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Original Research

Swainsonine induces apoptosis of rat cardiomyocytes via mitochondria-mediated pathway

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Abstract: Swainsonine is an Astragalus membranaceus extract. It is indole, alkaloid, and soluble in water. Its effect on rat cardiomyocytes apoptosis, and the mechanisms underlying that effect, were investigated by inducing apoptosis in H9c2 cells. This was detected by MTT assay, Annexin V-FITC/propidium iodide double staining and western blotting. Flow cytometry and fluorescence microscopy were used to confirm swainsonine's effect on mitochondrial membrane potential and levels of reactive oxygen species, while an ATP-dependent bioluminescence assay kit served to find the ATP contents. Assessment was also carried out for peroxisome proliferator activated receptor γ co-activator 1α (PGC-1α) expression levels as well as those of such apoptosis-associated proteins as Cytochrome c, Caspase-3, B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax). Overall, indications were that swainsonine may have the potential to inhibit viability of cells, decrease expression of PGC-1α, induce mitochondrial dysfunction, upregulate Cytochrome c, Bax and Caspase-3, and downregulate Bcl-2. The suggestion would be that apoptosis may be induced through signalling pathways in H9c2 cells mediated by mitochondria.

Key words: Swainsonine; Apoptosis; Rat cardiomyocytes; Mitochondria-mediated pathway.

Introduction

Swainsonine (SWA, Figure 1A) is indole, alkaloid, and soluble in water. It was originally an extract from the Swainsona canescens fruit in Australia and Locoweed in North America. Its inhibition of lysosomal acid, cytosolic a-mannosidases and Golgi a-mannosidase II is both specific and potent and leads to the accumulation of hybrid type oligosaccharides and a reduction in glycoproteins with complex side-chains (1-4). There is growing evidence to indicate that SWA prevents tumours from growing and metastasising, makes NK and LAK cells more lethal, reduces viability in human melanoma cells and encourages the proliferation and differentiation of bone marrow cells (5). Although this evidence suggests that abnormal processing could be induced by SWA in a range of cells and animal models, it is as yet unclear what impact it would have on apoptosis in cardiomyocytes.

Previous research has shown mitochondria to be involved in a variety of pathological and physiological processes. These include generating reactive oxygen species (ROS), oxidative phosphorylation producing ATP, and acting as metabolic pathways (6). One master mitochondrial transcriptional regulator, promoting mitochondrial biogenesis and energy metabolism, is PGC-1α (peroxisome proliferator activated receptor γ co-activator 1α) (7). A multiplicity of cardiomyopathies as well as cardiac hypertrophy and heart failure have shown evidence of a reduction in expression of PGC-1α (8), and so sustained research into SWA's possible impact on PGC-1α expression and mitochondrial function is indicated. The mitochondrial pathway is also a classic apoptosis pathway (9). Cytochrome c is part of the mitochondrial electron transport chain and an essential component not only in electron transfer but also in cell respiration (10). It is possible that executioner caspases are activated by release of Cytochrome c from mitochondria, with consequent cell apoptosis (11). While there has been reporting of SWA's multiple effects, little research has been done into SWA's ability to induce apoptosis in rat cardiomyocytes through an apoptotic pathway dependent on mitochondria.

For all these reasons, the H9c2 cell line was exposed in vitro to SWA to examine SWA's induction of rat cardiomyocytes apoptosis as well as the mitochondria-dependent apoptotic pathway and proteins related to apoptosis for SWA in vitro's apoptotic mechanism.

Materials and Methods

Materials

Swainsonine was bought from Chengdu Herbpurify Co., LTD (Chengdu, China), dissolved in ddH2O. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and carbonyl cyanide m-chlorophenylhydrazone (CCCP) were bought from Sigma Chemical Co. (St. Louis, USA).

Cell lines and cell culture

Embryonic rat ventricular myocardial H9c2 cells were obtained from KeyGEN BioTECH (Nanjing, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Invitrogen, Carlsbad, CA) with 10% added foetal bovine serum (FBS; Gibco; Invitrogen, Carlsbad, CA) at 37°C in the presence of 5%

CO2.

Cytotoxicity Assay by MTT

A slightly modified version of the MTT procedure described earlier was used to determine SWA's antiproliferative effect on H9c2 cells. Cells in logarithmic growth were seeded at 1 × 104 cells per well in 96-well microtiter plates, before being exposed to a range of concentrations of SWA for forty-eight hours. Four hours before the experiment ended, MTT dye (10 μL of 2.5 mg/mL in PBS) was added to each well in a 37 °C incubator containing 5% CO2. Next, the plates were centrifuged for fifteen minutes at 1500 rpm before discarding the supernatant with no disturbance to formazan crystals and cells in the wells. The MTT formazan crystals were dissolved in 150 μL of DMSO before agitating the plates on a plate shaker for five minutes. A microplate reader (Thermo, USA) was used to read the optical density (OD) at 490 nm. GraphPad Prism 6.0 software (San Diego, CA, USA) was used to calculate the IC50 values. Each experiment was conducted three times for each SWA concentration.

Annexin V-FITC/propidium iodide (PI) apoptosis assay

H9c2 cells were seeded at 1 × 105 cells per well in a 6-well microtiter plate.

After exposure to SWA (0, 10, and 20μM) for twenty-four hours, the cells were trypsinized before washing twice with cold PBS and then re-suspending them in a binding buffer. Next, the cells were treated with Annexin V-FITC/PI using the apoptosis detection kit (Nanjing Jiancheng Bioengineering Institute) in accordance with the protocol. Flow cytometry (BD FACSCalibur, USA) was used to analyse cellular fluorescence. Each experiment was carried out at least three times.

Reactive oxygen species assay

ROS Assay kit (Beyotime Institute of Biotechnology, Nanjing, China) was used to assess intracellular ROS in H9c2 cells. The non fluorescent probe DCFH DA can be hydrolysed to DCFH, after which further oxidisation is possible when ROS is present to DCF (dichlorofluorescein), which is extremely fluorescent. H9c2 cells were incubated with SWA for four hours before being treated with 10 μM DCFH-DA at 37˚ C for twenty minutes. The cells were washed three times with PBS and the fluorescence microscope showed changes in green fluorescence (excitation 488 nm, emission 525 nm). After harvesting, the cells were washed twice with PBS and then analysed by flow cytometry (BD FACSCalibur, USA).

ATP contents assay

An Enhanced ATP Assay kit (Beyotime) was used as instructed by the manufacturer to assess cellular ATP contents. Cells were seeded at 4x105 cells per well in six-well plates. After twelve hours incubation with SWA, the cells were rinsed and lysed by means of ATP lysis buffer on ice. Centrifuging samples for ten minutes at 12,000 rpm at 4˚ C produced supernatant for further examination. Immediately after adding ATP detection working dilution to the samples, luminescence was measured using a luminometer (GloMax 20/20; Promega Corporation, Madison, WI, USA). A standard curve of the ATP measurement was made for each assay, and then the intracellular ATP contents were normalised by each sample's protein contents.

Detection of mitochondrial transmembrane potential

Cells' mitochondrial membrane potential (ΔΨm) was examined using a Mitochondrial Membrane Potential Assay kit (JC-1; Beyotime Institute of Biotechnology) according to manufacturer's instructions. H9c2 cells were seeded at 1 × 105 per well in a 24-well microtiter plate before incubating for twenty-four hoursand then treating for four hours with 0, 10 or 20 μM SWA. A flow cytometer (BD FACSCalibur, USA) was used to assess variations in mitochondrial membrane potential (ΔΨm) of the H9c2 cells.

Western blotting analysis

Changes in protein expression relating to this study were analysed using western blotting analysis. H9c2 cells were harvested following a range of treatments, and lysed before using a BCA protein assay kit (Thermo Fisher Scientific, MA, USA) to measure protein concentrations. Western blotting analysis was carried out in accordance with previous studies. Primary antibodies for Bax and Cytochrome c and β-actin were bought from Cell Signalling Technology (Beverly, MA, USA) and the primary antibodies of PGC-1α, Bcl-2, Caspase-3 from Abcam (Cambridge, UK), with β-actin acting as internal reference. Chemiluminescence was measured with an ECL Kit (Pierce Biotechnology) and a Bio-Rad molecular imager first visualised and then captured the immunoreactive bands. ImageJ was used to calculate the band's total intensity for each protein, with the results normalised to that of β-actin.

Statistical analysis

GraphPad Prism 6.0 statistical software (San Diego, CA, USA) was used to carry out statistical analysis, with results expressed as the mean of arbitrary values ± standard deviation (SD). One-way ANOVA and then Tukey's multiple comparison, with statistical significance $P < 0.05$, were used to assess statistical significance.

Results

In vitro cytotoxicity of SWA against H9c2 cells

The MTT method was used to find SWA's cytotoxicity on H9c2 cells. Figure 1B shows that forty-eight hours SWA incubation significantly reduced the viability of cells; the degree by which viability was reduced depended on the concentration (0, 0.625, 1.25, 2.5, 5, 10, 20, 30 and 40 μM). Apart from a control group (0 μM), cells treated with more than 10 μM of SWA showed viability reduced to below 57.9%, which would indicate that SWA has an anti-proliferation effect against H9c2 cells that depends on the level of concentration.

Cell apoptosis by flow cytometry

Annexin V-FITC/PI double staining was used to search for a connection between cell apoptosis and the SWA-induced reduction in cell viability. As Figure 2

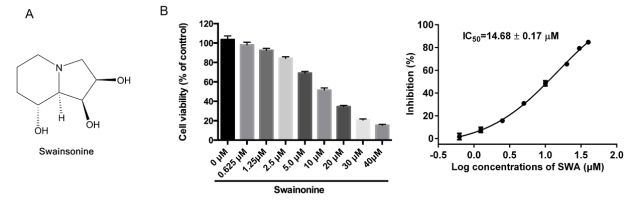

Figure 1. A) The structure of swainsonine; (B) The anti-proliferation effects of SWA against H9c2 cells at different concentrations (0, 0.625, 1.25, 2.5, 5, 10, 20, 30 and 40 μM). Each data point is presented as mean ± SD for three independent tests.

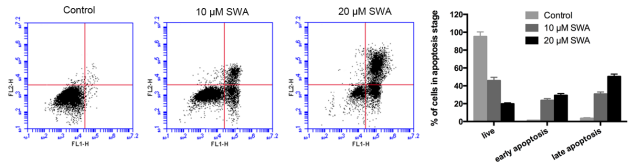

Figure 2. The effect of SWA on H9c2 cells apoptosis with Annexin-FITC/PI staining analysed by flow cytometry and the quantitative analysis of SWA-induced apoptosis. Each group's data were presented as the mean ±SD between three independent tests.

shows, the rates of early and late cell apoptosis after twelve hours exposure to 0, 10, and 20 μM SWA were respectively 3.52, 30.74, and 50.31%, and cells treated with varying SWA concentrations showed a significant increase in rates of cell necrosis.

Effects of SWA on reactive oxygen species

For the most part, ROS is the product of mitochondria and associated with cell apoptosis. An excess of ROS can have an influence on the viability of cells and can change the function of mitochondria. To examine the possibility of cellular ROS levels being increased by SWA, H9c2 cells were treated for four hours with SWA in concentrations of 0, 10 and 20 μM. As Figure 3A shows, there was a marked increase in green fluorescence after SWA treatment. Flow cytometry analysis showed relative content of ROS after treatment with 10 or 20μM of SWA to have increased more than twice when compared with the control cells to which no SWA had been added, and the fluorescence's intensity compared with the positive control (2.0 μM CCCP) (see Figure

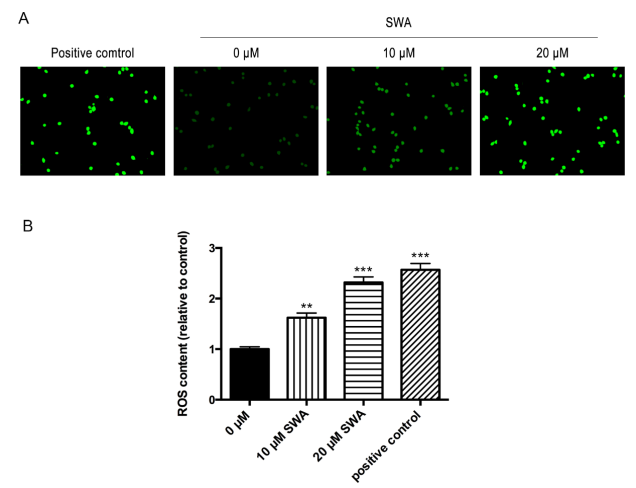

Figure 3. Effects of SWA on ROS in H9c2 cells. (A) ROS fluorescence images visualised by a fluorescence microscope (magnification, x200; scale bar, 10 μm); (B) Quantitative analysis of the level of ROS by flow cytometry. In each case, data are presented as mean ± SD for three independent tests.

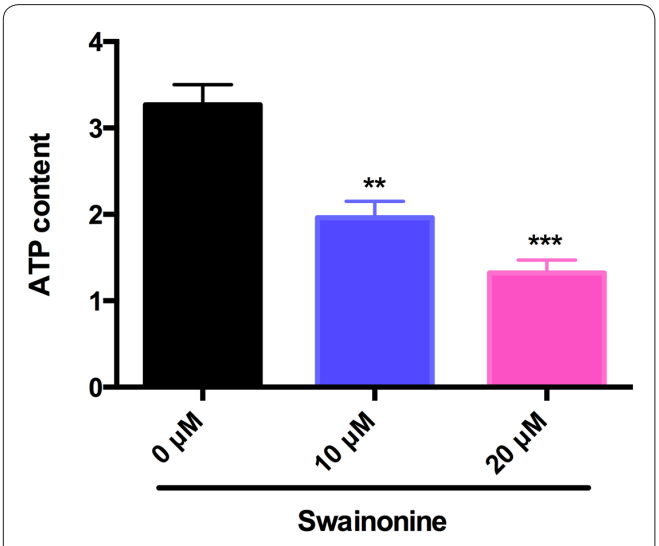

Figure 4. The inhibitory effect of SWA on the generation of intracellular ATP. Data were expressed as the mean ± SD for three independent tests. **P<0.01, ***P<0.001 vs. control group (0 μM).

3B). The flow cytometry results were in line with the fluorescent images, showing SWA's ability to promote ROS production of ROS in H9c2 cells to a significant degree compared with the control group (**P<0.01 for 10μM group, ***P<0.001 for 20μM group).

Effects of SWA on intracellular ATP generation

To evaluate changes in ATP content during SWA-induced H9c2 cell apoptosis, an enhanced ATP assay kit was used to detect ATP content. As Figure 4 shows, after twenty-four hours of exposure to SWA, cells' ATP contents reduced according to the percentage concentration. Compared with the control to which no SWA had been added, SWA concentrations of 10 or 20μM produced a remarkable decrease in ATP contents.

Effects of SWA on mitochondrial transmembrane potential and PGC 1α expression

To investigate how SWA afected H9c2 cells' mitochondrial functions, JC-1 staining was used to measure mitochondrial transmembrane potential. In a process facilitated by intact ΔΨm, JC-1 accumulates in normal mitochondria to form J-aggregates observable as red fluorescence. In a depolarised mitochondrial membrane,

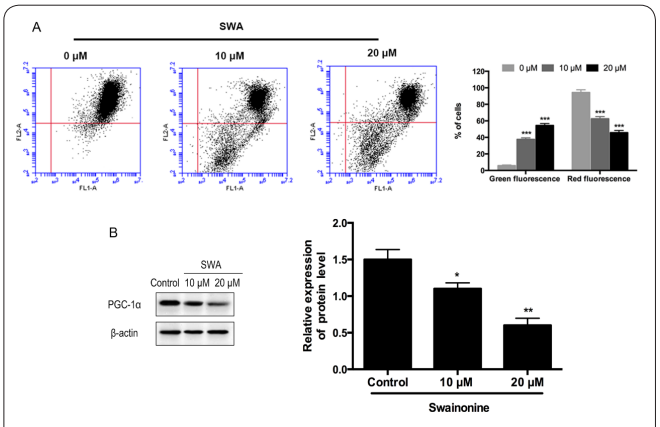

Figure 5. (A) SWA's effect on ΔΨm in H9c2 cells analysed by JC-1 kit and the fluorescence analysis by flow cytometry; (B) The effect of SWA on the expression of PGC-1α and its quantitative analysis. In each case, data are presented as mean ± SD for three independent tests. *P<0.05, **P<0.01 vs. control group (0 μM).

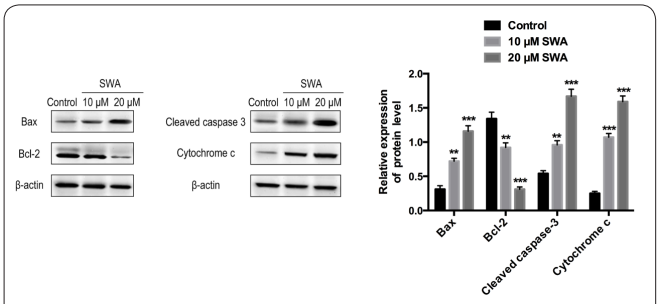

Figure 6. SWA's effect on expression of apoptosis-related proteins including Bcl-2, Bax, Cytochrome c, and cleaved Caspase-3, and their quantitative analysis. β-actin acted as internal control. In each case, data are presented as mean ± SD for three independent tests. **P<0.01, ***P<0.001 vs. control group (0 μM).

ΔΨm's collapse causes the release from the mitochondria of JC-1, which appears as green fuorescence. As Figure 5 shows, treating the cells with 10 or 20 μM of SWA caused a significant reduction in red fluorescence and an equally significant increase in green fluorescence compared with the control. Dependent on the concentration, SWA also suppressed PGC-1α expression to a remarkable degree.

Effects of SWA on apoptosis related proteins expression

Cell apoptosis's signalling pathway was verified by western blotting. Figure 6 shows determination of proteins related to apoptosis, including Caspase-3, Bcl-2, Bax and Cytochrome c, and the results show SWA's ability to up-regulate Bax and cleaved Caspase-3, and to down-regulate Bcl-2. SWA also significantly increased expression of Cytochrome c.

Discussion

Swainsonine is a trihydroxy indolizidine alkaloid and the principle toxic component isolated from many species of flowering plants and some fungi for the treatment of cancers including glioma and gastric carcinoma (5, 12). Its highly toxic nature limits its clinical application, examples being impaired host immune response in pregnant BALB/c mice, damaged cerebral cortical neurons and serious reproductive function disorders in livestock (13-15). There is little evidence on SWA's impact on cardiomyocytes. Mitochondria is known to be an essential component in the regulation of energy production and metabolism, particularly in such high energy demanding cells as cardiomyocytes and neuronal cells (16). Increased research has shown the mitochondrial structure's integrity and function to be essential if normal cardiac function is to be maintained (17, 18). Our attention therefore focussed on SWA's effects on mitochondria and on exploring the apoptosis signalling pathways involved in rat cardiomyocytes H9c2 cells.

An MTT assay was first carried out to assess SWA's cytotoxicity on H9c2 cells. Depending on the concentration, forty-eight hours of exposure to SWA inhibited cell viability to a pronounced degree. Double staining with Annexin V-FITC/PI produced consistent results, showing SWA's ability to induce significant cell apoptosis in early and late apoptosis. The conclusion was that SWA is able to induce apoptosis in H9c2 cells. Mitochondrial membrane potential, which is measured by the difference between the inner and outer mitochondrial membrane, plays an essential part in the maintenance of mitochondrial function (19). ROS is produced by the product of aerobic mitochondria, is involved early in apoptosis, and is the cause of the ΔΨm being lost (20). PGC-1α, in its critical role coactivating nuclear receptors, provides the stimulus for mitochondria biogenesis and energy metabolism. PGC-1α's ectopic expression can be the cause of ultrastructural mitochondrial abnormalities and dysfunction (21). Previous research has shown that ΔΨm dissipation can affect ATP and ROS generation, initiating pro-apoptotic factor release and causing mitochondrial dysfunction and cell apoptosis (22). This study confirmed the role of SWA in enhancing generation of ROS, reducing ATP contents and ΔΨm, and downregulating expression of PGC-1α, indicating that damage to mitochondria could be at the root of developing SWA-induced apoptosis in H9c2 cells. Apoptosis is programmed cell death and generally works through one of the mitochondrial-dependent, endoplasmic reticulum, or death receptor pathways (22). The first of those pathways has been seen as a major signalling pathway in the apoptosis of various cells (23) and it is associated with mitochondrial transmembrane potential, reactive oxygen species, Bcl-2 family members, and caspases. This research then used western blotting to analyse the expression of such proteins associated with apoptosis as the Bcl-2 family (Bcl-2, Bax), Caspase 3, and Cytochrome c to confirm possible mechanisms associated with connected with apoptosis induced by SWA. SWA treatment led to a decrease in anti-apoptotic protein Bcl-2 and an increase in Bax, so that the Bcl-2/Bax ratio, crucial in activating the mitochondrial apoptotic pathway, showed a marked decrease in cells exposed to SWA. Proteins in the Bcl-2 family play a part in apoptosis and have a key role in the regulation of the mitochondrial-dependent apoptotic pathway (24), the initiation of which requires release of Cytochrome c (25). Exposure to SWA at 10 and 20 μM concentrations produced a significant increase in Cytochrome c and Caspase-3 levels, consonant with cell apoptosis severity. There is also growing evidence that mitochondria have an important role to play in Cytochrome c and pro-apoptotic proteins release, which then activate caspases and bring about apoptosis (26). Previous research has shown downregulation of PGC-1α was downregulated in breast and colon cancers in humans (27), which suggests an association of PGC-1α with cancer prognosis. Reduced PGC-1α expression has also been found in human epithelial ovarian cancer and its over-expression can induce Ho-8910 cell apoptosis by the PPARγ-dependent pathway (28). On the other hand, this research found an association between decreases in PGC-1α and cell apoptosis, probably caused by differences between cancer cells and normal cells that allow uncontrolled growth, gene mutations and an invasive tendency in cancer cells. Doxorubicin significantly decreased the expression of PPARα and PGC-1α in primary cardiomyocytes in vitro, suggesting that PGC-1α could bring about energy metabolism remodelling and induce cell apoptosis (29). The speculation must be that there may be a correlation between a reduction in PGC-1α and apoptosis when H9c2 cells are exposed to SWA,

but more research is needed into PGC-1α's functional mechanisms in cell apoptosis induced by SWA. This research suggest a possible partial involvement of the mitochondrial-dependent pathway in apoptosis induced by SWA in H9c2 cells.

To summarise, this research showed apoptosis of H9c2 cells to be induced by SWA at least partially via the mitochondria-dependent apoptotic pathway. As already discussed, triggers for the apoptotic pathway were reduced expression of PGC-1α, induced mitochondrial dysfunction, upregulated Cytochrome c, Bax, cleaved Caspase-3, and downregulated Bcl-2, leading to H9c2 cell apoptosis. Although in our preliminary study H9c2 cells provided a unique model in vitro for investigation of the cell apoptosis mechanisms, limitations remain and they include single cell line. Animal models and primary cardiomyocytes will therefore be used in the following research to provide further validation of our conclusions and it is possible our findings will suggest a possible mechanism for SWA-induced apoptosis in part through the mitochondria-mediated pathway in H9c2 cells.

Interest conflict

The authors have no conflicts of interest to declare.

Author's contribution

This work was done by the authors named in this article and the authors accept all liabilities resulting from claims relating this article and its contents. The study was conceived and designed by Xiang Zheng and Xiaoming Yang; Shushu Wang collected and analysed the data; Dakai Chen wrote the manuscript and all authors read and approved the manuscript for publication.

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