\mu\text{g/ml}$ .

and RPMI1640 culture medium was purchased from GIBCO (USA).

### *AO-EB staining*

Cholangiocarcinoma cells RBE in the logarithmic growth phase were digested by trypsin and prepared into cell suspensions. Cell density was adjusted to  $5 \times 10^4$  cells/ml, and 2 ml of cell suspensions were added into each well of the 6-well plate with preset coverslips. After culturing for 24 hours, cells attached to the wall and grew into the logarithmic growth phase, and the original medium was discarded. RPMI 1640 culture medium containing different concentrations of NCTD (5, 10, 20 and 40  $\mu\text{g/ml}$ , respectively) were added into wells for the experimental groups; while, equal volumes of 1640 culture medium were added into wells for the control group, and intervened for 24 hours. Then, the culture medium was discarded, cells were rinsed with phosphate-buffered saline (PBS) three times and stained with 50 ml of AO/EB at 37°C for one minute in the dark. Cells were observed under fluorescence microscope at an excitation wavelength of 510 nm.

### *Observation of ultra-microstructural changes in the RBE cell membrane surface induced by NCTD under scanning electron microscope*

Cholangiocarcinoma RBE cells in the logarithmic growth phase were inoculated in a 6-well plate with preset coverslips on the bottom at a density of  $5 \times 10^4$  cells/well and conventionally cultured for 24 hours until cells well adhered and grew into the logarithmic phase. Then, cells were rinsed with serum-free RPMI 1640 medium, then in the treatment groups 5  $\mu\text{g/ml}$  or 20  $\mu\text{g/ml}$  NCTD was added, respectively; and equal volumes of RPMI 1640 medium were added in the control group. Intervention was carried out for 12 hours. Next, cells were washed with PBS, fixed with 1% glutaraldehyde

for five minutes, washed again with PBS three times, and dehydrated with 50% ethanol, 70% ethanol, 80% ethanol, 90% ethanol, and anhydrous ethanol, respectively, step by step; and each step was performed for five minutes. Then, cells were dehydrated with anhydrous ethanol twice, and vacuum dried at a critical point after being fixed on a copper specimen table. After cells were gold-plated (by an ion plating instrument, IB-3), they were observed under scanning electron microscope (Hitachi S-3500N).

### *Observation of ultrastructural changes in RBE cells induced by NCTD under transmission electron microscope*

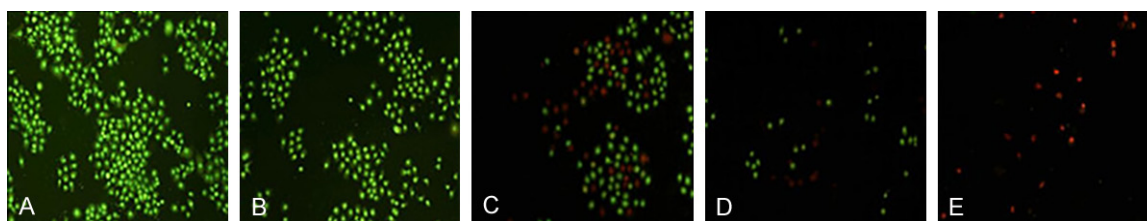
In the logarithmic growth phase, cholangiocarcinoma RBE cells were intervened with 20  $\mu\text{g/ml}$  NCTD for 12 hours in the treatment group; and in the control group, equal volumes of RPMI 1640 culture medium were added instead. Cells were digested into cell suspension by trypsin, washed with PBS twice, centrifuged at 1,500 rpm for 10 minutes, and the supernatant was discarded. Next, the cell mass was fixed with 2.5% glutaraldehyde at 4°C for 24 hours, and fixed with 1% diacid for two hours after the glutaraldehyde was washed away. Then, cells were washed with PBS, gradually dehydrated with acetone, substituted with propylene oxide, embedded with epoxy resin, and polymerized at 70°C for eight hours. Then cell mass was prepared into ultrathin sections and stained with uranyl acetate and lead citrate, cells were observed under transmission electron microscope.

## **Results**

### *Effects of NCTD on cholangiocarcinoma RBE cell structure*

Changes in cell structure were observed through AO/EB staining. In the control group,

## Effect of norcantharidin on human cholangiocarcinoma cells line RBE



**Figure 2.** Effects of norcantharidin on apoptosis of RBE cells, AO/EB fluorescence staining (100×). A: Control group; B-E: Norcantharidin group; B: 5 µg/ml; C: 10 µg/ml; D: 20 µg/ml; E: 40 µg/ml.



**Figure 3.** Influence of norcantharidin on the surface ultrastructure of cholangiocarcinoma RBE cell. A: Control (3.0 K×); B: 20 µg/ml (3.0 K×); C: 40 µg/ml (4.0 K×).

cells were irregular in shape and had clear boundaries. Furthermore, cells were polygonal with long filament protrusions; and the cytoplasm was abundant, a uniform distribution of green fluorescence was found in the cytoplasm, the nuclei were large and round, the nuclear membranes were clear and obvious, the chromatin distributed evenly and the nucleoli were prominent, and the mitotic figures were common (**Figure 1A**). When cells were treated with low doses (5-20 µg/ml) of NCTD, cells became round, cell membranes shrunk and filament protrusions were reduced, the cytoplasm was reduced and nuclear membranes were thickened and shrunk, and the orange fluorescence was visible in the nucleus (**Figure 1B-D**). When cells were treated with high doses (40 µg/ml) of NCTD, cells became round, envelope protrusions were reduced, the nuclei shrunk and disintegrated, and the chromatin were clump shaped and showed bright red fluorescence (**Figure 1E**).

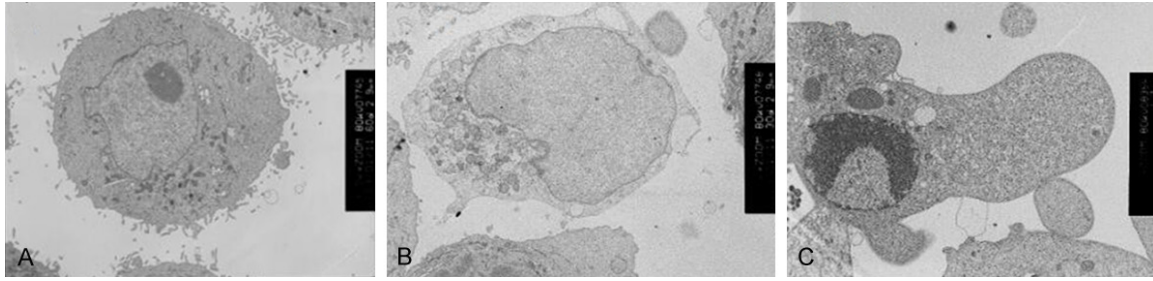
*AO/EB staining method was used to calculate cell apoptosis*

RBE cells were treated with NCTD for 24 hours, stained with AO/EB fluorescent dying reagent, and observed under a 100× low magnification microscope (**Figure 2A**). In the control group, all

cells showed green fluorescence; while in the low dose group (5 µg/ml), majority of the cells showed green fluorescence. However, some apoptotic cells with orange fluorescence sparsely distributed ( $29.5 \pm 5.9\%$ , **Figure 2B**). When treated with 10 µg/ml or 20 µg/ml NCTD, orange fluorescence appeared in clusters in cells; and the proportion of these cells in the field of vision increased ( $37.1 \pm 14.5\%$  and  $68.5 \pm 27.2\%$ , respectively; **Figure 2C** and **2D**). When treated with a dose of 40 µg/ml, orange cells in the field of vision accounted for  $83.1 \pm 15.6\%$  (**Figure 2E**); which were significantly higher than that in the negative control group (correction continuity for the  $\chi^2$ -test,  $P < 0.01$ ).

*Observation of RBE cell surface ultrastructural changes induced by NCTD under scanning electron microscope*

In the control group, cells were polygonal and the capsules were intact, the surface was rough with shrinkage and microvilli structures, a plurality of elongated projections could be observed, and the cellular junctions were visible between cells (**Figure 3A**). When cells were treated with 20 µg/ml NCTD, cell projections became fine and fractured, the microvilli on the surface disappeared, and the surface was deformed (**Figure 3B**). When cells were treated



**Figure 4.** Influence of norcantharidin on the surface ultrastructure of RBE cells. A: Control group (3.5 K $\times$ ); B: 20  $\mu$ g/ml (3.5 K $\times$ ); C: 40  $\mu$ g/ml (6.0 K $\times$ ).

with 40  $\mu$ g/ml doses of NCTD, cells became round, the microvilli and protrusions on the surface completely disappeared, holes appeared on the membrane structure, and vesicle-like structures formed and protruded out off the cell surface (**Figure 3C**).

#### *Observation of ultrastructural changes in RBE cells induced by NCTD under transmission electron microscope*

In the control group, microvilli protrusions were found on the cell surface, the cytoplasm was abundant, mitochondria, free ribosomes, and a rough endoplasmic reticulum were visible, the nucleus were large, nuclear membranes were smooth, the chromatins distributed evenly, and the nucleolus was obviously visible (**Figure 4A**). When cells were treated with 20  $\mu$ g/ml NCTD, microvilli on the cell surface disappeared, the cytoplasm was reduced, the nuclear membrane was sharp and protruded outwards, spherical bulges located outside the cell membrane were visible, and small vacuoles appeared in the cytoplasm (**Figure 4B**). When cells were treated with 40  $\mu$ g/ml NCTD, the microvilli on the cell surface disappeared; the chromatins in the nucleus were condensed, its volume was reduced, its electron density was increased and the chromatins concentrate in the cell border and presented a crescent shape. Furthermore, the endoplasmic reticulum swelled and degranulated, cytoplasmic vacuoles increased, the mitochondrial cristae disappeared, the envelope showed an outward spherical bulge, and part of cytoplasm filled in this space (**Figure 4C**).

#### **Discussion**

Cholangiocarcinoma is usually occult at onset due to its special anatomical location and the

characteristics of early invasion into the vascular, nerve, lymphatic tissues and adjacent liver tissues; and it has become a surgical problem due to its great difficulty in operation and poor prognosis [5]. The chemotherapeutic sensitivity of cholangiocarcinoma is low, and there are no standard chemotherapeutic regimens. However, NCTD can play a role in anti-cancer in multiple aspects through inducing apoptosis and inhibiting metastasis; arresting cell cycle and inhibiting tumor angiogenesis [1]. As an anti-tumor drug that can elevate the numbers of white blood cells [6], NCTD has continuously gained more attention. In this study, we investigated the effects of NCTD on the microstructure and ultrastructure of cholangiocarcinoma cells, and discussed the mechanism with the RBE cell line of cholangiocarcinoma as the model.

AO/EB double fluorescence staining is a staining method for detecting cell apoptosis, and can reflect the overall trend of changes in cells. Normal cancer cells can be stained into green fluorescence with AO. When apoptosis occurs, the cell membrane becomes damaged, and the chromatins can be stained with an orange fluorescence by EB [7]. Apoptotic cells could reach 47.8% of the total number of cells when treated with 40  $\mu$ g/ml NCTD, which can be determined by calculating the proportion of apoptotic cells through AO/EB staining. Changes in cells could be further observed under high magnification microscope. Compared with the control group, typical changes in damaged cells could be observed in the experimental group. The most significant changes were found in the cell nucleus, including changes in chromatin condensation and margination, and nuclear fragmentation; which are consistent with the characteristics of apoptosis.



When observed under scanning electron microscope, morphological changes of cholangiocarcinoma RBE cells revealed a reduction in pseudopodia when treated with low concentrations of NCTD [8]. In tumor cells, the main means of cell movement is the movement of pseudopodia; which is formed depending on the correct actin assembly [9]. As drug concentration increases, these cells became round, filopodia protrusions disappeared, and cells lost their microstructure for transferring; which lead to the suppression of cancer cell invasion [10]. When treated with high concentrations of NCTD, vesicle-like changes were found in part of the cells, which may be apoptotic bodies. This can be confirmed by observation under transmission electron microscope. However, in this experiment, holes on the membrane structures of some cells were also observed; and part of the cytoplasm were spilled over from these holes. This cell damage accompanied by the destruction of the integrity of the membrane structure could lead to the occurrence of inflammatory response [11].

When observed under transmission electron microscope, the nuclei of cholangiocarcinoma RBE cells were large, the number of nucleoli increased, and microvilli and other typical tumor cell structures appeared on the membrane surface [12]. In the experiment, when cholangiocarcinoma RBE cells were treated with NCTD, the microvilli vanished, the cytoplasm was condensed, and chromatin condensation and margination were observed. Furthermore, in the high dose group, a half-moon like chromatin was formed, which is a typical change of chromatin damage in early apoptosis [13]. In addition, vacuole like changes could also be observed in the cytoplasm. Previous studies have shown that NCTD could induce apoptosis through the activation of the bax/bcl-2 pathway on the mitochondrial membrane, which release cytochrome C into the cytosol, in order to activate cascades reactions of the caspase mitochondrial pathway [3, 4]. In this process, mitochondrial membrane permeability is changed, which leads to mitochondrial swelling [14]; and vacuole-like and pulpiform structures could be observed under transmission electron microscope.

Results in this study revealed that NCTD can cause microscopic and ultrastructural changes

in cholangiocarcinoma RBE cells, and these changes may have a close relationship with changes in apoptosis and the invasiveness of cholangiocarcinoma cells.

## Disclosure of conflict of interest

None.

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