

Aconitine-induced cardiac arrhythmia in human induced pluripotent stem cell-derived cardiomyocytes

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Abstract. Pre-clinical evaluation of cardiac dysfunction is important for assessing the safety of traditional or novel medicines due to the universality of potential drug-induced heart failure and irreversible arrhythmia. Aconitine (ACO), a traditionally used anti-pyretic, analgesic and anti-rheumatic drug, has been reported to have arrhythmogenic effects. In the present study, the Real-Time Cellular Analysis Cardio system was applied to evaluate the arrhythmogenic effects of ACO in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs). The results indicated that ACO is capable of increasing the frequency and decreasing the amplitude of hiPSC-CM contraction in a dose- and time-dependent manner. ACO at 0.25 μ M increased the beating rate of hiPSC-CMs by 3.7-fold within 30 min, while 3.0 μ M of ACO increased the beating rate by 7.3-fold. The present study also evaluated the potential pro-apoptotic effects of ACO by using caspase-3 and caspase-9 kits. To the best of our knowledge, the present study was the first to record the ACO-induced cardiac arrhythmia of hiPSC-CMs in real-time. The results also indicate that ACO-induced cell death is mediated, at least in part, by caspase-dependent apoptotic pathways.

Introduction

Aconitum carmichaelii Debeaux is widely used in traditional Chinese medicine, which has been used in China and other countries for >2,000 years due to its antipyretic, antirheumatic and analgesic activities (1-3). There are 32 prescriptions

containing aconitum in the Chinese Pharmacopoeia (4). However, the use of aconitum has been associated with severe cardiovascular toxicities, including tachyarrhythmia and hypotension (5). Aconitine is a key active component of aconitum plants. A recent study has reported that when combined with quercetin, aconitine synergistically inhibited the proliferation of HeLa cells at a wide range of concentrations (6). Identifying the toxic effects of ACO is important for the safe clinical application of aconitum species. The arrhythmogenic effects of ACO include the induction of ventricular tachycardia (VT) and ventricular fibrillation (VF), which result in a high mortality in affected patients (7,8). In isolated sheep heart Purkinje fibers, ACO has been demonstrated to act as a cardiac Na⁺ channel agonist that opens the Na⁺ channels during the depolarization/repolarization phase of an action potential, leading to a delayed repolarization and early after-depolarization (7,9). Similar effects of ACO were also obtained with isolated ventricular myocytes of mice, rats and guinea pigs (10). A previous study has reported that L-type calcium channel (LTCC) inhibition is a major mechanism of the arrhythmogenic action of ACO on human cardiomyocytes (5). While the cardiotoxic effect of ACO has been comprehensively documented in animal cardiomyocytes, the effects of ACO in human cardiomyocytes and the underlying mechanisms have remained to be assessed due to the lack of human cardiomyocyte models and suitable methods.

Human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs), which have a high similarity with native human cardiac myocytes in their structure and in function, have provided useful models to help elucidate cardiovascular function and diseases (11,12). HiPSC-CMs have been successfully adopted for modeling various cardiac diseases and for drug testing. A recent scientific breakthrough, namely the development of the Real-Time Cellular Analysis (RTCA) Cardio system, provides a homogeneous population of relatively pure single cells *in vitro* (13). The RTCA Cardio system allows for the real-time, label-free and non-invasive analysis of cardiomyocyte function. This platform has been used for cardiovascular toxicity screening, drug-induced cardiac contractility evaluation and estimating the risk of drug-induced arrhythmia (14-17).

The present study aimed to monitor the cardiotoxic effects of ACO in hiPSC-CMs by using the RTCA cardio system. It was observed that ACO was capable of triggering arrhythmogenic

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Abbreviations: CMs, cardiomyocytes; RTCA, real-time cell analysis system; hiPSC-CMs, human induced pluripotent stem cell-derived cardiomyocytes; CI, cell index

Key words: aconitine, arrhythmia, human induced pluripotent stem cell-derived cardiomyocytes, real-time cell analysis system

effects in hiPSC-CMs, as indicated by an increased beating frequency and a decreased amplitude. Such changes were accompanied by dose- and time-dependent intensive temporal profiling and gradually decreased cell index (CI). The potential pro-apoptotic effects of ACO on hiPSC-CMs were also evaluated. The resulting enhanced caspase-3 and caspase-9 activities indicated that ACO-induced cell death was mediated, at least in part, via caspase-dependent apoptotic pathways.

Materials and methods

Reagents and materials. ACO was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and dissolved in dimethyl sulfoxide (DMSO). Fetal bovine serum (FBS) for cell culture was purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). PSCeasy® pluripotent stem cell culture medium (PSCM; cat. no. CA1001500) was provided by Cellapbio (Beijing, China).

Cell culture of hiPSC-CMs. HiPSC-CMs, provided by a domestic manufacturer (cat. no. CA4024106; Cellapbio, Beijing, China), were cultured in fibronectin-coated wells of 96-well plates according to the manufacturer's protocols. In brief, the plates were prepared by coating each well with 50 μ l 0.1% gelatin overnight at 4°C. Frozen vials of cells were thawed at 37°C and diluted with pre-warmed PSCM at 500,000 cells/ml. After removing the excess coating solution, 100 μ l of the cell suspension (containing 50,000 cells) was added per well. The plates containing cells were kept at room temperature for 30 min and then maintained at 37°C in a humidified incubator containing air with 5% CO₂. The culture medium was refreshed daily without disturbing the attached cells.

MTT assay. The cell viability was determined by using the MTT assay. In brief, hiPSC-CMs were seeded into 96-well plates at a density of 50,000 cells/well. The cells were treated with ACO at the concentration of 0.125, 0.25, 0.5, 1, 2, 4, 8 μ M or DMSO as a vehicle control (final concentration of DMSO, 0.3%). After incubation for the indicated durations, 20 μ l 0.5 mg/ml MTT solution (Sigma-Aldrich; Merck KGaA) was added to each well, followed by further incubation for 4 h. The medium was then removed and the formazan crystals were dissolved in 100 μ l DMSO. The absorbance was measured at 570 nm on a microplate reader (Tecan Infinite M1000; Tecan Group, Ltd, Maennedorf, Switzerland). The relative cell viability was expressed as a percentage of the control group.

Functional data recording and analysis. The xCELLigence RTCA Cardio instrument (ACEA Biosciences, Inc., San Diego, CA, USA) was used to monitor the cardiomyocyte contractility. Impedance signals were recorded, from which the CI value was calculated, a measure of relative changes in electric impedance that represents the cell status. Hence, the number of attached cells and their morphology were reflected by the CI value. The E-Plate 96 (ACEA Biosciences, Inc.) was coated with 50 μ l 0.1% gelatin overnight at 4°C. The solution was then replaced by 150 μ l pre-warmed PSCM and incubated at 37°C for 4 h. The background impedance of the media was determined prior to seeding the cells to ensure that all wells were

functional. The software automatically informs the researcher if any connection problems arise. Following harvesting and counting, hiPSC-CMs were seeded onto the plate at a density of 50,000 cells per well. The E-plate was monitored every 15 min on the RTCA Cardio Instrument at 37°C in a 5% CO₂ incubator after incubation for 15 min at room temperature for an initial cell adhesion at the bottom of each well. The plate was then incubated in a 5% CO₂ incubator at 37°C. Typically, drug treatment was initiated 60-100 h after cell seeding, when the contraction was continuous and stable.

ACO treatment of hiPSC-CMs. ACO was dissolved in dimethyl sulfoxide (DMSO). Once the hiPSC-CMs generated robust and regular beating signals (usually occurring on day 3 after seeding), drug treatment was initiated. The culture medium was replaced with 90 μ l fresh PSCM 4 h prior to drug treatment. Subsequently, 90 μ l drug solution at the two-fold final concentration (dissolved in PSCM) was added to each well. HiPSC-CMs were treated with ACO at the final concentrations of 0.25, 0.3, 0.5, 1, 1.5, 2, 2.5 and 3 μ M or with a vehicle control. The cellular response to the drug treatments was recorded over 20-sec every 5 min in the first 30 min and every 15 min thereafter. After the data acquisition, the RTCA Cardio software 1.0 was used to calculate the parameters, including such as the normalized beating rate and amplitude with statistical analysis. Experiments were performed in 6 wells in parallel and repeated three times.

Measurement of the activities of caspase-3 and caspase-9. Caspase-3 and caspase-9 activities in the lysates of cells were determined using the CaspACE™ Assay System (Promega Corp., Madison, WI, USA) and the Caspase-Glo® 9 Assay kit (Promega Corp.) following the manufacturer's instructions. In brief, control- or ACO-treated cells were lysed, incubated on ice for 30 min and then centrifuged at 16,000 x g for 10 min at 4°C. The supernatant fraction was collected. For the colorimetric caspase-3 activity assay, the supernatant was incubated with 200 μ M DEVDpNA substrate at 37°C for 4 h and the absorbance was measured at 405 nm using a microplate reader. Caspase-9 activity was measured using a luminescent assay with the samples mixed with the respective aliquot of Caspase-Glo® 9 reagent and incubated for 3 h at room temperature. The luminescence was measured with a plate-reading luminometer (GloMax® Navigator; Promega Corporation, Madison, WI, USA) according to the protocol of the luminometer manufacturer.

Statistical analysis. For data analysis, the cellular impedance index, beating rate and amplitude were measured off-line using RTCA Cardio software 1.0 and normalized for each well to the baseline (pre-dose) values measured prior to the drug treatment. Statistical analysis was performed using SPSS 21.0 software (IBM Corp., Armonk, NY, USA). Values are expressed as the mean \pm standard deviation. Statistical significance of differences was estimated by one-way analysis of variance with Tukey-Kramer corrections. Comparisons of continuous variables between two groups were performed using unpaired two-tailed Student's t-tests. Multiple group comparisons were performed using Student-Newman-Keuls

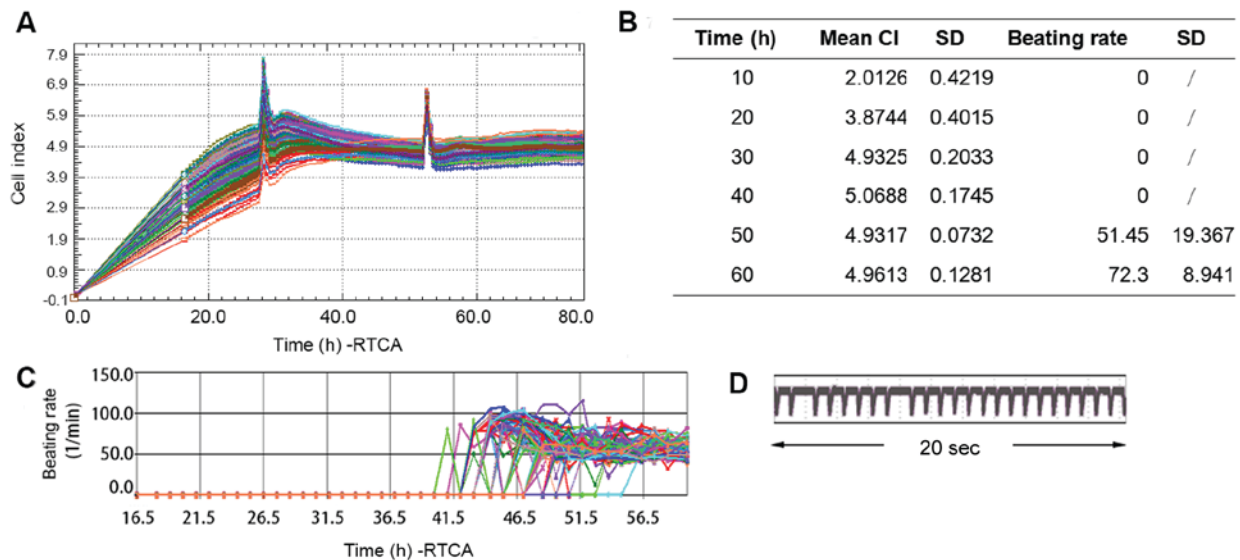


Figure 1. Growth curve and typical contraction of hiPSC-CMs. (A) Growth curve of hiPSC-CMs. (B) Statistical chart of CI and beating rates of hiPSC-CMs. (C) Beating rates of hiPSC-CMs. (D) Typical transient pulse patterns of the hiPSC-CMs over 20 sec. Values are expressed as the mean \pm SD (n=3). hiPSC-CMs, human induced pluripotent stem cell-derived cardiomyocytes; RTCA, real-time cellular analysis; CI, cell index; SD, standard deviation.

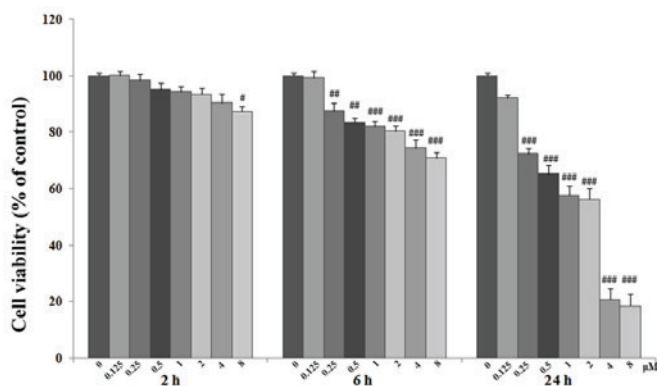


Figure 2. Viability of human induced pluripotent stem cell-derived cardiomyocytes after treatment with ACO at the specified concentrations/durations. The cell viability was assessed by an MTT assay. Values are expressed as the mean \pm standard deviation (n=3). #P<0.05, ##P<0.00, ###P<0.001 vs. control group. ACO, aconitine.

following analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Cultured hiPSC-CMs exhibit a functional cardiomyocyte phenotype on the xCELLigence Cardio platform. Based on previous studies (18), cells were seeded on the E-Plate 96 at 50,000/well. As presented in Fig. 1A and B, after 40 h of culture, the CI reached a stable value of 5.0688 ± 0.1745 , which was representative of viable hiPSC-CMs. Spontaneous contraction was observed at ~ 42 h after and stable contraction was achieved at ~ 60 h with a beating rate of 72.3 ± 8.941 bpm. Fig. 1C represents the beating rates of different wells of hiPSC-CMs from the beginning until stable contraction. Typical transient pulse patterns of hiPSC-CMs over a duration of 20 sec is presented in Fig. 1D. Based on these results, the subsequent experiments were performed at 60 h after seeding.

Time- and dose-dependent effects of ACO on hiPSC-CMs. In the clinical setting, ACO induces cell death of cardiomyocyte in certain patients within hours of intravenous administration (19,20). The present study investigated ACO-induced cytotoxicity by an MTT assay. Cells were grown on fibronectin-coated plates and exposed to ACO at 0.125, 0.25, 0.5, 1, 2, 4 or $8 \mu\text{M}$ for 2, 6 or 24 h. As presented in Fig. 2, exposure to 0.125– $4 \mu\text{M}$ ACO for 2 h only had a marginal effect on the cell viability. However, exposure for longer periods and/or to higher concentrations of ACO induced a dose- and time-dependent decrease in viability compared with that of the control cells (P<0.05 or P<0.01). ACO at 2– $4 \mu\text{M}$ had a significant but not excessive cytotoxic effect. Therefore, ACO was used at the concentrations of 0.25, 0.3, 0.5, 1, 1.5, 2, 2.5 and $3 \mu\text{M}$ in the subsequent experiments.

Time- and dose-dependent arrhythmogenic effects of ACO on hiPSC-CMs. The clinical use of ACO has been associated with various arrhythmias by affecting the electrophysiological characteristics of cardiomyocytes (5). To gain insight into the electrophysiological effects of ACO, the beating properties of hiPSC-CMs were assessed by using the xCELLigence Cardio platform. At 85 h after seeding, when the cardiomyocyte contraction was continuous and stable, ACO at the concentration of 0.25, 0.3, 0.5, 1, 1.5, 2, 2.5 or $3 \mu\text{M}$ was added to the hiPSC-CMs. As presented in Fig. 3A, the spontaneous beating rate of hiPSC-CMs was rapidly increased by ACO. All concentrations of ACO were capable of markedly enhancing the beating rate, and the effect of ACO was time- and dose-dependent. ACO at $0.25 \mu\text{M}$ increased the beating rate of hiPSC-CMs by 3.7-fold within 30 min, while $3.0 \mu\text{M}$ ACO increased the beating rate by 7.3-fold. As presented in Fig. 3B, amplitudes of hiPSC-CMs were reduced in parallel with the increase of the beating rates. A reduction of the CI was also observed within 6 h culture. In comparison with the control group, ACO treatment induced a time- and dose-dependent decrease in cell viability as shown by the cell index (Fig. 3C).

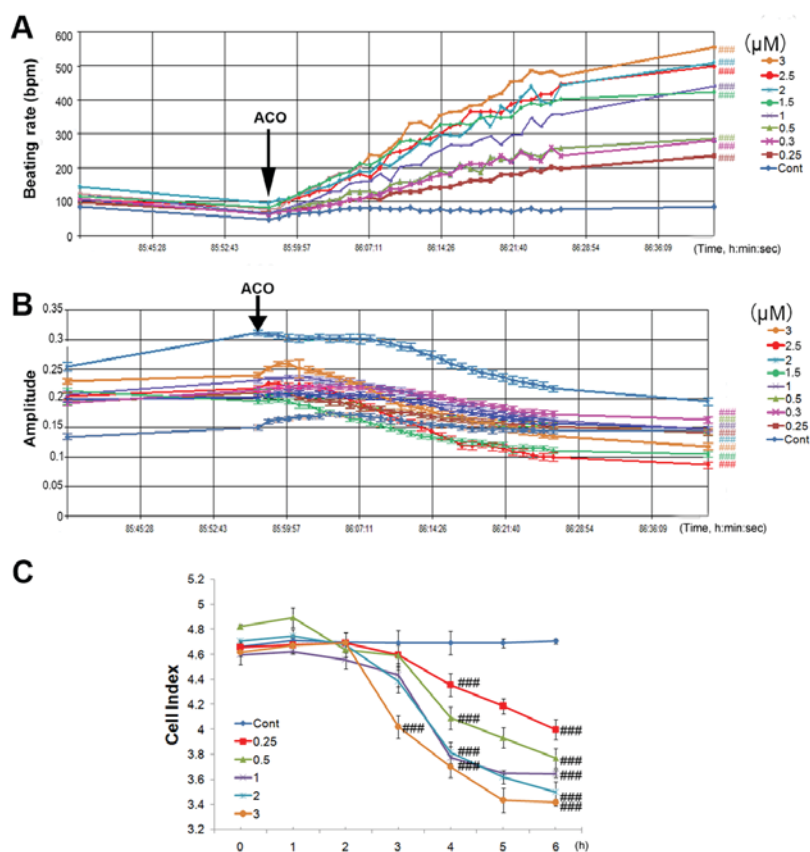


Figure 3. Dose effects of ACO on the hiPSC-CMs at the specified concentrations. (A) Effect of ACO on the beating rate of hiPSC-CMs. (B) Effect of ACO on the amplitude of hiPSC-CMs. (C) Cell index fluctuation of hiPSC-CMs in the presence of various doses of ACO. Values are expressed as the mean \pm standard deviation (n=3). ###P<0.001 vs. control group. ACO, aconitine; hiPSC-CMs, human induced pluripotent stem cell-derived cardiomyocytes; Cont, control.

ACO at 3.0 μM significantly decreased the CI after 3 h of incubation, while low concentrations of ACO (0.25, 0.3, 0.5, 1.0, 1.5, and 2.0 μM) also significantly decreased the CI within 4 h (P<0.001). Of note, after 6 h of incubation, 3.0 μM ACO reduced the cell index by 27.2% (P<0.001).

Effects of ACO on the typical temporal profiling and beating rates of hiPSC-CMs. The transient pulse patterns of hiPSC-CMs treated with various doses of ACO were then assessed. Fig. 4A presents typical temporal profiles at 5 time-points prior to and after ACO treatment. ACO had time- and dose-dependent stimulatory effects on the beating pattern of hiPSC-CMs. Accelerated beating patterns were observed with all concentrations of ACO immediately after addition of the drug. Of note, 3.0 μM ACO caused a serious disorder within 5 min, followed by an intense temporal profiling and relative low amplitude, which may be associated with lethal cardiac arrhythmias. Furthermore, the intensive beating patterns were gradually further compressed as time went on. The beating rate of hiPSC-CMs after 30 min of ACO treatment is presented in Fig. 4B.

Effects of ACO on caspase-dependent apoptosis of hiPSC-CMs. The activation of caspase is a unique feature of apoptotic cell death. In order to investigate whether ACO-induced cytotoxicity is associated with caspase-dependent apoptosis, the activities of caspase-3 and caspase-9 were analyzed using assay kits. hiPSC-CMs were

grown onto fibronectin-coated plates and exposed to 3.0 μM ACO, which was selected according to the aforementioned results. The activities of caspase-3 and caspase-9 were detected at different time-points. As presented in Fig. 5, ACO treatment resulted in a time-dependent increase in caspase-3 and caspase-9 activities after 4-6 h of treatment when compared with those in the control group.

Discussion

Along with the extensive use of aconitum, the cardiotoxic effect of ACO has received increasing attention (7). Studies have reported that ACO is capable of inducing VT and VF by opening the Na^+ channels of isolated cardiac myocytes from mice, rats, guinea pigs and rabbits (21). In addition, ACO-induced LTCC inhibition has been reported (5,22). In the present study, the RTCA Cardio system was applied, which is able to monitor the contractility of cardiomyocyte in real-time, to evaluate the arrhythmogenic effects of ACO in hiPSC-CMs. The results indicate that ACO is capable of triggering arrhythmogenic effects in hiPSC-derived cardiomyocytes in a dose- and time-dependent manner. Furthermore, the results suggested that ACO-induced cardiomyocyte death is mediated, at least in part, by inducing apoptosis. Overall, these results are in agreement with those of previous studies from cellular and human biopsy studies demonstrating ACO-induced cardiotoxicity (7,9,22).

Due to the high incidence of drug-induced heart failure and irreversible arrhythmia, the pre-clinical evaluation of the

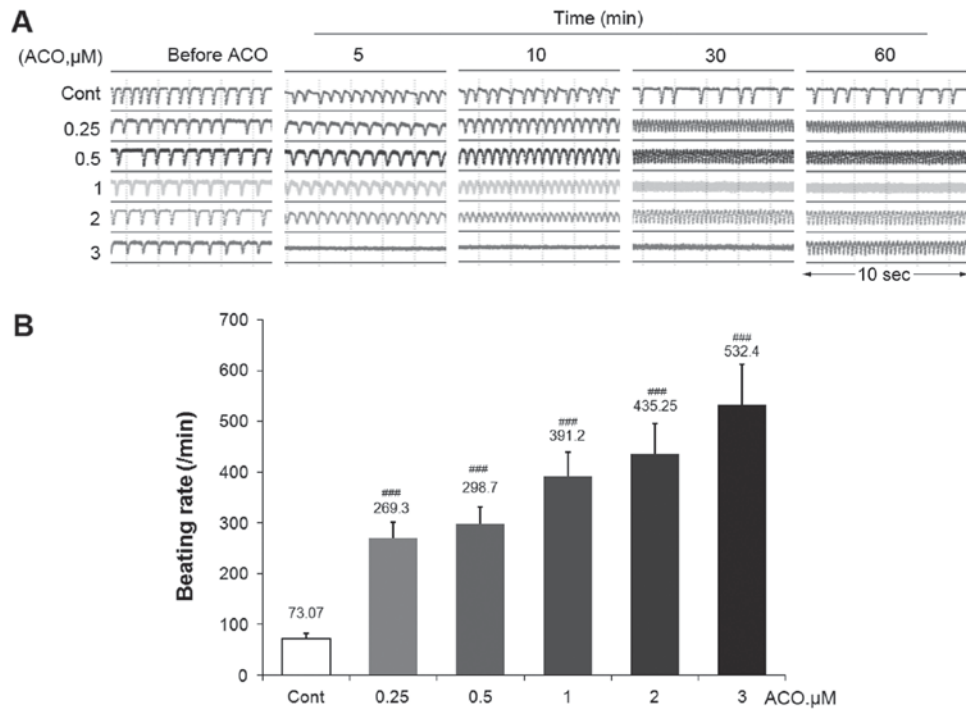


Figure 4. Effects of ACO on the typical temporal profiling and beating rates of hiPSC-CMs. (A) The transient pulse patterns of hiPSC-CMs treated with various doses of ACO; (B) The beating rate of hiPSC-CMs after 30 min of treatment with various doses of ACO. Values are expressed as the mean ± standard deviation (n=3). ###P<0.001 vs. control group. ACO, aconitine; hiPSC-CMs, human induced pluripotent stem cell-derived cardiomyocytes; Cont, control.

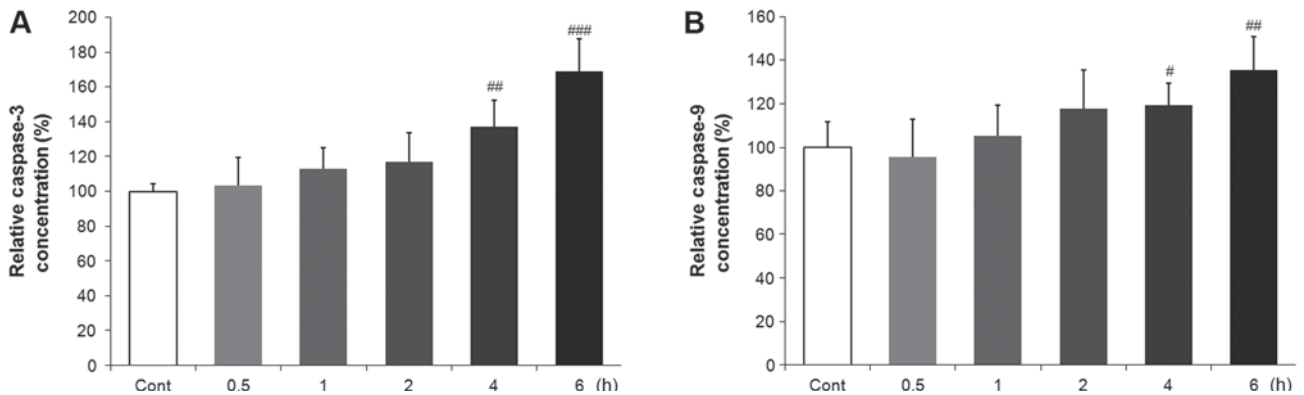


Figure 5. Effect of ACO on the apoptosis of hiPSC-CMs. hiPSC-CMs were incubated with ACO (3.0 μM) for different durations. (A) Caspase-3 and (B) caspase-9 were measured to determine the apoptosis of hiPSC-CMs. Values are expressed as the mean ± standard deviation (n=3). *P<0.05, **P<0.00, ###P<0.001 vs. control group.

cardiotoxicity of drugs is important (23). However, efficient approaches to evaluate the cardiotoxicity of traditional or novel treatments have been lacking. The development of drug evaluation systems remains difficult due to the lack of human cardiomyocyte models and suitable methods. hiPSC-CMs, which have predefined contractile characteristics of native cardiomyocytes, a genetically relevant background and the scope of serving as a model of relevant cardiac disease phenotypes, have provided an opportunity for pre-clinical drug evaluation (14,24). More importantly, hiPSC-CMs also have stable electrophysiological and contractile characteristics, which allows for a wide range of applications, including drug discovery, toxicity testing and cardiac disease research (12,25). A sensitive tool with accurate recording capacity is also required, since most of the previous techniques

only defined end-point measurements, for example MTT assays and flow cytometry, which can be performed at specific time points but not continuous. The impedance-based system RTCA has provided an opportunity to reassign a variety of assays, including pre-clinical drug evaluation, compound validation, monitoring of compound effects and exploration of cardiac disease models (13,15). The RTCA Cardio system has provided a more sensitive tool to detect potential cardiac side effects when compared to cytotoxicity assays using the H9C2 cell line. Furthermore, the sensitive label-free assay made it possible to detect the regular beating pattern of cardiomyocytes under physiological or pathological conditions. The RTCA Cardio system uses regular beating patterns to reflect the detailed beating status and to assess the beating activities of cardiomyocytes, which are diverse

and comprehensive (16,26). To analyze the detailed beating status, multiple parameters require assessment. Thus, in the present study, using the RTCA Cardio system, the dose and time responses to ACO were characterized by four parameters, including the CI, beating rate, amplitude and beating pattern in hiPSC-CMs. Compared to the H9C2 cell line, the spontaneous rhythm of the hiPSC-CMs tended to be more consistent and less irregular after cultivation for a certain duration.

The present results illustrated that ACO has time- and dose-dependent stimulatory effects on the beating pattern of hiPSC-CMs. The spontaneous beating rates of hiPSC-CMs were rapidly increased by ACO. At all concentrations (0.25, 0.3, 0.5, 1, 1.5, 2, 2.5 and 3.0 μM), ACO was capable of markedly enhancing the beating rate. Accelerated beating patterns were observed immediately upon addition of the drug. Of note, 3.0 μM ACO caused an intensive temporal profiling and relative low amplitude, which may be associated with lethal cardiac arrhythmias. Furthermore, the intensive beating patterns were gradually further compressed as time went on. ACO at 0.25 μM increased the beating rate of hiPSC-CMs by 3.7-fold within 30 min, while 3.0 μM of ACO increased the beating rate by 7.3-fold when compared with the control group. The resulting rapid collapse of the beating pattern due to excessive acceleration indicated that ACO exerts a significant stimulatory effect on cardiac contraction. The amplitudes of the beating of the hiPSC-CMs were reduced in parallel with the increase of the beating rates. A reduction of the CI and cell viability was also observed within 6 h of incubation with ACO. In comparison with the control group, ACO treatment induced a time- and dose-dependent decrease in cell viability and CI. Treatment with ACO at 3.0 μM significantly decreased the CI at 3 h, while low concentrations of ACO (0.25, 0.5, 1 and 2.0 μM) also significantly decreased the CI within 4 h.

Irreversible cell death, which includes apoptosis and necrosis, is another aspect of ACO-induced cardiotoxicity. Apoptosis (programmed cell death), which may be activated by a caspase-dependent or -independent pathway, is one of the crucial factors for cardiomyocyte loss. In order to investigate whether the ACO-induced cytotoxicity is associated with caspase-dependent cell apoptosis, the activities of caspase-3 and caspase-9 were analyzed with specific assay kits. ACO caused a significant increase in caspase-3 and caspase-9 activities after 4 and 6 h of treatment when compared with those in the control group. These results suggest that the ACO-induced cell death is mediated, at least in part, by caspase-dependent cardiomyocyte apoptosis. However, necrosis, involving swelling of mitochondria, irreversible damage to cellular membranes, potentially includes in ACO-induced cell death (27). The cytotoxicity of ACO on the hiPSC-CMs was evident at dose as low as 0.25 μM . The present results support the use of the hiPSC-derived cardiomyocytes as a human cellular model to assess the potential cardiotoxicity of pharmaceutical agents.

In conclusion, the present study suggested that ACO dose- and time-dependently induced arrhythmia and cardiotoxicity. An experimental *in vitro* cell model to mimic regular cardiac contraction was explored as a tool to provide the novel insight into the cardiac safety of ACO *in vitro*. More importantly, the present study introduced an efficient and effective

approach to evaluate the potential cardiac risk of the tested compounds.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FZ and LC conducted the study. JZ and FZ designed the experiments. XQ, FZ and CL performed experiments, and collected and analyzed the data. All authors commented on the study and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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