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Original Article

Effects of aconitine on the proliferation and apoptosis of human cholangiocarcinoma cells

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Abstract: Objectives: The aim of the study was to investigate the effect of aconitine on the proliferation and apoptosis of human cholangiocarcinoma QBC-939 cells and to explore its possible mechanism. Method: Human cholangiocarcinoma QBC-939 cells were treated with various concentrations of aconitine. Cell viability was detected by the CCK8 assay; Cell apoptosis was evaluated by flow cytometry analysis. The expression levels of stathmin and cyclinD1 were examined by quantitive PCR and western blotting. Results: We found that aconitine could significantly inhibited the proliferation of QBC-939 cells in a dose-dependent manner, meanwhile, the apoptotic rates of QBC-939 cells were also significantly enhanced by aconitine treatment with increasing concentration. Additonally aconitine made the cell cycle shift to a high G1 phase, especially at high dose. The quantitative PCR results showed that aconitine decreased the expressions of stathmin and cyclinD1 at mRNA level. The following Western blot analysis came to similar conclusion to PCR at protein level in aconitine treated cells. Conclusion: Aconitine could significantly inhibit cell proliferation and induce cell apoptosis of human cholangiocarcinoma cell line QBC-939 and the mechanism may be related to cell cycle arresting at G1 phase.

Keywords: Cholangiocarcinoma, aconitine, apoptosis, stathmin, CyclinD1

Introduction

Cholangiocarcinoma (CC) is one of malignant tumors that arises from bile ducts or ductules, and according to localization lesions divided into intrahepatic cholangiocarcinoma (ICC) a nd extrahepatic cholangiocarcinoma (ECC) [1]. As an advanced malignant disease hazard to human life and health, cholangiocarcinoma is characterized by difficulty in early diagnosis, quick metastasis, weak response to conventional treatments and poor prognosis [4]. Since the mid-1990s, incidence and morality of cholangiocarcinoma increase rapidly annually, more and more people have died of the aggressive disease [2, 3]. Therefore, to find new methods to achieve early diagnosis and improve survival of patients is particularly important.

Aconitine is an AC19 norditerpenoid alkaloid extracted from the root of aconitum plants, has been used as an essential drug in Traditional Chinese Medicine (TCM) over years. The tubers and roots of aconitum are widely applied to treat various diseases, such as collapse, syncope, rheumatic fever, painful joints, gastroenteritis, diarrhea, oedema, bronchial asthma, various tumors, and some endocrinal disorders like irregular menstruation [7, 8]. Previous studies found aconitine could induct apoptosis and inhibit cell growth in several tumors [5, 6]. While the the role of aconitine in proliferation and apoptosis of cholangiocarcinoma cells remains unknown.

Since excessive proliferation and abnormal apoptosis constitute main mechanisms of pathogenesis of malignances, it is necessary to discuss the possible antitumor effects of aconitine in cholangiocarcinoma cells based on these two approach. For the first time, our study focused on the effects of aconitine on the proliferation and apoptosis of cholangiocarcinoma cell line QBC-939 and explored the related mechanisms. The results demonstrated that aconitine could inhibit the proliferation and

induce the apoptosis of cholangiocarcinoma cells in a concentration dependent manner.

Materials and methods

Cell lines and cell culture

The human cholangiocarcinoma cell lines QBC-939 were maintained as suspension cells at 37°C in 5% carbon dioxide in humidified atmosphere. QBC-939 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (both from Hyclone, Logan, UT, USA). QBC-939 cells in the exponential growth phase were seeded to the 96-well plates or culture flasks.

Antibodies and reagents

Aconitine was purchased from Jinan Yanbio Technology Company (Shandong, China). Anti-actin monoclonal and anti-mouse and anti-rabbit were purchased from Santa Cruz, Anti-stathmin, and-cyclinD1 monoclonal antibodyies were purchased from ProteinTech. Radio-Immunoprecipitation Assay (RIPA) buffer was purchased from Thermo Fisher, 0.02% complete Protease Inhibitor EASY packs EDTA-Free was purchased from Roche.

Assessment of the cytotoxic effect of aconitine by cell counting Kit-8 (CCK-8)

Cell proliferation was measured by the CCK-8 assay (Beyotime, China). QBC-939 cell lines (1×10^4 cells/100 μL/well, respectively) were seeded into 96-well plates, treated with various concentrations of aconitine in a humidified atmosphere in a 5% carbon dioxide incubator. All experiments were performed in triplicate and repeated 3 times. Cells were incubated with 10 μL of CCK-8 at 37°C for 4 h. Then the optical density (OD) for each well was measured at 450 nm using ELISA reader. The inhibitory concentration of 50% of cells (IC50) was obtained using probit regression analysis method. The cell viability rate was calculated according to the following equation: Cell viability rate = (OD experiment-OD blank)/(OD control-OD blank) ×100%.

Assessment of apoptosis by annexin V and propidium iodide

Induction of apoptosis was assessed using the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (KeyGen Biotech, China). QBC-939 cells were treated with aconitine at 1, 10 and 100 μM. Cell culture medium without As_2S_2 was added to the untreated control. Dual staining with Annexin V-FITC and propidium iodide (PI) was carried out according to the manufacturer's instructions. The cells ($5\sim10\times10^5$) were analyzed by flow cytometry (Becton-Dickinson, USA). The acquired data were processed with FlowJo 7.6 software. Annexin V-FITC and PI-negative cells were identified as viable cells. Cells exhibiting Annexin V-FITC positive and PI-negative staining were considered to be early apoptotic cells while those with both Annexin V-FITC and PI-positive staining were considered to be late apoptotic cells. The sum of early and late apoptotic cells constituted the apoptotic cell population.

Gene expression study by quantitative real-time PCR

Total RNA was extracted from aconitine-treated and untreated QBC-939 cells using Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA). Then reverse transcription reaction was conducted by means of RevertAid First Strand cDNA (Thermo Fisher). Specific primers for RT-PCR were obtained from Sangon Biotech (Shanghai, China), The primers of the cyclinD1 used were forward (5'-GCTGCGAAGTGGAAACCATC -3') and reverse (5'-CCTCCTTCTGCACACATTTGAA-3'). The primers of the Stathmin used were forward (5'-TCAGCCCTCGGTCAAAAGAAT-3') and reverse (5'-TTCTCGTGCTCTCGTTTCTCA-3'), β-actin forward (5'-CATGTACGTTGCTATCCAGGC-3') and reverse (5'-CTCCTTAATGTCACGCACGAT-3'). Expression data were normalized to the geometric mean of housekeeping gene β-actin to control the variability in expression levels. For data analysis, the $2^{-\Delta\Delta Ct}$ method was used. Real-time PCR for each gene of each cDNA sample was assayed in triplicate.

ΔCt = Ct (target gene) -Ct (β-actin gene)

$\Delta\Delta Ct = \Delta Ct$ (As_2S_2-treated cells) -ΔCt (untreated control)

Protein expression study by western blot analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were performed to evaluate the protein levels of Stathmin and cyclinD1. Total protein

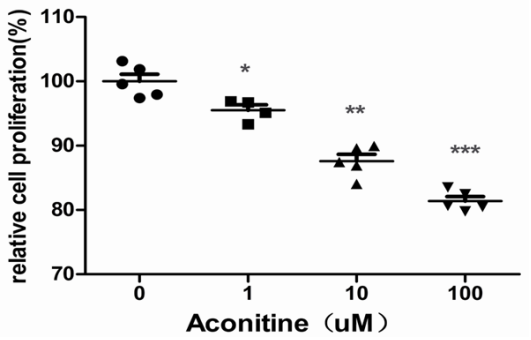

Figure 1. The effect of aconitine on the cell viability of QBC-939 cells.

was extracted from aconitine-treated and untreated QBC-939 cells using RIPA and 1% PMSF (Shenergy Biocolor, Shanghai, China). The protein concentration of the samples was determined by the BCA assay (Shenergy Biocolor). Proteins were detected using the chemiluminescence detection kit (Millipore, Billerica, MA, USA). Western blotting results were analyzed using the Las-4000 Image software and Multi Gauge Ver.3.0 software (Fujifilm Life Science, Japan).

Statistical analysis

Statistical analysis was performed using SPSS 17.0. The data are reported as mean ± standard deviation (S.D). ANOVA was used to evaluate data from the cell viability assays and for the cell apoptotic rates. Other statistical analyses of data were performed using the Student's t test. Statistical significance was defined as $P<0.05$.

Results

Aconitine inhibits the proliferation of QBC-939 cells

The effect of aconitine on the cell viability of QBC-939 cells was evaluated by CCK-8 assay. A significant decrease in cell viability was observed after the QBC-939 cells were incubated with different doses of aconitine (1, 10, and 100 μM) (**Figure 1**). Compared with the untreated control, treatment with 1, 10, 100 μM aconitine for 48 h resulted in a viability of 95.51 ± 0.83, 87.58 ± 1.06, 81.38 ± 0.69 in QBC-939 cells, respectively ($P<0.05$). The inhibitory effect on cell viability was enhanced along with increasing aconitine doses.

Aconitine induces apoptosis of QBC-939 cells

To determine the effect of aconitine -induced apoptosis in QBC-939 cells, Annexin V-FITC/PI dual staining followed by flow cytometric analysis was assessed. As shown in **Figures 2** and **3**, aconitine induced apoptosis in QBC-939 cells in a concentration dependent manner. Exposure to aconitine at concentrations of 1~100 μM for 24 h led to relatively low rate of apoptosis. After treatment with various doses of aconitine, the maximal apoptosis rate was 22.06 ± 6.54% in QBC-939 cells at 24 h. When the treatment time increased to 72 h, the maximal apoptosis rate was 58.94 ± 0.79% in QBC-939 cells. In contrast, not more than 5% of the untreated control QBC-939 cells underwent apoptosis under the same conditions. After treatment with high and middle dosages of aconitine, especially the high dosage, the cell cycle shifted to a high G1 phase, These results indicate that aconitine blocked the G1/S transition.

Aconitine decrease the stathmin and CyclinD1 expression at the mRNA level

To further investigate whether the aconitine-induced apoptosis is dependent on mitochondria mediated apoptosis pathway, the effect of aconitine on the mRNA levels of stathmin genes was measured by quantitative real-time PCR. Following treatment with 10 μM aconitine for 48 h, the expression of stathmin and cyclinD1 mRNA in QBC-939 cells was was reduced (**Figure 4A** and **4C**).

Aconitine decrease the stathmin and CyclinD1 expression in QBC-939 cells at the protein level

We also investigated whether the protein expression levels of stathmin and cyclinD1 were altered after the aconitine treatment. As shown in **Figure 4B** and **4D**, after treatment with different doses of aconitine (1 and 10 μM) for 48 h, the stathmin and cyclinD1 expression was markedly downregulated in the QBC-939. Western blotting also demonstrated that after exposure to 2 μM aconitine, the levels of stathmin and cyclinD1 expression were decreased.

Discussion

In the present study, we found that aconitine significantly inhibited proliferation and promot-

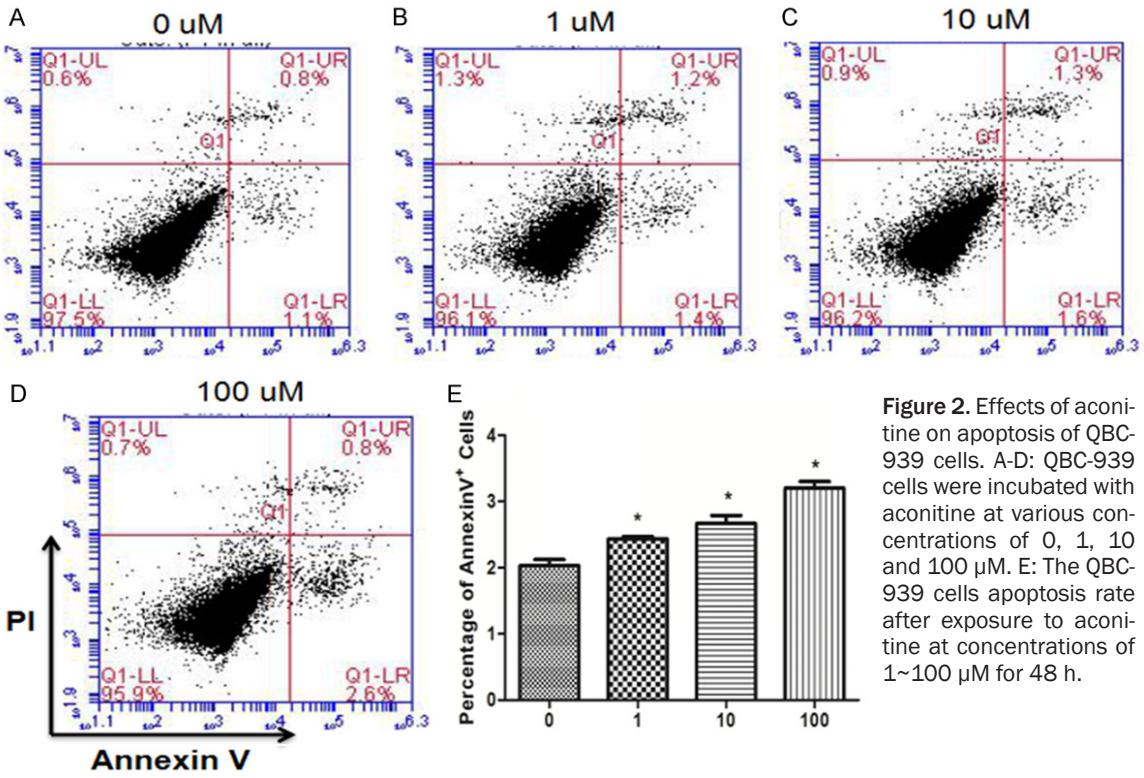

Figure 2. Effects of aconitine on apoptosis of QBC-939 cells. A-D: QBC-939 cells were incubated with aconitine at various concentrations of 0, 1, 10 and 100 μM. E: The QBC-939 cells apoptosis rate after exposure to aconitine at concentrations of 1~100 μM for 48 h.

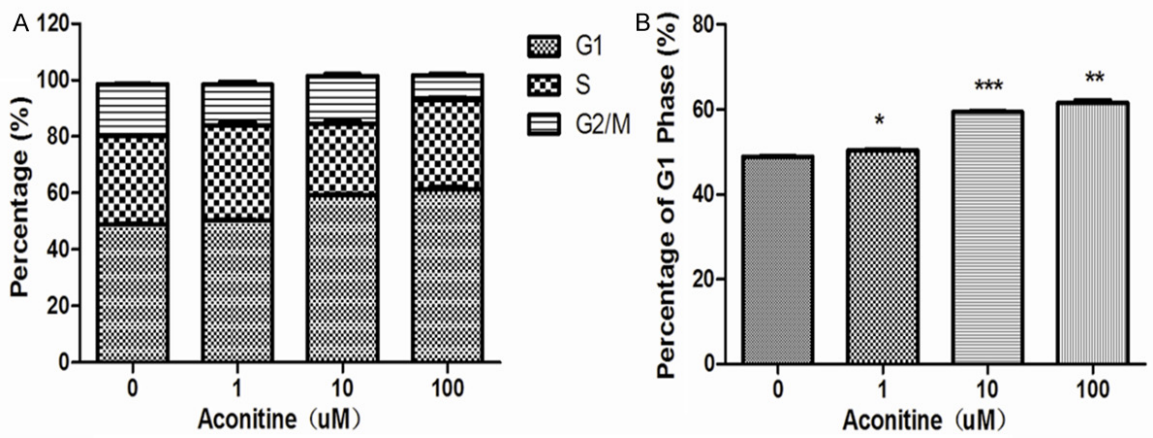

Figure 3. Effect of aconitine on the QBC-939 cell cycle and percentage of G1 phase. A: Effect of aconitine on the QBC-939 cell cycle. B: Percentage of G1 phase after aconitine effect on the QBC-939 cell.

ed apoptosis in the QBC-939 cells in a dose-dependent manner. The study results aslo confirmed aconitine induced cell cycle arrested in G1 phase. Additonally concomitant stathmin and cyclinD1 cleavage which involved in the mitochondria-mediated pathway might participate in the suppressing tumor survival effects of aconitine. Therefore, this is the first study concerning the effects of aconitine on cholangiocarcinoma cells *in vitro*.

Cyclin D1 is the most important checkpoint in the mammalian cell cycle, an essential G1 cyclin is involved in regulating the G1 ± S transition [9, 10]. Overexpression of cyclinD1 has been reported in many human tumors [11, 12], Nishida N founded that in hepatocellular carcinoma amplification and overexpression of the cyclin D1 gene can stimulate cancer cell growth [13]. Cyclin D1 forms a complex with cdk4 or cdk6 and phosphorylates Rb protein, which

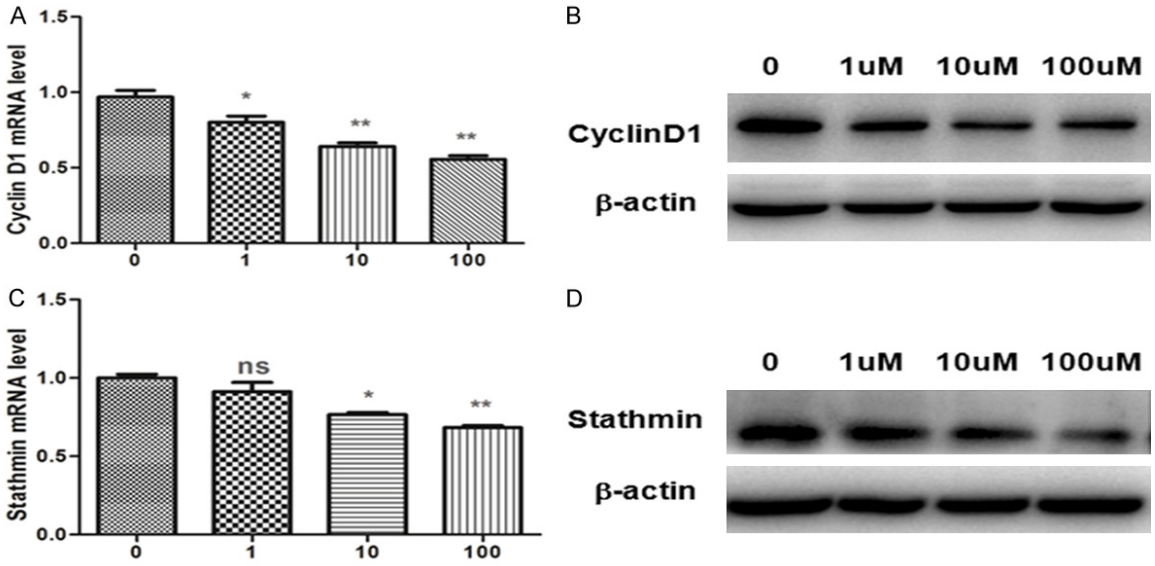

Figure 4. Effects of aconitine on protein levels of cyclinD1 and stathmin proteins in QBC-939 cells (A and C). Western blotting was used to analyze whole cell lysates for cyclinD1 and stathmin expression following aconitine treatment (B and D).

allows the cells to enter the S phase [14]. Some studies blocked cell access to S phase by inhibiting the activity of cyclinD1, eventually leading to cell cycle arrest [15], In this study, the expression of cyclinD1 mRNA in QBC-939 cells was reduced. Notably, western blot analysis of cyclinD1 protein expression showed that when cholangiocarcinoma QBC-939 cell were exposed to aconitine the cyclinD1 expression was markedly downregulated, cell cycle arrest in G1 phase.

Stathmin is a microtubule depolymerization protein, participate in the regulation of cell cycle and migration, signal transduction and transporter [16, 17]. Some studies confirm that depletion of stathmin1 can leads to cell cycle arrest [18-21] and increased apoptosis [22, 23]. Inhibition Stathmin expression and activity can make tumor cells cleavage arrest in G2/M phase and induce apoptosis of cells into the program [24], indicating that Stathmin may play an important role in cell proliferation, apoptosis and cell cycle extremely. Our results showed that aconitine treatment downregulated that of stathmin, increased QBC-939 cell apoptosis. This suggests that aconitine acts on the stathmin to exert its apoptotic effect.

In conclusion, the search for novel and effective treatments for QBC-939 cells remains a challenge. Our research found that aconitine inhibited the proliferation and induced the apoptosis of QBC-939 cells *in vitro* through the mitochondria pathway. In addition, stathmin and cyclinD1 was critical for the initiation of apoptosis in aconitine-treated QBC-939 cells. Thus, Aconitine may be useful as a potential therapeutic agent against CC and further research is warranted.

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Disclosure of conflict of interest

None.

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