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Original Article

Cantharidin exhibits promising inhibitory effect on cell viability in oral cancer cells through mitochondrial pathway

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Abstract: Present study aimed to investigate the effect of cantharidin on reduction in cell viability and induction of apoptosis in KOSC-2 oral cancer cells. Cantharidin treatment for 48 h caused a concentration dependent reduction in KOSC-2 cell viability. The IC_{50} of cantharidin against KOSC-2 cells was found to be 50 nM after 48 h. KOSC-2 cells showed reduction in size, protrusion of membrane and condensation of nuclear material on treatment with 50 nM concentration of cantharidin for 48 h. Flow cytometry using propydium iodide staining revealed significant ($P<0.05$) increase in the population of cells in G0/G1 phase of cell cycle in cantharidin treated compared to the control cells. Results from western blot analysis showed increase in the expression of pro-apoptotic BAD and decrease in the expression of anti-apoptotic protein Bcl-2 on treatment with cantharidin in KOSC-2 cells. Cantharidin treatment for 48 h also resulted in a significant increase in the expression of Apaf-1 and AIF as well as translocation of cytochrome c into the cytosol. The activation of caspase-3 in KOSC-2 cells was enhanced significant ($P<0.05$) in cantharidin treated compared to the control cells. Thus cantharidin treatment inhibits KOSC-2 cell viability and induces apoptosis through mitochondrial pathway. Therefore, cantharidin can be used for the treatment of oral cancer.

Keywords: Apoptosis, nuclear material, inhibition, cantharidin, terpenoid

Introduction

Oral cancer represents a cancerous tissue present in the oral cavity and is considered to be one of the types of head and neck cancer [1]. It has been observed that in most of the oral cancer cases squamous cell lining the mouth is affected [2]. The currently used treatment strategies for the oral cancer involve use of chemotherapeutic agents, radiations and surgical removal. However, despite availability of these treatment techniques the average five years survival rate of oral cancer patients is poor [2]. Thus the discovery of novel and efficient treatment methods for oral cancer is highly desired.

Apoptosis is the controlled death of cells which is regulated by mitochondrial or non-mitochondrial pathways. The process of apoptosis is responsible for the removal of unwanted cells for the body in multicellular organisms [3]. Induction of apoptosis is characterized by reduction in cell size, protrusion of the cell membrane and condensation of the nuclear material [4]. Many of the chemotherapeutic agents inhibit cell viability through induction of the apoptosis [5, 6]. Apoptosis induction leads to inhibition of the cell viability and therefore the strategy can be used for the cancer treatment. Apoptosis is regulated at the genetic level by several genes which act in a cascade [7, 8]. There are two types of proteins in the Bcl-2 family which also play important role in the regulation of apoptosis. The pro-apoptotic proteins included Bax and Bid facilitate apoptosis and the anti-apoptotic proteins included Bcl-2 and Bcl-xL, which inhibited apoptosis [7, 9]. Expression of pro-apoptotic factors and their translocation to nucleus leads to cytochrome c release into the cell cytosol which then induces [10].

Cantharidin, a natural isolate from Blister Beetles has been used in the traditional Chinese medicine for very long time [11, 12].

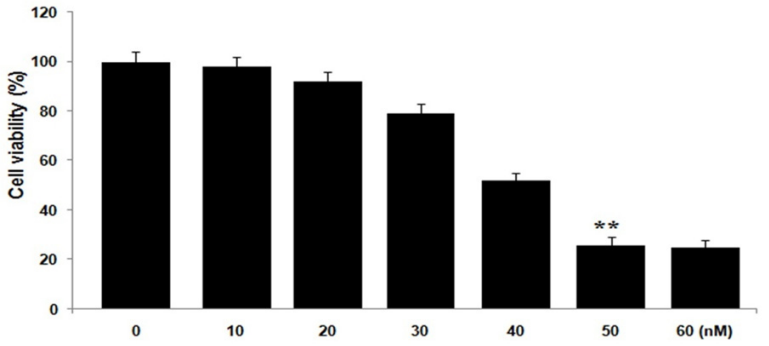

Figure 1. Reduction in viability of KOSC-2 cells by cantharidin treatment in dose dependent manner. The cells were incubated with range of cantharidin concentrations for 48 h and then viability was determined. Viability of cantharidin treated cells was compared with the untreated control cells. The results presented are the mean ± SD of three experiments performed independently. *P<0.05 compared with the control group.

Treatment of the carcinoma cells with cantharidin results arrest of cell cycle and induction of apoptosis [13]. Cantharidin treatment inhibits growth of various cancer lines including, colon, hepatoma, bladder and breast cancer cells [14-16]. In bladder cancer cells exposure to cantharidin leads induction of apoptosis and arrest of cell cycle in the G2/M phase. Another mode of action of cantharidin involves promotion of the COX2 and PGE2 expression in T24 cells [17]. The present study was designed to investigate the effect of cantharidin on inhibition of cell viability and induction of apoptosis in KOSC-2 oral cancer cells. The results showed that cantharidin treatment inhibited cell viability and induced apoptosis through mitochondrial pathway.

Materials and methods

Chemicals and reagent

Cantharidin, dimethyl sulfoxide (DMSO) and propidium iodide (PI) were purchased from the Sigma-Aldrich Corp. (St. Louis, MO, USA). Stock solution of cantharidin was prepared in DMSO and stored at -15°C prior to use in the experiments.

Cell lines and culture

Human oral cancer cell line KOSC-2 was obtained from the American Type Culture Collection center (Manassas, VA, USA). The cells were cultured in DMEM Gibco/Life Technologies (Carlsbad, CA, USA) supplemented with 10% FBS in humidified atmosphere of 5% CO_2 and 95% air at 37°C. The media contained antibiotics penicillin (100 U/ml) and streptomycin (100 µg/ml).

Cell viability

Cell viability was measured by tetrazolium salt (WST)-1 assay (EZ-CyTox Enhanced Cell Viability Assay kit; Daei-Lab Service, Seoul, Korea). Briefly, KOSC-2 cells were incubated with various concentrations of cantharidin for 48 h and then washed with PBS. After PBS washing, WST-1 solution was added to each well of the plate and incubation was continued for 4 h more. Absorbance for each well of the plate was measured at a wavelength of 475 nm using BIOTEL™ (EL-800) according to the manufacturer's instructions. All the measurements were performed in triplicates.

Determination for cell morphology

KOSC-2 cells were distributed at a density of 2×10^6 cells per well into 24-well plates. Various concentrations of cantharidin were added to each well of the plate and the cells were incubated for 48 h. Alterations in the morphology of cells was observed according to the earlier reported protocol (18) by phase-contrast microscope. For chromosomal condensation cantharidin treated cells were fixed on cover slips using 70% ethyl alcohol and then subjected to DAPI staining. The cells were then observed under a fluorescence microscope.

Apoptosis

For the analysis of apoptosis induction in KOSC-2 cells Annexin V-FITC Apoptosis Detection kit (BD Bioscience, San Jose, CA, USA) was used. The cells seeded at a density of 2×10^6 per well were treated for 48 h with cantharidin. After incubation, the cells were washed in ice cold PBS and resuspended in binding buffer (100 µl). Then the cells were incubated for 20 min with 5 µl Annexin V-FITC (BD Bioscience) and 10 µl propidium iodide (PI; BD Bioscience). Flow cytometer (Bacton Dickson San Jose) was used for the determination of fluorescent intensities. The experiments were carried out in triplicates.

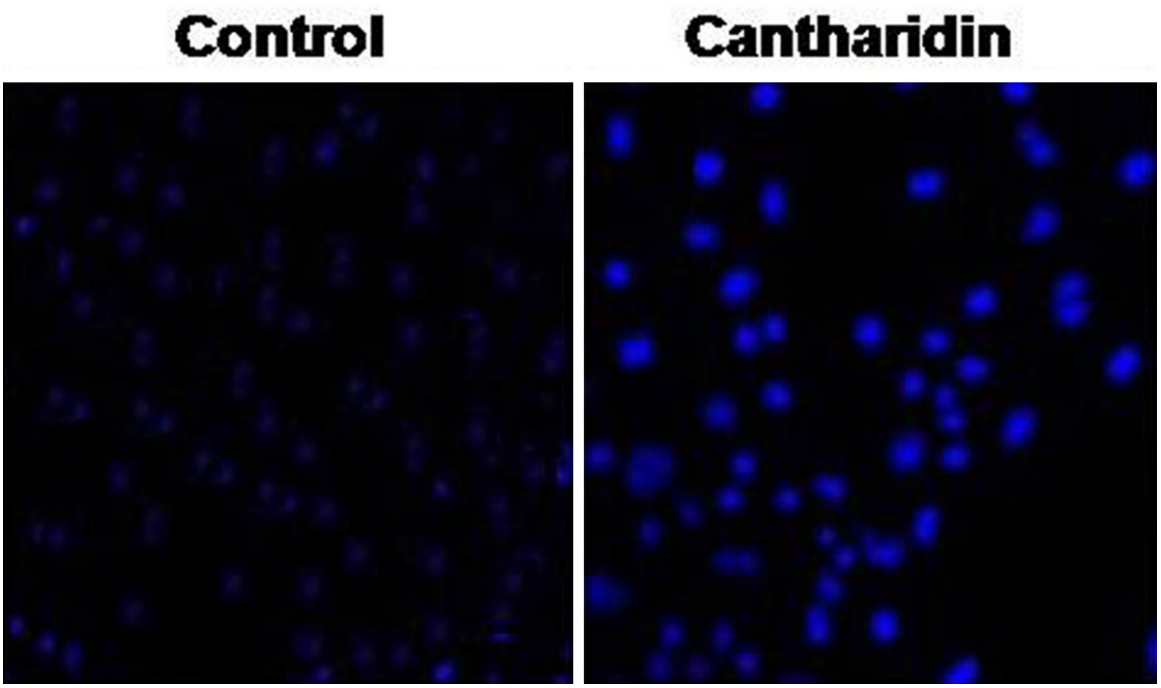

Figure 2. Cantharidin treatment for 48 h altered the morphological features of KOSC-2 cells. The microphotographs of KOSC-2 cells were captured after 48 h of cantharidin treatment at 200× magnification.

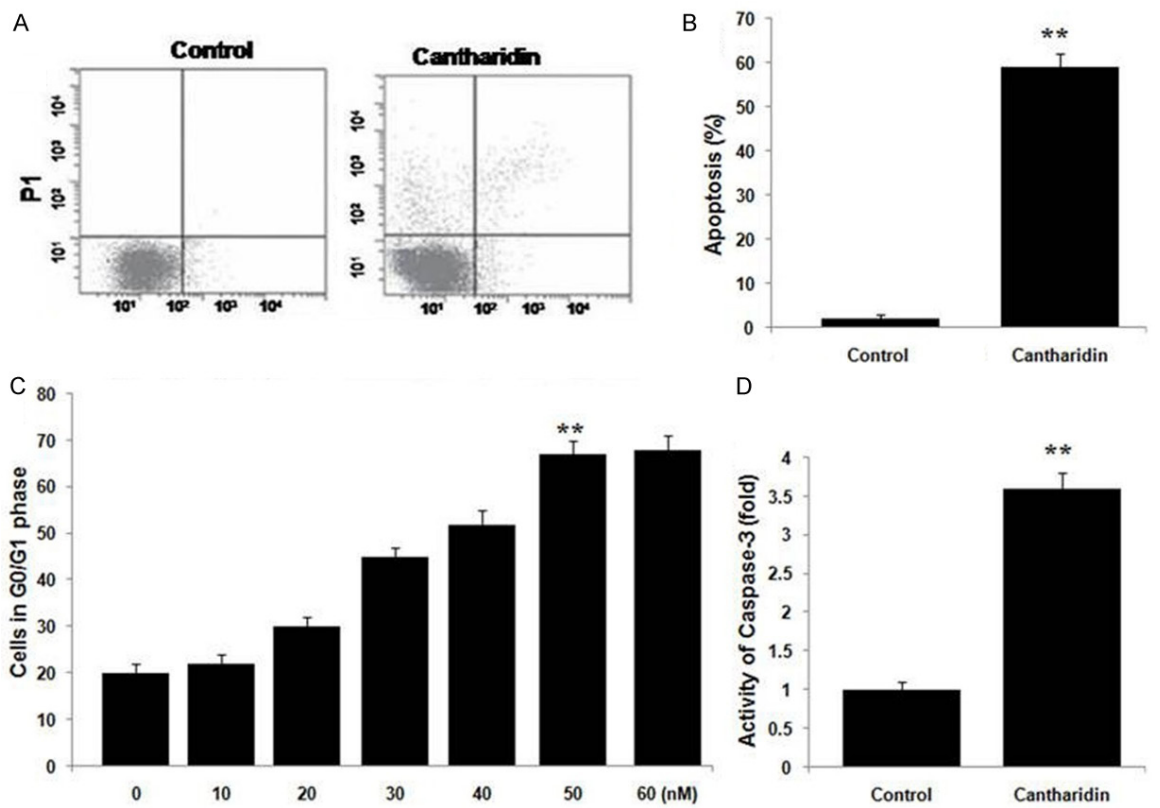

Figure 3. Cantharidin treatment for 48 h caused arrest of cell cycle in KOSC-2 cells in G0/G1 phase. A-C. The cells were incubated with various concentrations of cantharidin for 48 h. Flow cytometry was used to measure the distribution of cells in cell cycle. The data presented are representative of experiments performed independently in triplicates. *P<0.05 compared to the control cells. D. Effect of cantharidin on activation of caspase-3 in KOSC-2 cells. The cells were treated for 48 h with cantharidin and then analyzed for caspase-3 activation.

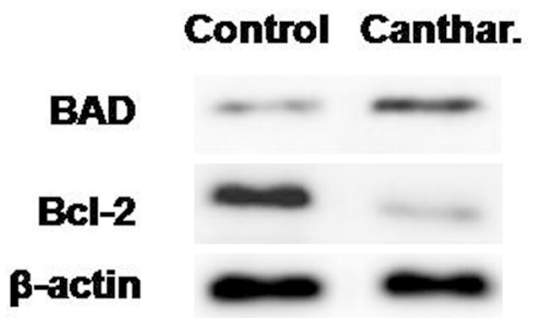

Figure 4. Effect of cantharidin on expression of BAD and Bcl-2 using western blot analysis. β-actin was used as the loading control and the bands were quantified by ImageJ software.

Cell cycle assay

The cells were seeded at a density of 2×10^6 cells per dish onto 60-mm dishes and cultured overnight. Then media was replaced by the new media containing cantharidin and the cells were incubated for 48 h. Following incubation, cells were harvested, fixed in 70% ethyl alcohol for overnight at 4°C. Then 100 µg/ml PI and 100 µg/ml RNase A in PBS was added to each of the dish and incubated for 1 h more at 37°C. Flow cytometry (BD Biosciences, FACSCalibur flow cytometer, San Jose, CA, USA) was used for analysis of cell cycle distribution. The experiments were carried out in triplicates independently.

Analysis of caspase-3 activity

The cells were seeded at a density of 2×10^5 cells in the flasks and incubated for 48 h with cantharidin. Following incubation, the cells were collected and treated with cold lysis buffer for 4 h. Cell lysates were subjected to incubation with Ac-IETD-pNA antibody for 45 min at 37°C. Activation of the caspase-3 was determined by examining the pNA cleavage by microplate spectrophotometer (BioTek Instruments Inc.) 405 OD.

Western blot analysis

The expression of the apoptosis-associated proteins Bak, Bax, p53, caspase-3 and -9 were detected in hepatic cancer cell lines. Cantharidin treated or control cells were treated with ice cold lysis buffer for 45 min. The cell lysates were subjected to centrifugation at 12,000× g and 4°C for 15 min. Concentration of the proteins was determined in the supernatants by bicinchoninic assay. The proteins were separated using SDS-PAGE and subsequently transferred to polyvinylidene difluoride membranes. The non-specific sites in the membranes were blocked with 5% non-fat milk and Tween 20 (TBST). The membranes were then washed with primary antibodies against BAD, Bcl-2 and β-actin at room temperature for overnight. Following washing with TBST three times the membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. The enhanced chemiluminescence blotting detection system (FluorChem E; Proteinsimple, Santa Clara, CA, USA) was used for analysis of the bands.

Statistical analysis

All the data were obtained from three experiments performed independently and are presented as the mean ± standard deviation. Student's t-test was used for the assessment of differences between the groups. The differences were considered significant statistically at $P<0.05$.

Results

Effects of cantharidin on the proliferation and viability of KOSC-2 cells

Cantharidin treatment reduced KOSC-2 cell viability in a dose dependent manner after 48 h (**Figure 1**). Preliminary screening revealed that IC_{50} of cantharidin against KOSC-2 cells was 50 nm for 48 h of the treatment.

Effects of cantharidin on apoptosis induction in KOSC-2 cells

The results from phase contrast microscopy showed reduction in cell size, protrusions in cell membrane and formation of apoptotic bodies in KOSC-2 cells on treatment with cantharidin for 48 h (**Figure 2**). However, no such alteration was observed in the control cell cultures after 48 h.

Effects of cantharidin on cell cycle arrest in KOSC-2 cells

Analysis of cell cycle in KOSC-2 cells following 48 h of cantharidin treatment showed a significant increase in the population of cells in G0/G1 phase (**Figure 3A**, **3B**). The proportion of cells was increased significantly in the sub-G1 phase. Thus cantharidin treatment inhibits

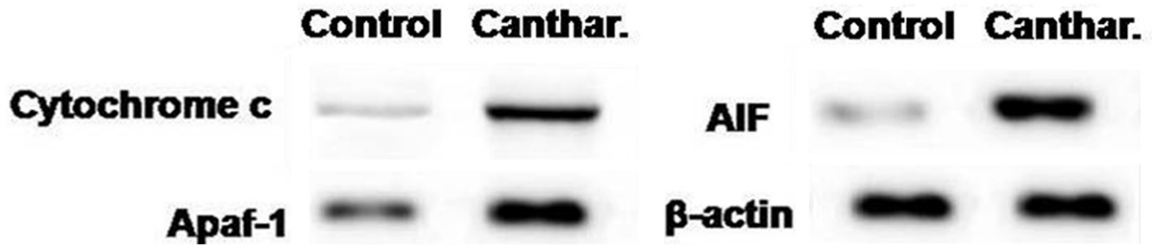

Figure 5. Effect of cantharidin on expression of cytochrome c, Apaf-1 and AIF using western blot analysis. β-actin was used as the loading control and the bands were quantified by ImageJ software.

KOSC-2 cell viability through cell cycle arrest in the G0/G1 phase after 48 h (**Figure 3C**).

Effects of cantharidin on activation of caspases in KOSC-2 cells

The results from western blot analysis revealed a significant increase in the caspase-3 activation in KOSC-2 cells on treatment with cantharidin for 48 h (**Figure 3D**). In control KOSC-2 cells the caspase-3 activation was significantly lower compared to cantharidin treated cells.

Effects of cantharidin on expression of BAD and Bcl-2 in KOSC-2 cells

Western blot analysis showed that cantharidin treatment for 48 h caused a significant increase in the expression of BAD proteins (**Figure 4**). However, the expression of Bcl-2 protein was decreased by exposure of KOSC-2 cells to cantharidin for 48 h (**Figure 4**).

Effects of cantharidin on expression of cytochrome c, Apaf-1 and AIF in KOSC-2 cells

Cantharidin treatment for 48 h significantly increased the expression of cytochrome c in KOSC-2 cells. Furthermore, the expression of Apaf-1 and AIF was also enhanced significantly compared to the control cells (**Figure 5**).

Discussion

Cantharidin, isolated from the natural source because of the no harmful side effects has a long traditional medicinal importance in China [11, 12]. Cantharidin exhibits its effect in carcinoma cells through induction of cell cycle arrest and apoptosis [13]. Induction of apoptosis in colon, bladder, breast and hepatoma carcoinoa cells by cantharidin treatment has also been reported [14-16]. In the present study effect of cantharidin on cell viability and induction of apoptosis in human oral carcinoma cells was investigated.

Apoptotic induction in the cells is evident by condensation of chromatin, reduction in cell size, formation of cell protrusions, activation of caspase-3 and release of cytochrome c [19-21]. It is reported that activated caspases play an important role in the induction of cell apoptosis [21]. Results from the present study showed that cantharidin treatment inhibited the viability of CAL 27 cells in a dose dependent manner. Analysis of the cause of inhibition in cell viability revealed induction of apoptosis in CAL 27 cells on exposure to cantharidin. CAL 27 cells showed a significant increase in the activation of caspase-3 and cytochrome c on treatment with cantharidin. Thus our results demonstrated that cantharidin inhibited CAL 27 cell viability and induced apoptosis through mitochondrial pathway. Cantharidin treatment for 48 h led to arrest of CAL 27 cell cycle in the G0/G1 phase.

Release of cytochrome c into the cell cytosol is followed by its interaction with Apaf-1 resulting in the activation of caspase-3 [19, 21]. Our results revealed a significant increase in the expression of Apaf-1 in CAL 27 cells treated with cantharidin for 48 h. Induction of apoptosis through mitochondrial pathway is regulated by the proteins, BAD and Bcl-2. The pro-apoptotic factor BAD induces cell apoptosis whereas the anti-apoptotic factor Bcl-2 prevents cells from undergoing apoptosis [22]. Present study revealed that cantharidin treatment increased the expression of BAD and reduced the expression of Bcl-2 in CAL 27 cells.

In conclusion cantharidin treatment reduces viability of oral carcinoma cells by inducing apoptosis through mitochondrial pathway. Thus

cantharidin has a scope to be investigated further for the treatment of oral cancer.

Disclosure of conflict of interest

None.

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