Aconitine induces apoptosis in H9c2 cardiac cells via mitochondria-mediated pathway

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Received July 10, 2017; Accepted October 13, 2017

DOI: 10.3892/mmr.2017.7894

Abstract. Aconitine, a diterpenoid alkaloids derived from Aconitum plants, is widely employed to treat various diseases. The aim of the present study was to investigate the apoptotic effect of aconitine in H9c2 cardiac cells. H9c2 cell apoptosis induced by aconitine was detected by a Cell Counting kit-8 assay, DAPI staining, Annexin V-FITC/propidium iodide double staining and western blotting. The effects of aconitine on reactive oxygen species levels and mitochondrial membrane potential were confirmed by fluorescence microscopy and flow cytometry. In addition, ATP contents were determined using a ATP-dependent bioluminescence assay kit. The levels of peroxisome proliferator activated receptor γ co-activator 1α (PGC-1α) expression and apoptosis-associated proteins including Caspase-3, B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax) and Cytochrome c were also assessed. Taken together, the results indicated that aconitine may inhibit cell viability, decrease PGC-1α expression, induce mitochondrial dysfunctions, upregulate Cytochrome c, Bax and Caspase-3, and downregulate Bcl-2, suggesting that aconitine may induce apoptosis through mitochondria-mediated signaling pathways in H9c2 cells.

Introduction

For centuries, Aconitum plants have been extensively applied to treat various diseases, including inflammation, pain, neurologic and cardiovascular diseases in China and some other countries (1,2). Aconitine, one of the major bioactive alkaloids derived from Aconitum plants, belongs to genus *Aconitum* of Ranunculaceae family and is frequently employed to treat rheumatoid arthritis, cardiologic disorders and tumors (3,4). The ester combining with C8 and C14 is mainly responsible for its high toxicity, while hydrolysis of esters can reduce the toxicity effectively (5). Aconitine poisoning incidents have happened occasionally caused by misuse, suicide, or homicide and toxic symptoms include dizziness, numbness, nausea, vomiting, ventricular arrhythmias, or even death (6-8). Thus, the application of aconitine in clinics has been severely limited due to its toxic effects.

In recent years, accumulating evidences have demonstrated the pharmacological and toxicological characteristics of aconitine (9-11). Previous studies confirmed that aconitine could block the inactivation of voltage-dependent sodium channels causing persistent Na^+ influx at resting potential, thereby leading to arrhythmia (12,13). Moreover, aconitine diminishes the amplitude of delayed rectifier K^+ current in Jurkat T-lymphocytes, which probably affects the function of immune cells (14). Ca^{2+} is well known to play crucial roles in the pathogenesis of heart dysfunctions and disruption of intracellular Ca^{2+} homeostasis may cause arrhythmia (15,16). It is well established that aconitine increases intracellular Ca^{2+}, triggers arrhythmia, and finally induces apoptosis through activation and phosphorylation of p38 MAPK signaling pathway in rats (17). In addition, recent study revealed aconitine inhibits tumor growth and induces cell apoptosis via NF-κB signaling pathway in human pancreatic cancer (18). Based on above findings, it is convinced that aconitine could induce abnormal electrical activities in various cell and animal models. However, the impact of aconitine on cardiomyocytes apoptosis remains unclear.

Mitochondria are well known to be involved in various physiological and pathologic processes, including generation of reactive oxygen species, ATP production by oxidative phosphorylation, and metabolic pathways (19,20). The peroxisome proliferator activated receptor γ co-activator 1α (PGC-1α) is a master mitochondrial transcriptional regulator that promotes mitochondrial biogenesis and energy metabolism in various cells (21). It has been demonstrated that PGC-1α expression is significantly decreased in cardiac hypertrophy and heart

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Key words: aconitine, H9c2 cells, mitochondria, apoptosis

failure (22-24). Considering that PGC-1α plays a vital role in multiple cardiomyopathies, extensive efforts should be made to explore the potential effects of aconitine on PGC-1α expression and mitochondrial function. Meanwhile, recent evidences have also suggested that the mitochondrial pathway is one of classic apoptosis pathways (25). Cytochrome c, as a member of electron transport chain in mitochondria, is known to act important roles in the electron transfer and cell respiration (26). Furthermore, the release of Cytochrome c from mitochondria might subsequently activate the executioner caspases, resulting in cell apoptosis (27). Therefore, we turned our attention to aconitine-induced cell apoptosis.

The multiple effects of aconitine have been reported, however, whether aconitine could induce H9c2 cells apoptosis through mitochondria-dependent apoptotic pathway has been little studied. In our present research, H9c2 cell line was used as a model *in vitro* exposed to aconitine and further investigated its possible apoptosis mechanisms.

Materials and methods

Materials. Aconitine was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The molecular weight of aconitine is 645.74. HPLC analysis showed the purity was >98%. Aconitine was dissolved in the ethyl alcohol and diluted to corresponding concentration.

Cell culture and cell viability assay. Rat embryonic ventricular myocardial H9c2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Gibco; Invitrogen), at 37˚C in a humidified incubator of 5% CO_2. Cell viability was measured using Cell Counting kit-8 (CCK-8; Dojindo, Shanghai, China), according to the suppliers' instructions. H9c2 cells were seeded at densities of $2x10^4$ cells/ml into 96-well plates and then treated with different concentrations of aconitine (0-250 μM) for 24 h. CCK-8 was added to each well and then incubated for 4 h. The optical density was read at 450 nm with ELx808 Absorbance Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA) and cell viability was calculated. The experiments were repeated at least three times.

Assessments of nuclear morphology by DAPI staining. Cells were seeded onto 24-well plates and treated with aconitine (0, 100, 200 μM) for 24 h. Cells were washed with PBS thrice, fixed in 4% paraformaldehyde for 30 min, then nuclei were stained with DAPI staining solution for 10 min at room temperature in the dark. Morphologic changes were observed and images were acquired by an inverted fluorescence microscope (Nikon Corporation, Tokyo, Japan).

Detection of apoptotic rate of H9c2 cells by flow cytometry. The Annexin V-FITC/PI Apoptosis Detection kit (Beyotime Institute of Biotechnology, Haimen, China) was used to determine apoptosis of cells. In brief, cells were seeded into 6-well plates. After 24 h exposure to aconitine (0-200 μM), cells were trypsinized, washed twice with cold PBS and re-suspended in 195 μl binding buffer. The cells were then incubated with 5 μl Annexin V-FITC and 10 μl PI working solution for 15 min in the dark at room temperature, according to the manufacturer's instructions. Cellular fluorescence was measured by flow cytometry (FC500; Beckman Coulter, Inc., Brea, CA, USA). Each experiment was repeated at least three times.

Reactive oxygen species assay. Intracellular reactive oxygen species (ROS) in H9c2 cells was assessed using Reactive Oxygen Species Assay kit (S0033; Beyotime Beyotime Institute of Biotechnology, Haimen, China. DCFH-DA, a non-fluorescent probe, can be hydrolyzed to DCFH, and further oxidized to the highly fluorescent compound dichlorofluorescein (DCF) in the presence of ROS. H9c2 cells were incubated with aconitine for 4 h, subsequently, treated with 10 μM DCFH-DA for 20 min at 37˚C. The cells were washed with PBS three times and changes of green fluorescence were observed by fluorescence microscope (excitation 488 nm, emission 525 nm). In addition, the cells were also harvested and washed twice with PBS, then analyzed using flow cytometry (FC500; Beckman Coulter, Inc.).

ATP contents assay. Cellular ATP contents were assessed by Enhanced ATP Assay kit (S0027; Beyotime) according to the manufacturer's instructions. Briefly, cells were seeded at $4x10^5$ cells/well in six-well plates. After incubated with aconitine for 24 h, cells were rinsed and lysed using ATP lysis buffer on ice. Samples were collected and centrifuged at 12,000 rpm for 10 min at 4˚C to acquire supernatant for further determination. Samples and ATP detection working dilution were added and luminescence activity was measured immediately using luminometer (GloMax 20/20; Promega Corporation, Madison, WI, USA). Standard curve of ATP measure was made in each assay. Subsequently, the intracellular ATP contents were normalized by the protein contents in each sample.

Detection of mitochondrial transmembrane potential. Mitochondrial Membrane Potential Assay kit (JC-1; Beyotime Institute of Biotechnology) was used to evaluate mitochondrial membrane potential ($\Delta\Psi$m) of cells following the manufacturer's instructions. Briefly, cells were seeded onto 24-well plates and then treated with aconitine (0-200 μM) for 4 h. Cells were incubated with the medium containing JC-1 (molecular probes) for 20 min at 37˚C. CCCP was used as a positive control to induce the decrease of mitochondrial membrane potential. After washing with ice-cold JC-1 (1x) for two times, images were observed by a fluorescent microscope (Nikon Corporation). Additionally, cells were collected and rinsed with PBS, then analyzed using flow cytometry (FC500; Beckman Coulter, Inc.). The ratios of red fluorescence intensity over green fluorescence intensity represented the levels of $\Delta\Psi$m.

Western blot analysis. Cells treated with different concentrations of aconitine were lysed with the RIPA Lysis Buffer (Beyotime Institute of Biotechnology) and protein concentrations were measured by BCA Protein assay kit (Beyotime Institute of Biotechnology). Equal amount of protein extracts (80 μg) from each sample was separated by 10 or 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane.

The membranes were blocked with 5% BSA and then incubated overnight at 4˚C with primary antibodies against Bax (1:1,000; 2772S; Cell Signaling Technology, Inc., Danvers, MA, USA), Bcl-2 (1:1,000; ab13285; Abcam, Cambridge, UK), PGC-1α (1:1,000; ab191838; Abcam), Caspase-3 (1:1,000; ab179517; Abcam), Cytochrome c (1:1,000; 136F3; Cell Signaling Technology) and β-Actin (1:5,000; AT0001; CMCTAG). Membranes were washed three times and incubated with secondary antibody at a dilution of 1:1,000 in the same buffer for 2 h at room temperature. After washed three times, membranes were visualized using the ECL chemiluminescence system (ChemiScope, Shanghai). The intensity of specific brands was quantified and normalized to a loading control. All the experiments were conducted at least three times.

Statistical analysis. All experiments were repeated at least three times. Data were expressed as means ± SD. All statistical analyses were performed using GraphPad Prism software version 5.0. The statistical significance of differences were evaluated using one-way ANOVA followed by Bonferron's post hoc correction. P<0.05 was considered to indicate a statistically significant difference.

Results

Aconitine suppresses cell viability in cultured H9c2 cells. The cytotoxic effect of aconitine on H9c2 cells was detected by CCK-8 kit. As shown in Fig. 1, aconitine treatment for 24 h potently suppressed cell viability in a concentration-dependent manner (0-250 μM). Compared with the control group, cells were treated with 100, 200 μM of aconitine, cell viability were significantly decreased to 58±2.91 and 10±0.5%, respectively. The results indicated that aconitine could remarkably inhibit cell viability in a concentration-dependent manner in H9c2 cells.

Assessments of nuclear morphology by DAPI staining. To verify the effects of aconitine on apoptosis in H9c2 cells, DAPI staining was used to observe the morphological changes of aconitine-treated H9c2 cells. The fragmentation of cell nuclei exposed to aconitine for 24 h was observed, and cell nuclei shrinkage and chromatin condensation were increased slightly with the concentration of aconitine in H9c2 cells as compared to the control (Fig. 2).

Detection of aconitine-induced cell apoptosis by flow cytometry. In attempt to illustrate whether the decreased cell viability induced by aconitine was associated with cell apoptosis, Annexin V-FITC/PI double staining was further performed. As shown in Fig. 3, after exposed to 0, 100, and 200 μM aconitine for 24 h, cell apoptosis rates of both early and late apoptosis were 4.61±0.76, 22.16±0.64, and 44.64±3.23%, respectively, and the cell necrosis rate significantly increased. Our data indicated that treatment with aconitine not only reduced the numbers of surviving cells but also increased the cell numbers in early and late apoptosis.

Effects of aconitine on reactive oxygen species. Reactive oxygen species is mainly produced by mitochondria, which

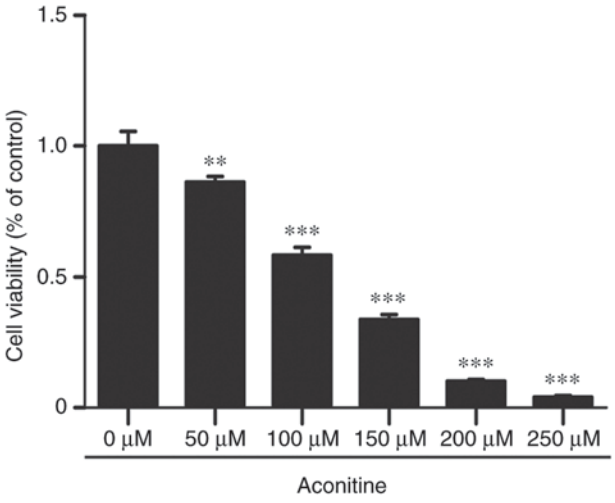

Figure 1. Aconitine suppresses cell viability in cultured H9c2 cells. H9c2 cells were incubated with different concentrations of aconitine (0, 50, 100, 150, 200, and 250 μM). Cell viability was assessed after treatment for 24 h by the Cell Counting kit-8. Data are presented as the mean ± standard deviation. **P<0.01 and ***P<0.001 vs. control group (0 μM).

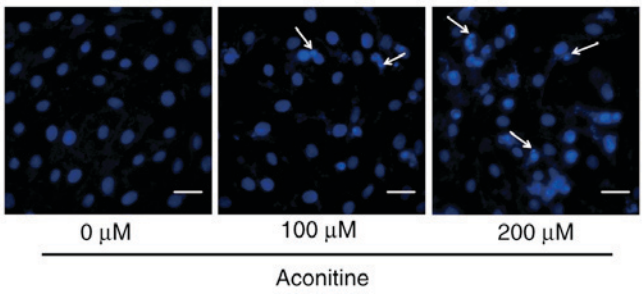

Figure 2. Effects of aconitine on nuclear morphology in H9c2 cells. H9c2 cells morphological changes were revealed by DAPI stain and visualized under fluorescence microscopy upon treatment with different concentrations of aconitine (0, 100, and 200 μM). Scale bar, 10 μm. Arrow indicated aconitine-induced cell nuclei shrinkage and chromatin condensation (magnification, x400).

is associated with the process of cells apoptosis. Meanwhile, excessive ROS can influence cell viability and alter mitochondrial function. In order to clarify whether aconitine could increase cellular ROS levels, H9c2 cells were treated with aconitine (0-200 μM) for 4 h. As seen in Fig. 4A, green fluorescence distinctly increased after treatment with aconitine. Flow cytometry analysis revealed that the levels of ROS after treated with aconitine (200 μM) were obviously increased to approximately two folds compared with the control cells (Fig. 4B and C). The results of flow cytometry were consistent with the fluorescent images, indicating that aconitine could effectively promote the production of ROS in H9c2 cells compared with the control group.

Inhibition the generation of intracellular ATP induced by aconitine. To evaluate the changes of ATP contents during apoptosis of H9c2 cells induced by aconitine, ATP contents were detected by enhanced ATP assay kit. As depicted in Fig. 5, the cellular ATP contents were decreased in a concentration-dependent manner after exposure to aconitine for

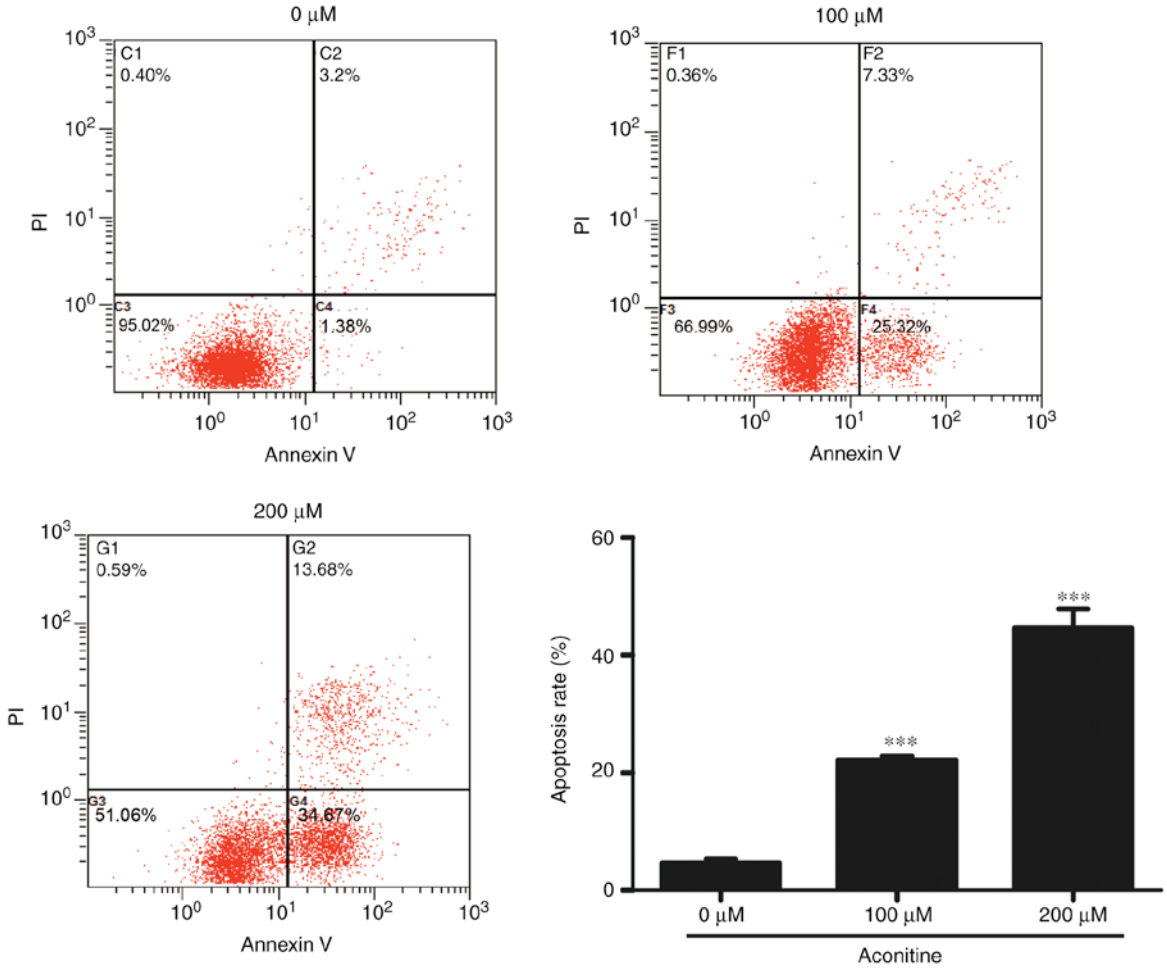

Figure 3. Detection of cell apoptosis in H9c2 cells by Annexin V-PI staining assay. Cells treated with different concentrations of aconitine (0, 100 and 200 μM) for 24 h. (A) H9c2 cells were stained with FITC-Annexin V-PI and analyzed by flow cytometry. (B) Quantitative analysis of aconitine-induced apoptosis. Values of apoptotic ratios for each group are presented as the mean ± standard deviation. ***P<0.001 vs. control group (0 μM). PI, propidium iodide.

24 h compared with the control. Aconitine (200 μM) notably decreased ATP contents, as compared to control.

Decreased mitochondrial transmembrane potential and PGC-1α expression induced by aconitine. To investigate the effects of aconitine on mitochondrial functions in H9c2 cells, mitochondrial transmembrane potential was measured by JC-1 staining. As shown in Fig. 6, fluorescent images of H9c2 cells stained with JC-1 showed that cells emitted red fluorescence with a little green fluorescence in control group, suggesting that mitochondrial membrane potential was normal. When ΔΨm is low, the JC-1 aggregates becomes monomer form green fluorescence with a little red fluorescence in cells treated with aconitine (100, 200 μM) for 4 h. The ratio of red and green fluorescence indicates the relative level of ΔΨm. The results showed that the ΔΨm was significantly lower compared with the control group. Moreover, aconitine decreased PGC-1α expression remarkably.

Aconitine induced apoptosis-related proteins expression in H9c2 cells. To confirm the signaling pathway involved cell apoptosis, western blotting was further performed. We examined the expression of Caspase-3, Bcl-2, Bax, Cytochrome c (Fig. 7). These results demonstrates aconitine could upregulate Bax and cleaved Caspase-3, as well as downregulate Bcl-2. In addition, aconitine significantly increased the expression level of Cytochrome c.

Discussion

Aconitum alkaloids are mainly used in China and other Asian countries to treat rheumatoid arthritis and cardiovascular disorders (28). However, the high toxicity restricts its clinical application (29). Previous studies have mainly focused on the aconitine-induced arrhythmia. However, little information is available about the impacts of aconitine on mitochondria. Mitochondria are known to have critical roles in regulating energy production and metabolism especially in high energy demanding cells, such as cardiomyocytes and neuronal cells (30). Emerging researches have demonstrated that the integrity of mitochondrial structure and function is essential for maintaining normal cardiac function (31,32). Therefore, we paid our attention to investigate the effects of aconitine on mitochondria and explore the relevant apoptosis signaling pathways in H9c2 cells.

To investigate the effects of aconitine on H9c2 cells, CCK-8 assay was firstly used to assess the cytotoxicity. It is intriguing that cell viability was potently inhibited after

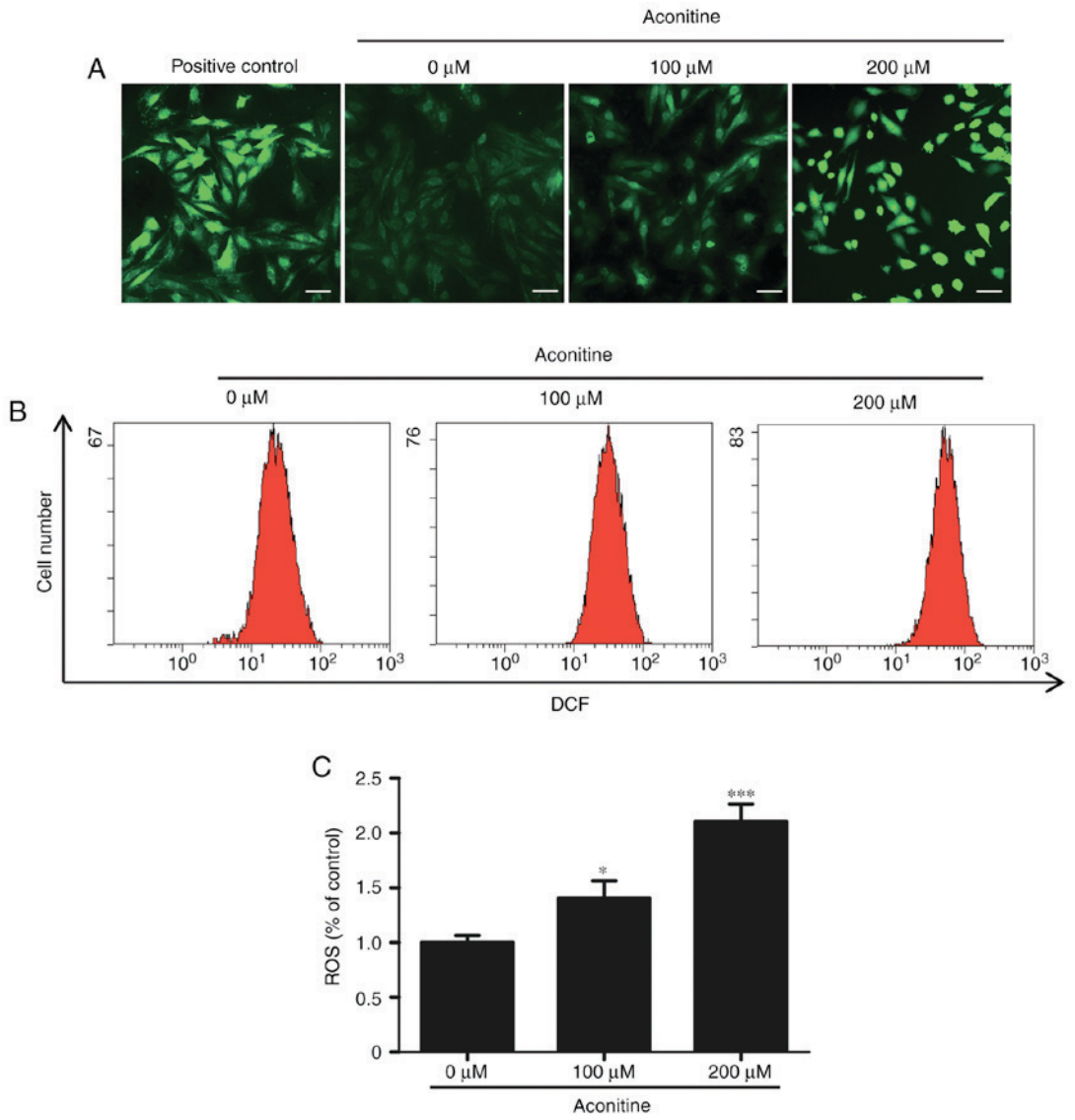

Figure 4. Effects of aconitine on ROS in H9c2 cells. Cells were treated with different concentrations of aconitine (0, 100 and 200 μM) for 4 h. (A) ROS fluorescence images visualized by a fluorescence microscope (magnification, x200; scale bar, 10 μm). (B) The fluorescent intensity was measured by flow cytometry. (C) Quantitative analysis of the level of ROS. Data are expressed as the mean ± standard deviation of three experiments. *P<0.05 and ***P<0.001 vs. control (0 μM). ROS, reactive oxygen species; DCF, dichlorofluorescein.

exposed to aconitine for 24 h. Nevertheless, the results of Annexin V-FITC/PI double staining indicated that aconitine could markedly induce cell apoptosis in early and late apoptosis. Consistent with the above results, DAPI staining showed that aconitine-exposed H9c2 cells exhibited obvious morphological changes of apoptosis including cell nuclei shrinkage and chromatin condensation. In conclusion, the results demonstrated aconitine could induce apoptosis in H9c2 cells.

Mitochondrial membrane potential determined by the difference of mitochondrial inner and outer membrane acts a vital role in maintaining mitochondrial function (33). ROS, a product of aerobic metabolism in mitochondria, is involved in early stages of apoptosis, and triggers the loss of the $\Delta\Psi$m (34,35). PGC-1α, as a crucial coactivator of nuclear receptors, stimulates mitochondrial biogenesis and energy metabolism. Ectopic expression of PGC-1α could result in mitochondrial ultrastructural abnormalities and mitochondrial dysfunction (36,37). Previous studies have demonstrated

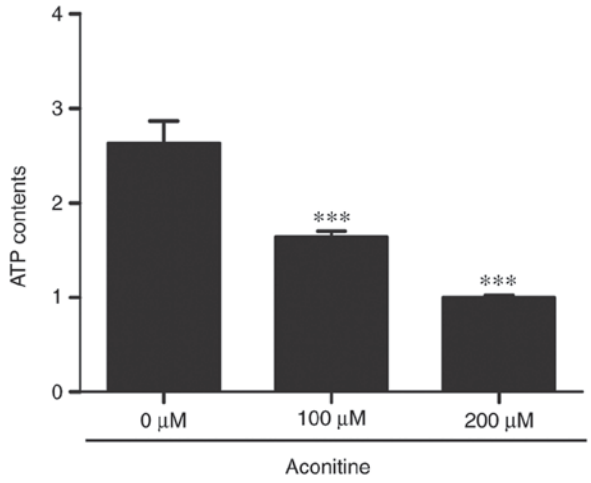

Figure 5. Aconitine inhibits the generation of intracellular ATP. Cells were treated with aconitine for 24 h and ATP levels in H9c2 cells were measured with an enhanced ATP assay kit. Data were expressed as the mean ± standard deviation. ***P<0.001 vs. control group (0 μM).

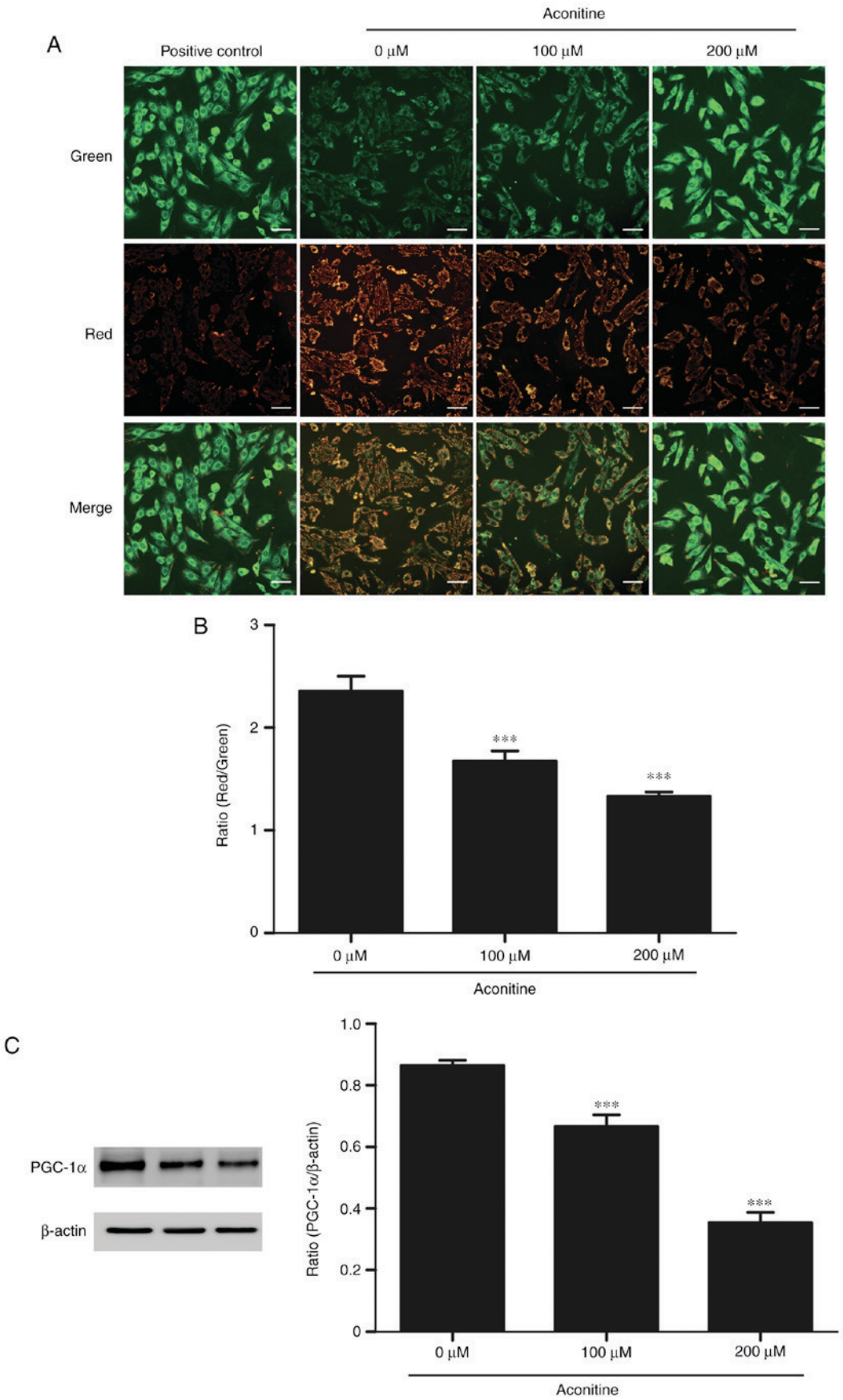

Figure 6. Aconitine treatment decreases mitochondrial membrane potential and PGC-1α expression in H9c2 cells. (A) Cells were treated with different concentrations of aconitine (0, 100 and 200 μM) for 4 h. Mitochondrial transmembrane potential fluorescence images observed by a fluorescence microscope (magnification, x200; scale bar, 10 μm). (B) Levels of mitochondrial transmembrane potential were detected by flow cytometry. The ratio of red/green fluorescent densities was calculated to assess the relative mitochondrial membrane potential. (C) Aconitine decreases PGC-1α expression in a concentration-dependent manner. Optical densities of the protein bands were quantitatively analyzed and normalized with loading control (β-Actin). Data are expressed as the mean ± standard deviation of three experiments. ***P<0.001 vs. control group (0 μM). PGC-1α, peroxisome proliferator activated receptor γ co-activator 1α.

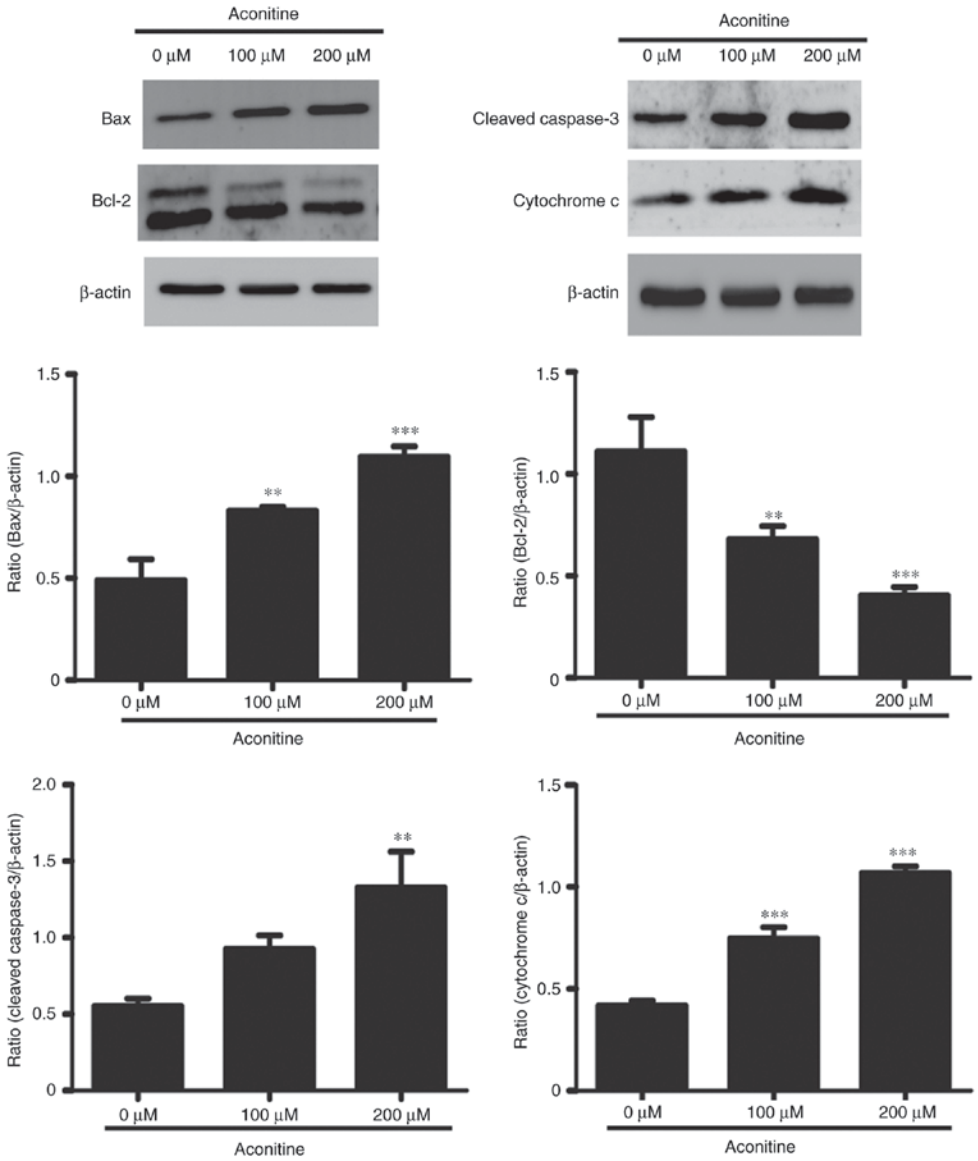

Figure 7. Aconitine induced the expression of apoptosis-related proteins in H9c2 cells. The expression of Bcl-2, Bax, Cytochrome c, and Caspase-3 were detected by western blot. Quantification of Bcl-2, Bax, Cytochrome c, and Caspase-3 expression. The β-actin was used as an internal control. Values for each group are presented as the mean ± standard deviation. **P<0.01 and ***P<0.001 vs. control (0 μM). Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

that the dissipation of ΔΨm could impact the generation of ATP and ROS, initiate the release of pro-apoptotic factors, thereby leading to mitochondrial dysfunction and cell apoptosis (38). In the study, we confirmed that aconitine enhanced ROS generation, decreased ATP contents and ΔΨm, and downregulated PGC-1α expression, indicating that mitochondrial damage might be concerned with the development of aconitine-induced apoptosis in H9c2 cells.

Apoptosis is a programmed cell death process, which is generally modulated via three major pathways: Mitochondrial-dependent pathway, endoplasmic reticulum pathway, and death receptor pathway (39-41). It seems increasingly evident that mitochondrial-dependent pathway has been considered as the one of the major signaling pathways in apoptosis of various cells (42). More importantly, mitochondrial transmembrane potential, reactive oxygen species, Bcl-2 family members, and caspases are associated with mitochondrial-mediated apoptosis pathway (43). We further analyzed the expression of apoptosis associated proteins including Bcl-2 family proteins (Bcl-2, Bax), Caspase-3, and Cytochrome c by western blotting to confirm the possible mechanisms connected with aconitine-induced apoptosis. Anti-apoptotic protein Bcl-2 decreased while Bax increased after treatment with aconitine. Thus, the ratio of Bcl-2/Bax, which plays a crucial role for the activation of the mitochondrial apoptotic pathway, was notably decreased in cells exposed to aconitine. Bcl-2 family proteins are involved in the process of apoptosis and play central role in regulating mitochondrial-dependent apoptotic pathway (44,45). It is worth mentioning that the release of Cytochrome c is essential to initiate mitochondrial-mediated cells apoptotic pathway (46,47). The levels of Cytochrome c, Caspase-3 were significantly increased at the concentrations of 100, 200 μM aconitine, which are in line with the severity of cell apoptosis. Increasing evidences also suggest that mitochondria play a crucial role in the release of Cytochrome c and

pro-apoptotic proteins, which subsequently active caspases and induce apoptosis (48,49). In addition, previous studies have demonstrated PGC-1α was downregulated in human breast cancer, colon cancer (50,51), indicating that PGC-1α may be associated with the prognosis of cancers. More recently, the expression of PGC-1α decreased was also found in human epithelial ovarian cancer and PGC-1α overexpression could induce Ho-8910 cell apoptosis by the PPARγ-dependent pathway (52). Contrary with the result, we found decreased PGC-1α was associated with the process of cell apoptosis, which is probably due to cancer cells differ from normal cells in many ways that allow them to grow out of control, accompany gene mutations and become invasive. More importantly, it has been demonstrated doxorubicin decreased significantly the expression of PPARα and PGC-1α in primary cardiomyocytes *in vitro*, indicating that PGC-1α could involve energy metabolism remodeling and induce cells apoptosis (53,54). We speculate decreased PGC-1α may correlate with apoptosis in H9c2 cells exposed to aconitine, however, the functional mechanisms of PGC-1α in aconitine-induced cells apoptosis still need to be further investigated. Our results suggested that mitochondrial-dependent pathway is partially involved in aconitine-induced apoptosis in H9c2 cells.

In summary, the present study demonstrated that aconitine induced apoptosis of H9c2 cells at least in part via mitochondria-dependent apoptotic pathway. As described already, the apoptotic pathway was triggered by decreased PGC-1α expression, induced mitochondrial dysfunction, upregulated Cytochrome c, Bax, cleaved Caspase-3, downregulated Bcl-2, ultimately leading to apoptosis of H9c2 cells. Although H9c2 cells provided a unique model *in vitro* to investigate the mechanisms of cells apoptosis in our preliminary study, there is still some limitations including single cell line. Thus, animal model and primary cardiomyocytes will be used to further validate our conclusion in the following research. Therefore, our findings may provide a possible mechanism of the aconitine-induced apoptosis partially through mitochondria-mediated pathway in H9c2 cells.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant no. 81571848) and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

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