

Aconitine induces cell apoptosis in human pancreatic cancer via NF- κ B signaling pathway

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Abstract. – OBJECTIVE: Pancreatic cancer is one of the leading causes of death from cancer in European countries and the United States. This study sought to investigate the effects of aconitine, a well-known aconitum plant-produced toxin, on pancreatic cancer cell growth and apoptosis and to explore the potential mechanisms.

MATERIALS AND METHODS: In this study, pancreatic cancer cell lines Miapaca-2 and PANC-1 were cultured, and cell viability was examined in these two cells treated with different doses of aconitine. Moreover, cell apoptosis was also analyzed upon aconitine treatment, and the specific mechanism was examined by Western blot assay and caspase activity detection.

RESULTS: The results showed that aconitine inhibited pancreatic cancer cell growth in a dose- and time-dependent manner. The administration of aconitine in Miapaca-2 and PANC-1 cells also induced cell apoptosis by upregulating the expression of pro-apoptotic factors Bax, cl-caspase-3, cl-caspase-9, and cleaved poly (ADP-ribose) polymerase 1 (PARP1), and by decreasing the anti-apoptotic Bcl-2 expression. More importantly, NF- κ B was also decreased upon aconitine treatment. In a xenograft mouse model of pancreatic cancer, aconitine suppressed tumor growth and increased cell apoptosis.

CONCLUSIONS: This study is the first report on the effects of aconitine on pancreatic cancer, and it reveals that aconitine may serve as a potent therapeutic strategy for clinical treatment of pancreatic cancer.

Key Words:

Aconitine, Pancreatic cancer, Apoptosis, NF- κ B, Caspases, PARP1, Bcl-2.

year has increased to 54% in recent years⁶. The most dreadful manifestation of pancreatic cancer is its strong invasion and metastasis to other tissues⁷⁻⁹. Although thousands of studies have been conducted, the specific mechanism involved remains unknown. Therefore, obtaining an in-depth understanding of pancreatic cancer and finding a novel therapeutic strategy to diagnose and treat this disease are necessary.

Apoptosis is the process of programmed cell death (PCD), which is highly regulated and controlled during the lifetime of an organism. Apoptosis is based on genetic programs that are indispensable parts of the development and function of all organisms. Multiple signaling pathways are involved in this process, and the two most direct of which are the intrinsic and extrinsic pathways¹⁰. Both pathways lead to the activation of the caspase family and dismantling and removal of dead cells. The extrinsic pathway begins outside the cells stimulated by cytotoxic stress. Death receptors belong to the tumor necrosis factor receptor (TNFR) family and are the initial components of extrinsic apoptosis. Many proteins are involved in this process, including TRADD, TRAF2, CIAP1, NF- κ B, PI3K, CytoC, Bcl-2, and Bax¹¹⁻¹⁶. In most individuals, anticancer medicines eventually result in the activation of cell apoptosis.

Aconitine, also known as monkshood or devil's helmet, is a kind of *Aconitum* plant-produced toxin. Despite its narrow therapeutic index, aconitine is used as a traditional medicine in China because of its analgesic and anti-inflammatory activities and currently has limited applications in herbal medicine¹⁷. The toxic effects of aconitine were demonstrated in multiple animals, including dogs and mice. The first symptoms of aconitine intoxication, which occur approximately 20 min to 2 h after oral intake, include sweating, paraesthesia, and nausea, which result in se-

Introduction

Pancreatic cancer is the fifth most common cause of cancer-related deaths in the world¹⁻³. It has a dismal prognosis with high annual morbidity and mortality. The five-year survival rate after the amendable resection at diagnosis ranges from 8% to 19%^{4,5}, and the recurrence rate within one

vere vomiting, intense pain, colicky diarrhea, and paralysis of skeletal muscles. Following the onset of life-threatening arrhythmia, including ventricular tachycardia and fibrillation, death finally occurs as a result of respiratory paralysis or cardiac arrest¹⁸. Although multiple pharmacological studies have been reported, few systemic studies on the effects of aconitine in tumorigenesis are available.

The aim of the present study was to explore the effects of aconitine in human pancreatic cancer. To this end, pancreatic cancer cell lines Miapaca-2 and PANC-1 cells were cultured, and cell viability was examined in these two cells treated with different doses of aconitine. Moreover, cell apoptosis was analyzed upon aconitine treatment, and the specific mechanism was examined by Western blot assay and caspase activity detection. Our study may pave the way for novel clues for the treatment of human pancreatic cancer.

Materials and Methods

Cell Culture and Reagents

Pancreatic cancer cell lines Miapaca-2 and PANC-1 were commercially purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in the recommended medium supplied with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) in a 5% CO₂ atmosphere. Aconitine was bought from Sigma-Aldrich (Sigma, St. Louis, MO, USA) and prepared in methanol at a concentration of 120 µM and diluted to the desired density.

Cell Viability Assay

Cell viability assay was performed with 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). 1×10^4 pancreatic cancer cells were seeded in 96-well plates and treated with different concentration of Aconitine in triplicate. After treatment (at different time intervals), 10 µl of MTT (5 µg/ml) was mixed with the medium in each well and incubated for another 3 h at 37 °C in the dark. Formazan crystal formed was dissolved again in 100 µl dimethyl sulfoxide (DMSO), and the optical absorbance of each well was collected at 570 nm with a TECAN reader.

Colony Formation Assay

Miapaca-2 and PANC-1 cells in 6-well plates were treated with different concentrations of Aconitine (0, 15, 30, 60 µM) and 24 h after treat-

ment, were seeded into 12-well plates (100 cells/well) in triplicate. The medium was changed very other day and after incubation for 10 days, the colonies were fixed with pre-iced methanol and stained with crystal violet. Colonies containing more than 50 cells were considered and counted as survivors under a microscope (Leica, Wetzlar, Germany) at a magnification of 200×. The following formula was used to calculate the rate of colony formation: colony formation rate = (number of colonies / number of seeded cells) × 100 %.

Cell Apoptosis Assay

Cell apoptosis was examined with Hoechst-33258 (Beyotime, Nanjing, China) according to the manufacturer's instruction. Miapaca-2 and PANC-1 cells were seeded into 12-well plates in triplicate and treated with different concentration of Aconitine (0, 15, 30, 60 µM) for additional 48 h. Afterwards, Hoechst-33258 was mixed with the medium in each well and incubated for 15 min at 37 °C. Fluorescence images were captured randomly with an inverted fluorescence microscope (Leica, Wetzlar, Germany). The percentages of apoptotic neurons were calculated with the following formula: apoptotic rate = (apoptotic cells/total cells) × 100%.

The Annexin V-FITC Apoptosis Detection Kits (BD, San Diego, CA, USA) were also included for assessing the cell apoptosis after Aconitine treatment. According to the manufactures' protocols, 5×10^5 cells were seeded into 12-well plates and treated with different doses of Aconitine for 24 h. Afterwards, cells were centrifuged and resuspended in binding buffer. 5 µl of Annexin V-FITC and 5 µl of PI were added into each well, respectively and incubated for 5 min in the dark. Cell percentage in each phase was determined by fluorescence-activated cell sorting. Cells in the early stage of apoptosis showed Annexin V-FITC positive and in late apoptotic assumed both annexin-V-FITC and PI positive.

Western Blot Analysis

Total proteins were extracted with NP40 lysis buffer (Beyotime, Shanghai, China) and quantified with the standard BCA method (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instructions. Equal amounts of proteins (50 µg) were loaded into each well of SDS-PAGE gels and electroblotted onto nitrocellulose (NC) membranes (Millipore, Billerica, MA, USA). After blocked with TBS supplied with 5%

milk, the membranes were incubated with primary antibodies at 4°C overnight. Primary antibodies against NF- κ B, Bax and Bcl-2 were purchased from Abcam (Cambridge, UK). Primary antibodies against caspase-9, caspase-3, PARP, cyto. C, β -actin and secondary antibodies were commercially from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Caspase Activity Detection

The activities of caspase-3, caspase-8 and caspase-9 were determined by the Caspase-3 Activity kit, Caspase-8 Activity kit and Caspase-9 Activity kit, respectively, according to the instructions from Beyotime (Nanjing, China). In brief, cell lysates were collected after treatment with different concentrations of aconitine. 10 μ l proteins from cell lysates were added into 96-well plates and mixed with 80 μ l reaction buffer containing caspase substrate (2 mM). After incubated for 4 h at 37°C, caspase activities were obtained with a Tecan reader at an absorbance of 405 nm.

Xenograft Mouse Model

30 (four-week-old) male athymic nude mice were purchased from SLRC Laboratory Animal (Shanghai, China) and randomly divided into three groups: vehicle (methanol)-administrated control group (n = 10), 50 mg/kg Aconitine-administrated group (n = 10), and 100 mg/kg Aconitine-administrated group (n = 10). For each mouse, 1×10^6 of Miapaca-2 cells were subcutaneously injected. After cancer cell injection, mice from treatment groups were administrated with corresponding doses of Aconitine once a day for 4 weeks. Tumor sizes were measured once every four days and tumor weights were obtained at the end of the experimental period after all mice were sacrificed. All efforts were made to minimize the sufferings.

Immunohistochemistry (IHC) Analysis

Tumor tissues from each mouse were fixed with 10% formalin, paraffin-embedded and cut into 4 μ m slices, then subjected to antigen retrieval by microwaving the slices at 900 W for 2.5 min and 150 W for 10 min in 0.1 M citric acid buffer (PH 6.0). After blocked with goat serum for 20 min, slides were thereafter incubated with primary antibodies at 37°C for 1 h and then 4°C overnight. On the second day, slides were washed with TBST three times and then incubated with secondary antibodies at 37°C for 45

min. The immunoreactivity was determined in 0.05% diaminobenzidine (DAB) containing 0.01% hydrogen peroxidise (H₂O₂). Images were captured with a Nikon microscope at a magnification of 400 $\times$.

Statistical Analysis

All data were presented as the means $\pm$ standard deviations (SD). Each experiment was repeated at least three times in triplicate except otherwise stated. Student's *t*-test was included to compare the difference between groups. Any value of *p* < 0.05 was considered as statistically significant.

Results

Aconitine Inhibits Cell Viability in Cultured Human Pancreatic Cancer Cell Lines

Miapaca-2 and PANC-1 cells were seeded into 24-well plates and treated with different concentrations of aconitine for various periods prior to cell viability analysis to test the effects of aconitine administration in pancreatic cancer. As shown in Figure 1, aconitine could potently suppress cell survival in a time- and dose-dependent manner. When the cells were co-incubated with aconitine for 24 h, the cell proliferation rates decreased by 90% and 75% for Miapaca-2 and PANC-1 cells, respectively, with aconitine concentrations of up to 80 μ M. The lethal dose 50 (LD50) for both cell lines was between 20 μ M and 40 μ M (Figure 1A). Then, the cells were treated with 30 μ M of aconitine; the inhibitory rate was increased to 44% on the second day and to 59% on the third day for Miapaca-2 cells compared with the control ones. A similar inhibitory effect was observed in PANC-1 cells (Figure 1B). These data show that aconitine could inhibit cell viability in a time- and dose-dependent manner in both Miapaca-2 and PANC-1 cells.

Aconitine Suppresses cell Proliferation in Miapaca-2 and PANC-1 Cells

Subsequently, we performed colony formation assay to assess the effects of aconitine in cell proliferation. We used three different concentrations of aconitine and a blank control one. For both cell lines, the colonies formed dramatically decreased with the increase of aconitine dose of aconitine increasing (Figure 2A). The clones formed on the

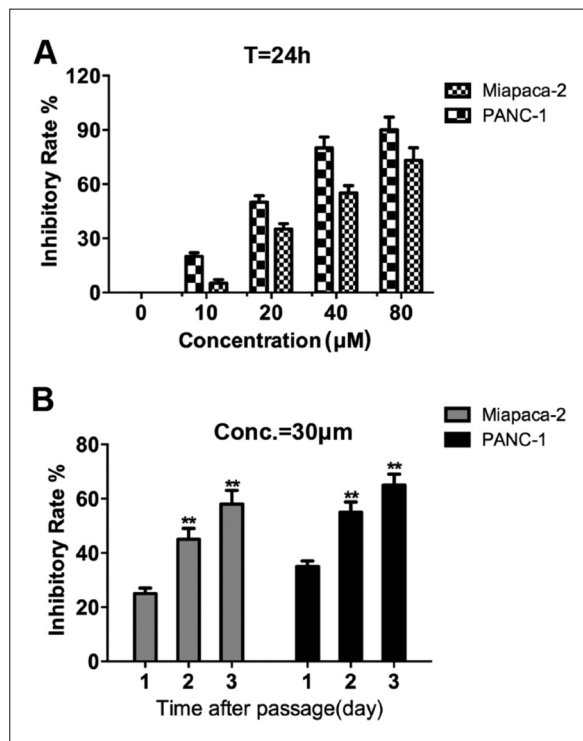


Figure 1. Aconitine inhibits cell proliferation in dose- and time-dependent manner in Miapaca-2 and PANC-1 cells. **A**, Both Miapaca-2 and PANC-1 cells were treated with different concentrations of aconitine (0, 10, 20, 40 and 80 μM). Cell viability was examined after treatments for 24 h by MTT kit. **B**, Both pancreatic cancer cell lines were administrated with 30 μM of aconitine for different time durations (1, 2, and 3 days). Cell viability was examined at each time point by MTT assay. ** $p < 0.01$, vs. Day 1.

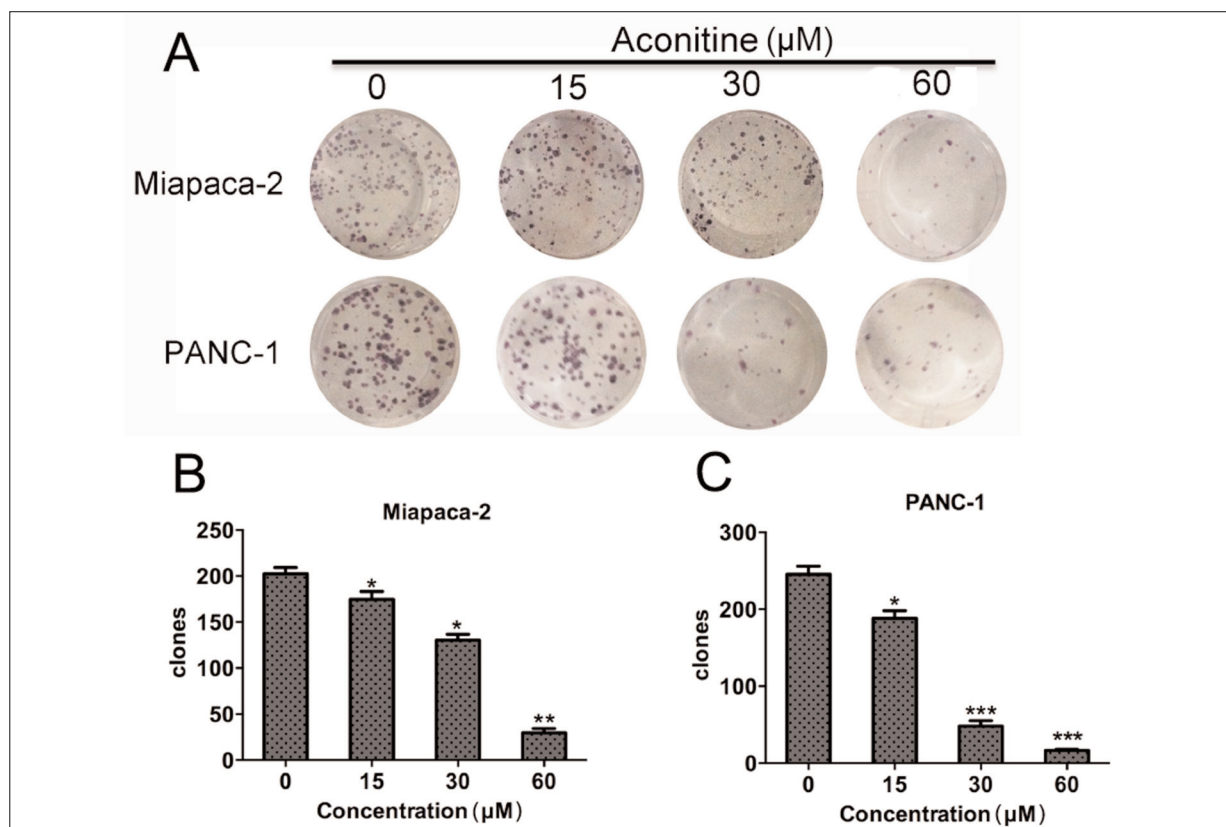


Figure 2. Aconitine suppresses colony formation in pancreatic cancer Miapaca-2 and PANC-1 cells. **A**, Colony formation analysis was performed to examine the effects of aconitine (0, 15, 30 and 60 μM) on both Miapaca-2 and PANC-1 cells. Images were acquired with crystal violet staining and representatives were shown. **B**, **C**, Quantification of colony formation assay in Miapaca-2 and PANC-1 cells treated with different concentrations of Aconitine. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, vs. control untreated one.

plates showed that aconitine inhibited cell proliferation in a dose-dependent manner. The Miapaca-2 clones decreased by 10%, 30%, and 80% with the treatment of 15, 30, and 60 μ M of aconitine, respectively, compared with the untreated cells (Figure 2B). The clones for PANC-1 cells also dropped to 80%, 20%, and 10%, respectively, as the aconitine concentrations increased (Figure 2C). These results suggest that aconitine decreased cell proliferation in a dose-dependent manner in both pancreatic cancer cells.

Aconitine Increases Cell Apoptosis in a Dose-dependent Manner in Pancreatic Cancer Cells

Next, we assessed cell apoptosis processes to examine how aconitine inhibited cell growth. After exposure to different concentrations of aconitine

(0, 15, 30, and 60 μ M) for 48 h, both Miapaca-2 and PANC-1 cells were subjected to Hoechst 33258 staining (Figure 3A). The fragmentation of nuclei of cells treated with 15 μ M of aconitine was visible, and the number of fragmented nuclei increased with the aconitine doses (30 μ M and 60 μ M) in both cell lines compared with the untreated control cells. The quantification of the morphologically changed nuclear cells further suggested that the apoptotic nuclei with condensed chromatin increased remarkably with the concentration of aconitine. The apoptosis rates were highly increased by 21% and 35% in Miapaca-2 and PANC-1 cells, respectively, with the administration of 60 μ M of aconitine (Figure 3B and 3C). These data suggest that aconitine could induce cell apoptosis in both pancreatic cancer cell lines.

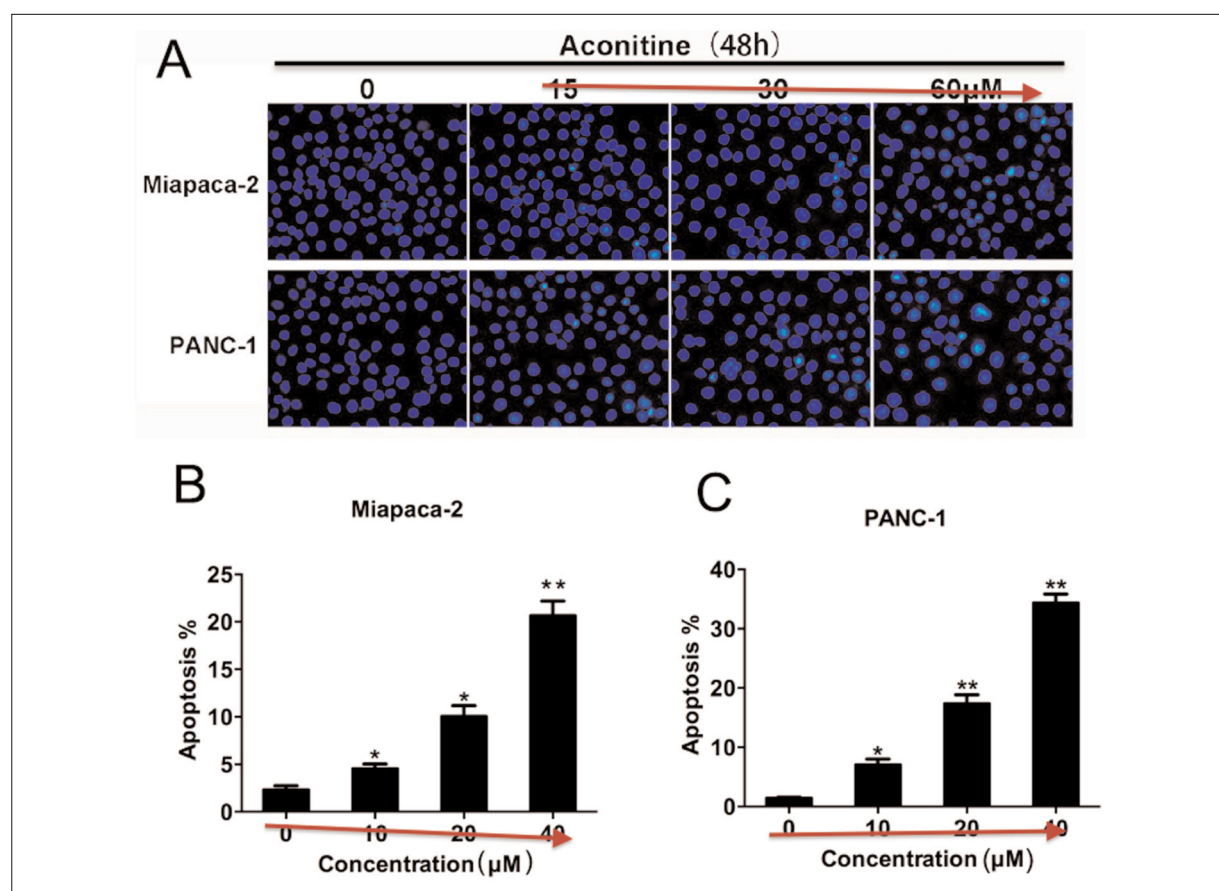


Figure 3. Aconitine induces cell apoptosis in both pancreatic cancer cell lines. **A**, Hoechst 33258 staining was performed to reveal the morphological changes of nuclei of Miapaca-2 and PANC-1 cells upon treatment with different concentrations of Aconitine (0, 15, 30 and 60 μ M). **B**, Quantification of Miapaca-2 nuclei that contained condensed chromatin and fragmentations upon cells treated with 0, 15, 30 and 60 μ M aconitine. **C** Quantification of apoptotic nuclei in PANC-1 cells when cells were co-incubated with different doses of Aconitine. * $p < 0.05$, ** $p < 0.01$, vs. control. Flow cytometry was conducted to further reveal cell survival and apoptosis. **D**, Treatment of aconitine reduced the numbers of survival cells. Instead, numbers of early and late apoptotic cells were synchronously increased in a dose-dependent manner.

Furthermore, flow cytometry analysis was performed to demonstrate cell survival and apoptosis (Figure 4A). Cells were pretreated with 0, 15, 30, and 60 μM of aconitine and collected for the subsequent assay. Figure 4A shows that the treatment of aconitine reduced the numbers of surviving Miapaca-2 and PANC-1 cells but increased the cell numbers in early and late apoptosis in a dose-dependent manner. In Miapaca-2 cells, the surviving cells decreased from 94% to only 72% with the increased concentration of aconitine (60 μM). Meanwhile, the untreated cells in the late apoptotic phase increased dramatically from 4% to 22% (Figure 4B). PANC-1 cells were also proven to undergo a similar apoptosis upon treatment with aconitine (Figure 4C). The data obtained from the cell flow cytometry assay were consistent with those from the Hoechst 33258 staining. All these data together revealed that the

aconitine-mediated inhibition of pancreatic cancer cell proliferation was closely related to increased cell apoptosis.

Aconitine-induced Pancreatic Cancer Cell Apoptosis Through NF- κ B Signaling Pathway

Cell apoptosis mainly involves two signaling pathways: intrinsic and extrinsic. To further confirm which signal is involved, we performed Western blot analysis. Caspases play a central role in cell apoptosis transduction. We examined the initiator caspases-9, effector caspase-3, and caspase substrate poly ADP-ribose polymerase (PARP). Figure 5A shows that the protein levels of cleaved caspase-9 (cl-caspase-9), cleaved caspase-3 (cl-caspase-3), and cleaved PARP (cl-PARP) were significantly upregulated

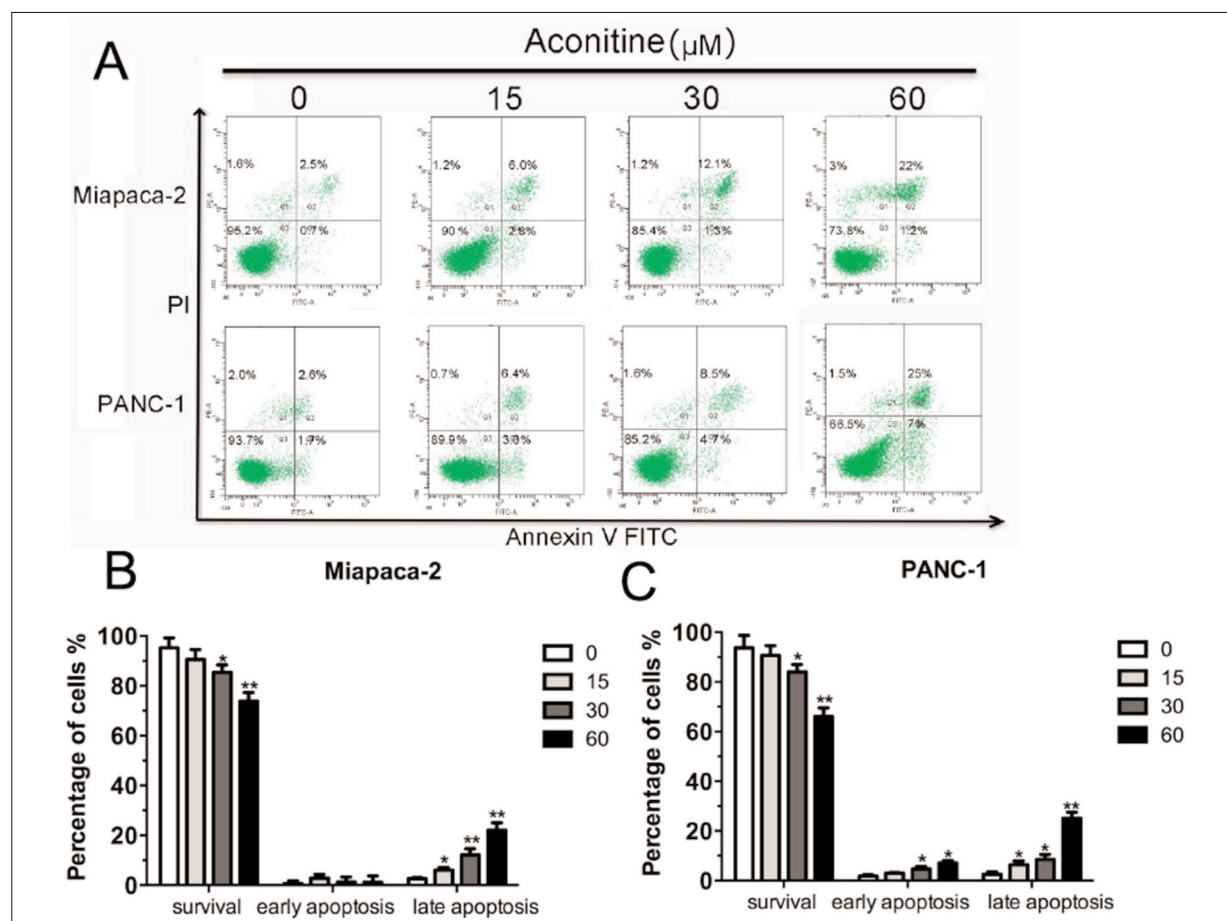


Figure 4. Aconitine causes cell apoptosis examined by cell flow cytometry. **A**, Miapaca-2 and PANC-1 cells were treated with different concentrations of aconitine and subjected to flow cytometry assay. **B**, Quantification of cell flow cytometry assay in Miapaca-2 cells. Top left quadrant was early apoptosis; top right quadrant was late apoptosis and lower left quadrant was survival. **C**, Quantification of cell flow cytometry assay in PANC-1 cells. * $p < 0.05$, ** $p < 0.01$, vs. control.

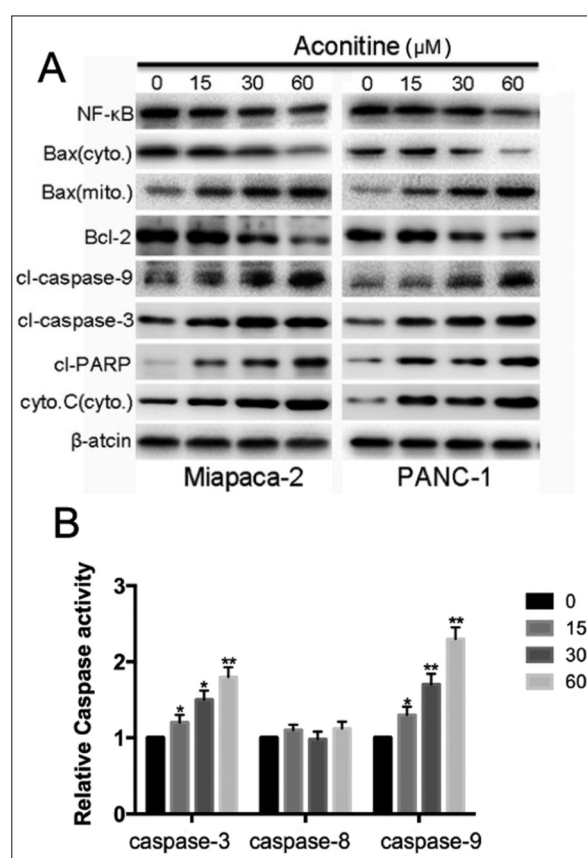


Figure 5. Aconitine induces cell apoptosis via NF- κ B signaling pathway. **A**, Western blot analysis was performed to detect the apoptotic pathway proteins after cells were treated with different doses of aconitine. β -actin was included as a loading control. **B**, Relative caspase activity was examined by specific caspase activity kits. The activity of caspase-3 and caspase-9 was highly increased upon aconitine administration, while caspase-8 remained unchanged. * $p < 0.05$, ** $p < 0.01$, vs. control.

as the aconitine concentration increased, indicating the promoting effects of aconitine on cell apoptosis. Bax is a representative of pro-apoptotic proteins and Bcl-2 is the main member of anti-apoptotic proteins. The Western blot assay also showed that the expression of Bax in the cytoplasm and total Bcl-2 notably decreased as the aconitine dose increased. However, the protein level of Bax in mitochondria was upregulated when the cells were treated with aconitine (15, 30, and 60 μ M). Cytochrome C (cyto. C) is another effective indicator of cell apoptosis that binds with Apaf-1 and procaspase-9, forming the apoptotic body that is the active form of caspase-9. Then, we detected the protein level of cyto. C in cytoplasm and found that it was highly upregulated when both cells were incubated

with aconitine. Most importantly, the expression of NF- κ B decreased with the pre-treated aconitine. NF- κ B is the key nuclear factor of the TNF α downstream gene and plays a central role in the entire apoptosis system. All of these data suggested that aconitine could increase cell apoptosis, which was highly related with the NF- κ B signaling pathway.

Moreover, we detected the relative caspase activity in Miapaca-2 cells with the aconitine administration by specific caspase-3/-8/-9 activity kits. As shown in Figure 5B, the relative activity of effector caspase-3 increased remarkably to 1.2-, 1.5-, and 1.8-fold, respectively, when the cells were treated with different doses of aconitine (15, 30, and 60 μ M) compared to the control ones. Meanwhile, the initiator caspase-8 remained stable and caspase-9 also increased to 1.4-, 1.6-, and 2.2-fold, respectively, as the aconitine concentration increased. The preceding data show that aconitine increased the activities of both effector caspase-3 and initiator caspase-9 through which the cell apoptosis was upregulated.

Aconitine Suppresses Tumor Growth and Induces Cell Apoptosis in a Xenograft Mouse Model of Pancreatic Cancer

Nude mice with subcutaneous Miapaca-2 tumor xenografts were intraperitoneally injected with different doses of aconitine (0, 50, and 100 mg/kg) to investigate the effects of aconitine treatment further on pancreatic cancer *in vivo*. The tumor volume in each group was monitored regularly during the four weeks of mouse culture. The tumor volume in the control group increased dramatically (Figure 6A). On day 28, the tumor size shrunk by 20% in the 100 mg/kg aconitine-injected mice compared to the control ones. The mice injected with 100 mg/kg aconitine showed lower tumor weight (mean weight = 0.25 g) than the control mice (mean weight = 1.5 g). Furthermore, the tumor weights decreased in a dose-dependent manner (Figure 6B). These data suggest that aconitine inhibits pancreatic cancer growth *in vivo*. The tumor tissues were then sectioned for HE staining and IHC analysis (Figure 6C). Consistently, cl-caspase-9 and cl-caspase-3 were also observed to be highly expressed in the tumor tissues from aconitine-treated mice. All these effects were dose-dependent *in vivo*. These data reveal the promoting effects of aconitine on cell apoptosis.

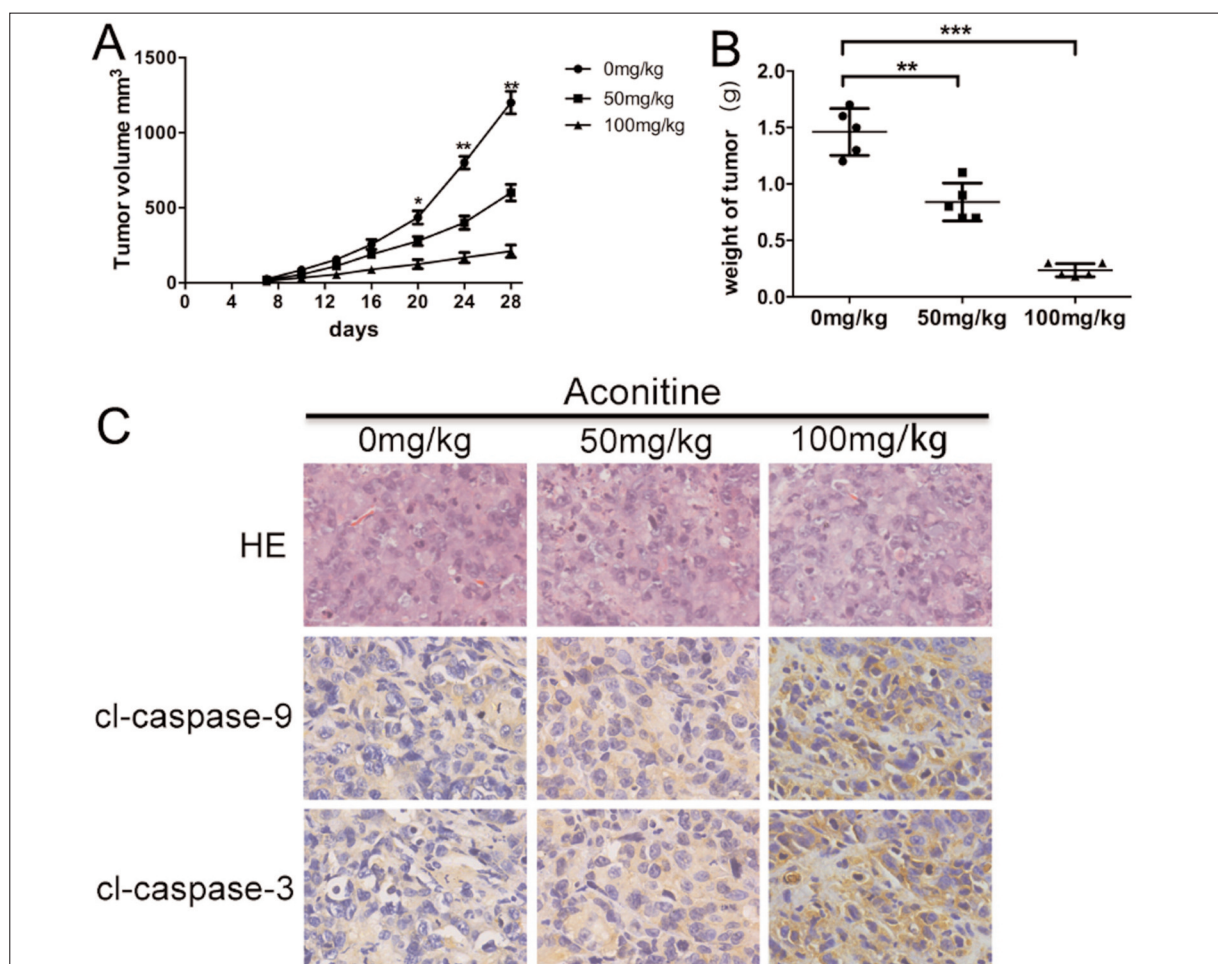


Figure 6. Aconitine suppresses tumor growth and induces apoptosis in a xenograft mouse model of pancreatic cancer. Tumor xenografts were established by subcutaneous inoculation of Miapaca-2 cells into the left flank of nude mice. Mice were then injected with methanol or aconitine (50 mg/kg and 100 mg/kg) daily for up to 4 weeks. **A**, Periodic monitoring of tumor dimensions showed that tumor volume was the largest in control mice, whilst aconitine-treated mice exhibited smaller tumor volumes since day 20. **B**, Dissection of tumors by the 4th week showed that tumor weights were decreased in aconitine-treated mice than the control mice. * $p < 0.05$, ** $p < 0.01$, vs. control. **C**, Tumor tissues were sectioned for HE staining or IHC analysis.

Discussion

Pancreatic cancer is one of the leading causes of cancer-related deaths in the world, resulting in 330,000 deaths in 2012, according to a World Health Organization report. This disease occurs most frequently in developed countries. The five-year survival rate is approximately 20% for early-diagnosed patients; otherwise, only 5% of patients can survive for five years^{19,20}. Although surgery, chemotherapy, and radiotherapy were explored in the treatment of pancreatic cancer, the outcome did not inspire optimism. Thus, finding novel clues for the early diagnosis and effective treatment of pancreatic cancer is a priority.

Aconitine belongs to the family of aconitum alkaloids and is reported to interact with voltage-dependent sodium-ion channels on the cell membranes of excitable tissues. For example, aconitine could bind to the receptor at the neurotoxin-binding site 2 on the α subunit of the channel protein²¹. Aconitine can be metabolized by cytochrome P450²². However, few studies have reported the effects of aconitine on tumorigenesis. Our research team is the first to explore the potential role of aconitine in pancreatic cancer. Aconitine could inhibit the growth of pancreatic cancer cell proliferation, as evidenced by the cell viability assay and colony formation assay, which are consistent with those in other studies^{23,24}. In a xenograft mouse model, aconitine

administration was shown to inhibit the growth of pancreatic cancer and induce cell apoptosis. All of these data demonstrate that aconitine could act as a potential therapeutic reagent to treat pancreatic cancer patients through its effective function against tumor growth.

The induction of cell apoptosis is a novel clue for cancer treatment²⁵. In this study, we found through Hoechst staining and cell cytometry analysis that aconitine administration could remarkably increase cell apoptosis in both Mia-paca-2 and PANC-1 cell lines. As mentioned, at least two pathways leading to apoptosis exist, namely, the extrinsic and intrinsic pathways. The extrinsic pathway begins outside a certain cell when stimulated by oxidant stress or chemical attack. Death receptors, which belong to the superfamily of TNFR, are the first stop when apoptosis happens. Stress-induced apoptosis involves changing the mitochondrial permeability, the subsequent cyto. C release, and the final activation of caspase-9. Multiple proteins are involved in this process, including Bcl-2 (anti-apoptotic factor), Bcl-2 associated X-protein (BAX, proapoptotic factor), Bcl-2-antagonist of cell death, and cleaved poly ADP-ribose polymerase 1 (cl-PARP1)^{13,14,16,26-30}. The expressions of Bax (cyto.), Bcl-2, and NF- κ B decreased as the aconitine concentration increased. Meanwhile, the protein levels of Bax (mito.), cl-caspase-9, cl-caspase-3, cl-PARP, and cyto. C were remarkably upregulated after aconitine administration, which were coincident with the process of cell apoptosis.

Conclusions

Overall, our study validated the inhibitory effects of aconitine on pancreatic cancer, which was demonstrated to be associated with cell apoptosis. Our findings may provide novel evidence of aconitine as a potential therapeutic reagent against pancreatic cancer.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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