Original Paper

Cardiac Shock Wave Therapy Attenuates H9c2 Myoblast Apoptosis by Activating the AKT Signal Pathway

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Key Words

Cardiac Shock Wave Therapy • Ischemia/Hypoxia • Apoptosis • AKT

Abstract

Background: Previous studies have demonstrated that Cardiac Shock Wave Therapy (CSWT) improves myocardial perfusion and cardiac function in a porcine model of chronic myocardial ischemia and also ameliorates myocardial ischemia in patients with severe coronary artery disease (CAD). Apoptosis plays a key role in ischemic myocardial pathogenesis. However, it remains unclear whether CSWT is beneficial for ischemia/hypoxia (I/H)-induced myocardial cell apoptosis and by which mechanism CSWT could improve heart function. We put forward the hypothesis that CSWT might protect heart function during ischemia/hypoxia by decreasing apoptosis. **Methods:** We generated ischemia/hypoxia (I/H)-induced apoptosis in the H9c2 myoblast cell line to examine the CSWT function and possible mechanisms. H9c2 cells were treated under hypoxic serum-starved conditions for 24 h and then treated with or without CSWT (500 shots, 0.06, 0.09, 0.12mJ/mm²). The apoptotic cell rate was determined by flow cytometry assay, cell viability was examined by the MTT assay, nuclear fragmentation was detected by Hoechst 33342 staining, and the mitochondrial-mediated intrinsic pathway of apoptosis was assessed by the expression of Bax and Bcl-2 protein and Caspase3 activation. **Results:** First, apoptosis could be induced by ischemia/hypoxia in H9c2 cells. Second, CSWT attenuates the cell death and decreases the H9c2 cell apoptosis rate induced by ischemia and hypoxia. Third, CSWT suppresses the expression of apoptosis molecules that regulate the intrinsic pathway of apoptosis in H9c2 cells. Fourth, CSWT increases the phosphorylation of AKT, which indicates the activation of the PI3K-AKT pathway. **Conclusions:** These results indicate that CSWT exerts a protective effect against I/H-induced cell death, potentially by preventing the activation of components of the mitochondrial-dependent intrinsic apoptotic pathway. We also demonstrate that the PI3K-Akt pathway may be involved in the CSWT effects on apoptosis.

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Introduction

Coronary artery disease (CAD) is a leading cause of morbidity and mortality worldwide [1]. The current therapeutic options are dependent on medical treatment, percutaneous coronary intervention (PCI), and coronary artery bypass grafting (CABG). With these three therapies, we have achieved significant improvement in morbidity and mortality [2]. However, the prognosis of patients with severe ischemic heart disease but without efficacious revascularization remains poor [3]. Therefore, it is crucial to develop alternative therapeutic strategies for these patients.

Cardiac Shock Wave Therapy (CSWT) is currently the most advanced technology developed in recent years. CSWT induces angiogenesis through the up-regulation of VEGF in ischemic myocardium and ameliorates myocardial ischemia and dysfunction in a porcine model of chronic myocardial ischemia, increasing myocardial perfusion and improving the patient's symptoms of angina and heart function [4-6].

Apoptosis plays a key role in the pathogenesis of CAD due to the loss of myocardial cells. It has been reported that patients with end-stage congestive heart failure and myocardial infarction have higher rates of myocardial cell apoptosis than normal subjects [7-10]. Apoptosis is a highly regulated program of cell death and can be mediated by two pathways, the extrinsic and intrinsic pathway, leading to Caspase activation. The cell death program is activated in myocardial cells by various apoptotic stimuli, including hypoxia, cytokines, increased oxidative stress, and DNA damage. Many studies have demonstrated that the inhibition of apoptosis can prevent the development of heart failure and improve heart function [11-14].

Recent studies have demonstrated that CSWT improves myocardial perfusion and cardiac function in a porcine model of chronic myocardial ischemia and also ameliorates myocardial ischemia in patients with severe CAD. Nonetheless, it is not clear whether CSWT has any beneficial effect on ischemia/hypoxia (I/H)-induced myocardial cell apoptosis and the mechanisms by which CSWT could improve heart function. Based on the promising results from animal and clinical studies, we put forward the hypothesis that CSWT can attenuate myocardial cell apoptosis. This study utilized an H9c2 cell ischemia/hypoxia model to test the effects of CSWT on apoptosis and to explore the possible mechanism of CSWT in improving myocardial function.

Materials and Methods

H9c2 cell culture

H9c2 myoblast cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD); all of the cell culture reagents were obtained from Invitrogen Corporation (Carlsbad, CA). The H9c2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 25 mM glucose, 2 mM glutamine, 100 µg/ml penicillin and 100 µg/ml streptomycin. The cells were maintained in a humidified incubator with 5% CO₂ at 37°C, passaged regularly, and subcultured to approximately 70% to 80% confluence pre-experimentally.

Simulated ischemia/hypoxia model

To induce apoptosis, the culture medium was replaced with serum-free Dulbecco's modified Eagle's medium, and the cells were transferred into a hypoxia chamber controlled by a ProOx system balanced with 5% CO₂/95% N₂ (Biospherix, Redfield, NY) for the indicated periods at 37°C. To determine the appropriate time to induce apoptosis, the H9c2 cells were treated under the ischemia/hypoxia conditions for 12, 24, 36, or 48 h.

Shock wave therapy

After incubation under I/H conditions for 24 h, the cells used for CSWT were transferred to special tubes (2ml tube, 5×10⁶ cells/ml). Shock waves were produced by the CSWT system (Modulith SLC, Storz

Medical, Switzerland), and the shock wave pulse was easily focused on the tube. We treated the H9c2 cells with 500 shots of shock wave at three different energy levels (0[control], 0.06, 0.09, and 0.12mJ/mm²) and then cultured the cells for 24h under normal conditions [4]. Moreover, two groups incubated under normal conditions and treated with CSWT (0.12mJ/mm²) or without CSWT were evaluated as another control.

Flow cytometry assay

The percentage of cells that underwent apoptosis or necrosis was determined using the Annexin V-FITC and propidium iodide (PI) Analysis kit according to the manufacturer's instructions. The cells were harvested, washed twice with cold 1X phosphate-buffered saline (PBS), and resuspended in 1 ml cold 1X binding buffer. A 100- μ l aliquot of the cell suspension was stained with 5 μ l of Annexin V-FITC for 10 minutes in the dark and at room temperature; 5 μ l of PI was then added for 5 min at room temperature, protected from light, and PBS was added to 500 μ l. Flow cytometry was carried out using a FACScan instrument, and the data were processed using WinMDI/PC software.

Cell viability assay

Cell viability was examined by the MTT assay according to the instructions of the manufacturer. H9c2 cells were plated in 96-well plates. The cells were pre-treated with I/H for 24h and then treated with CSWT. The cells were incubated with 0.5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylterazolium bromide for 4h, and the absorbance at 490 nm was measured, as described previously [15]. The mean optical density (OD) of six wells in the indicated groups was used to calculate the percentage of cell viability according to the following formula: percentage of cell viability = OD treatment group/OD control group $\times$ 100%. The experiments were performed in quadruplicate.

Hoechst 33342 staining

Nuclear fragmentation was detected by incubating fixed cells in 10 mmol/L Hoechst 33342, as previously described [15]. A total of 500-700 cells in 10 randomly chosen fields from each dish were counted to determine the percentage of apoptotic nuclei. The morphological features of apoptosis (chromatin condensation and nuclear fragmentation) were monitored by fluorescence microscopy. Each data point shows the results from 5000-7000 cells of 4-8 independent experiments.

Western blot analysis

Cell lysates (15-30 μ g of protein) were separated by 12% SDS-PAGE, transferred to PVDF membranes (Millipore), blocked with 5% nonfat dry milk, and probed with antibodies at 4°C overnight. The blots were incubated with HRP-conjugated anti-IgG, followed by detection with ECL (Millipore). Rat monoclonal antibodies against Bax, Bcl-2, Caspase-3, AKT, p-AKT, ERK, p-ERK, p38 MAPK, and p-p38 MAPK were purchased from Cell Signal Technology. The levels of staining for target proteins were normalized to those for β -actin as an internal control. ImageJ software was used to perform the densitometric analysis.

Statistical analysis

All of the statistical calculations were performed using the GraphPad Prism 5 software program. The data were expressed as the mean $\pm$ SEM. Student's t-test was used to compare two conditions, and a one-way ANOVA with Bonferroni correction was used for multiple comparisons. P values of less than 0.05 were considered significant.

Results

H9c2 cell apoptosis is induced by ischemia/hypoxia

To choose the appropriate time to induce apoptosis, H9c2 cells were cultured under I/H conditions for 12 h, 24 h, 36 h, or 48 h and showed a significant time-dependent reduction in cell viability compared to the control, as assayed by the MTT assay (Fig. 1A). The H9c2 cell viability was decreased by 26% after incubation under I/H conditions for 24 h, which is a medium degree of cell injury. Therefore, cells incubated under I/H conditions for 24 h were used in the subsequent experiments. After incubation under I/H conditions for 24 h, H9c2

Fig. 1. Ischemia/hypoxia induced H9c2 cell apoptosis. The H9c2 cells were incubated in standard DMEM (NC) or in ischemia/Hypoxia conditions (I/H). (A): The effects of simulated ischemia/hypoxia on the cell viability. I/H markedly increased H9c2 cells death assayed by MTT. (B): Ischemia/Hypoxia markedly increased H9c2 cell death assayed by Hoechst 33342 staining (white arrow indicates the apoptotic body or apoptotic nucleus) and the average data (C). $n=4$, Mean $\pm$ SEM. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs NC.

Fig. 2. The H9c2 cells were treated by Ischemia/Hypoxia for 12 h, 24 h, 36 h, and 48 h. (A): Western blotting was used to analysis of Caspase3 abundance in H9c2 cells. After incubate in I/H conditions for 24 h, the cleaved Caspase3 protein was begun to be detected. (B): The average data of Cleaved-Caspase3 in H9c2 cells. $n=5$, Mean $\pm$ SEM. ** $P<0.01$ vs NC.

cell apoptosis was markedly increased, as assayed by Hoechst staining (Fig. 1B-1C), and less full-length caspase3 and more cleaved caspase3 was found compared to the NC group (Fig. 2). This result suggested that it is appropriate to incubate under I/H conditions for 24 h to induce apoptosis.

Fig. 3. CSWT attenuates the cellular death induced by ischemia in the H9c2 cells. The H9c2 cells were incubated in standard DMEM (NC) or in I/H culture conditions with (I/H+CSWT) or without CSWT (I/H). (A): The H9c2 cell viability as shown by MTT assay. $n=4$. Mean $\pm$ SEM. $\#P<0.05$ vs NC-0, $*P<0.05$, $**P<0.01$ vs I/H-0. (B, C): CSWT markedly reduced H9c2 cell death, assayed by Hoechst 33342 staining. $n=4$. Mean $\pm$ SEM. $**P<0.01$, $***P<0.001$ vs I/H.

Fig. 4. CSWT decreased I/H-induced H9c2 cell apoptotic rate by Flow Cytometry Assay. The H9c2 cells were incubated in standard DMEM (NC) or in I/H culture conditions with (I/H+CSWT) or without CSWT (I/H). The cells were labeled with a combination of FITC-Annexin V and propidium iodide (PI). The percentage of early apoptotic cells (lower right quadrant) is indicated. $n=3$. Mean $\pm$ SEM. $*P<0.05$, $**P<0.01$ vs I/H.

CSWT attenuates the cell death induced by ischemia and hypoxia in H9c2 cells

CSWT significantly reduced I/H-induced cell death and increased cell viability. Compared to treatment with I/H alone, cell viability was strongly enhanced after CSWT treatment, with

Fig. 5. CSWT suppresses the expression of apoptosis molecules induced by I/H. The H9c2 cells were incubated in standard DMEM (NC) or in I/H culture conditions with or without CSWT. (A, B): Western blotting analysis of Bax, Bcl-2, Caspase3 abundance in H9c2 cells. (C, D, E): The average data of Bax, Bcl-2, Caspase3 in H9c2 cells. $n=3$, Mean $\pm$ SEM. $^{##}P<0.01$, $^{###}P<0.001$ vs NC-0, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs I/H-0.

a maximum effect at 0.12 mJ/mm² (Fig. 3A). Moreover, the apoptotic effect of I/H was further manifested by I/H-induced increases in chromatin condensation and fragmentation, which was demonstrated by Hoechst 33342 nuclear staining. As shown in Fig. 3B-3C, chromatin condensation was decreased and the cell density was increased compared to the I/H group.

CSWT decreases the I/H-induced H9c2 cell apoptosis rate by flow cytometry

H9c2 cells were incubated in standard DMEM (NC) or under I/H culture conditions with (I/H+CSWT) or without CSWT (I/H). In a previous study (Fig. 3A), there was a maximum effect noted at 0.12 mJ/mm²; thus, we choose 0.12 mJ/mm² as a treatment condition in subsequent experiments. FITC-Annexin V/propidium iodide staining showed that I/H treatment for 24 h induced apparent early apoptosis in H9c2 cells. As shown in Fig. 4A, the control and I/H groups showed early apoptosis at rates of 4.03 and 24.60%, respectively. After treatment with CSWT (0.12mJ/mm²), the early apoptosis rate was reduced to 14.74%, which was significantly different (Fig. 4B). The results indicate that CSWT could reduce the early apoptosis induced by I/H in H9c2 cells.

CSWT suppresses the expression of apoptosis molecules induced by I/H

To examine the mechanism of CSWT's protective effect on I/H-induced apoptosis in H9c2 cells, we analyzed the protein expression of Bax, Bcl-2, and Caspase3, which play important roles in regulating the intrinsic pathway of apoptosis in myocardial cells. Bcl-2 is an anti-apoptosis protein, and the expression of bcl-2 was significantly increased in the CSWT treatment group (0.06, 0.09, and 0.12mJ/mm²) compared to the I/H group, whereas its expression was increased in the NC group treated with CSWT (0.12mJ/mm²). CSWT (0.09, 0.12mJ/mm²) also decreased the expression of pro-apoptosis protein Bax compared to the

Fig. 6. CSWT's anti-apoptotic effects are mediated through AKT-dependent signaling pathways. The H9c2 cells were incubated in standard DMEM (NC) or in I/H culture conditions with or without CSWT. (A): Western blotting analysis of p-AKT, AKT, p-ERK, ERK, p-p38MAPK and p38MAPK abundance in H9c2 cells. (B, C, D): The average data of AKT phosphorylation, ERK phosphorylation and p38MAPK phosphorylation in H9c2 cells.

I/H group (Fig. 5A). Moreover, we detected cleaved Caspase3, as shown in Fig. 5B. CSWT (0.12mJ/mm²) could significantly reduce the activation of Caspase3. The results indicate that CSWT of the appropriate energy suppresses the expression of apoptosis molecules induced by I/H.

The AKT pathway may be involved in the CSWT effects on apoptosis

Because we found that CSWT inhibited the Bax and cleaved caspase3 expression induced by I/H, we speculated that CSWT might affect PI3K-AKT activation. Compared with the I/H group, CSWT (0.06, 0.09, 0.12mJ/mm²) increased the phosphorylation of AKT (Fig. 6A), though there was no significant difference between the NC and NC+CSWT groups with regard to the AKT ratio. We also determined whether other MAPK signal pathways, such as the p38 MAPK and ERK signaling pathways, could mediate the protective effect of CSWT on I/H-induced myocardial cell apoptosis. We found that there were no significant differences in the phosphorylation of p38 MAPK and ERK between the CSWT and I/H groups (Fig. 6A). The results indicate that CSWT's anti-apoptotic effects are mediated through AKT-dependent signaling pathways.

Discussion

The novel findings of this study are that CSWT can attenuate H9c2 myoblast apoptosis and that the activation of the AKT signaling pathway may be involved in the CSWT effects on apoptosis. To our knowledge, there are few reports that CSWT can attenuated the H9c2 cell apoptosis induced by I/H.

Primary cardiac myocytes are widely used in cardiac studies; however, the major disadvantage of these cells is the large number of animals that are required. H9c2 rat cardiomyocytes demonstrate many similarities to primary cardiomyocytes [16], and some previous studies have used hypoxia-induced H9c2 cell line models to replace myocytes

[17-19]. The H9c2 rat cardiomyocyte cell line is a reliable alternative for some studies and was used in all of our experiments. In the model of simulated ischemia-induced apoptosis herein, simulated ischemia significantly caused caspase3 activation, signs of nuclear apoptosis, and increasing amounts of dead cardiac myocytes. Although these results prove that this is a reliable model, further study *in vivo* should be carried out in the future.

CSWT is a new technology, and some studies have been made in European nations such as Switzerland, Germany, Italy, Russia, and Japan at present, achieved some good effect, and made a tendency to gradually expand the application [20-24]. Although many clinical studies have preliminarily confirmed that CSWT can treat myocardial ischemia, improving the patient's symptoms, the precise mechanism by which this occurs remains unclear. Apoptosis plays an important role in ischemic diseases, but there are few reports on whether CSWT treatment can attenuate apoptosis in myocardial cells. Due to this lack of studies, we attempted to establish a model of ischemia and hypoxic cells and use the myocardial cell injury model to explore whether CSWT could attenuate apoptosis.

Tanaka et al. [25] found that hypoxia causes apoptosis in cultured myocardial cells *in vitro*. Kajstura et al. [26] and Leri et al. [27] also found that hypoxia causes myocardial cell apoptosis *in vivo*, and several other studies have reported that the apoptosis induced by ischemia/hypoxia is mainly dependent on the activation of mitochondrial pathways in cells [28]. Apoptosis is regulated by Bcl-2 family proteins, the members of which are pro-apoptotic or anti-apoptotic. Bax is an important factor leading to apoptosis under hypoxic stress. In response to apoptotic stimuli, Bax undergoes conformational activation and then facilitates the release of cytochrome c from mitochondria [29]. In contrast, Bcl-2 has the ability to suppress this Bax-induced release of cytochrome c from mitochondria [30, 31]. Hypoxia decreases Bcl-2 expression in myocardial cells [32, 33], and the expression ratio of Bcl-2 to Bax was reported to determine cell survival or death following an apoptotic stimulus. Bax and Bcl-2 play important physiological roles in the protection or acceleration of apoptosis in human myocardial cells after ischemia and/or reperfusion. We adopted the method of ischemia and hypoxia experiments to induce apoptosis. In our experiment, the viability of myocardial cells decreased notably after ischemia and hypoxia for 24 h. The apoptotic cell rate began to increase compared with the control cells, as indicated by increased Bax and Caspase3 expression and reduced Bcl-2 expression.

It has been reported that the apoptosis induced by ischemia and hypoxia plays an important role in the development in CAD: the loss of myocardial cells causes abnormal loading conditions, which may induce LV dilatation and a gradual decline in LV contractility during the chronic phase [1]. The development of LV remodeling leads to heart failure. Recent studies have demonstrated that CSWT improves myocardial perfusion and cardiac function in a porcine model of chronic myocardial ischemia and also ameliorates myocardial ischemia in patients with severe CAD. In chronic ischemia animal experiments, it was observed that CSWT could induce a complete recovery of the left ventricular ejection fraction, wall thickening fraction, and regional myocardial blood flow. In AMI pig models [34], CSWT normalized the LV ejection fraction in the SW group but not in the control group. In the present study, we investigated whether CSWT had any effect on cardiomyocyte apoptosis *in vitro*. We observed that CSWT attenuated the cell death induced by I/H in H9c2 cells, the expression of bcl-2 was significantly increased, and the expression of Bax and cleaved Caspase3 were decreased. Our studies clearly demonstrate for the first time that CSWT attenuates the intrinsic pathway of apoptosis in cardiomyocytes.

To further explore the regulation mechanism by which CSWT attenuates intracellular myocardial cell apoptosis, three MAPK intracellular signal pathways were detected at the same time: the AKT, ERK, and p38MAPK pathways. We found that under the intervention of CSWT, AKT phosphorylation increased significantly, whereas ERK and p38MAPK phosphorylation had no significant change, which suggests that the AKT pathway may be involved in the effects of CSWT on apoptosis. The AKT signaling pathway is closely related to growth, proliferation, and apoptosis inhibition, playing an important role in maintaining cell survival [35]. AKT is the most important factor for mediating these PI3K-dependent

cell-survival responses. Previous studies have established the principle role of AKT in the regulation of cell survival in several cell types. The pro-apoptotic Bcl-2-family member BAD inhibits Bcl-2 and other anti-apoptotic Bcl-2 family members by direct binding when it is not phosphorylated [36], and AKT can phosphorylate BAD to regulate cell survival in IL-3-dependent myeloid cells [37] and in fibroblasts and primary neurons [38]. In our study, we found that the AKT pathway may be involved in the effects of CSWT on apoptosis, but more detailed mechanistic analyses are required.

To date, several reports about shock wave application in experimental myocardial ischemia have been published, showing effectiveness in CAD. However, there also has some conflicting viewpoint for CSWT, indicating that CSWT can cause irreversible myocardial damage and may cause an injury-and-repair process, which is expected to result in fibrosis and functional loss of the myocardium [39, 40]. Given the lack of safety studies, an Italy study evaluates the safety of CSWT on the adult heart and its effects on cardiac tissue function and morphology. Four-month-old Fisher 344 rats were treated by CSWT. Cardiac function was measured by echocardiogram at baseline and at 1 and 3 months after treatment. The left ventricular ejection fraction and fractional shortening remained stable at all time points. Immunohistochemistry was used to evaluate the signs of inflammation, apoptosis and fibrosis in the control and treated hearts. There were neither differences in the extracellular matrix collagen content nor the presence of fibrosis. Moreover, CSWT did not increase the apoptosis of cardiac cells in treated hearts *in vivo*, and there were no signs of inflammation at 3 months after CSWT treatment [41]. Meanwhile, myocardial ischemia mini-pigs were used in Fu's study, they found that there were lower mean fibrosis area and apoptotic nuclei in CSWT treated group compared to ischemia group by immunohistochemical staining at day 180 [42]. According to the results of the animal experiments and preliminary clinical trial observation, we think that CSWT is a safe and effective way to treat ischemic heart disease.

Several limitations should be mentioned for this study. First, although we were able to demonstrate that CSWT could attenuate myocardial cell apoptosis *in vitro*, the effects of CSWT *in vivo* require future studies. Second, the best strategy of SW delivery remains to be elucidated. Our strategy was to shoot 500 shots/spot for ischemia and hypoxia in myocardial cells, whereas Aicher et al. [43] shot 500-2,000 shocks in one session. Further studies are required to determine the best treatment strategy.

In conclusion, we show here that CSWT exerts a protective effect against I/H-induced cell death and demonstrate that this may occur by preventing the activation of components of the mitochondrial-dependent intrinsic apoptotic pathway. We also demonstrate that the PI3K-Akt pathway may be involved in the effects of CSWT on apoptosis. Together with further work on this topic, our study demonstrates that CSWT is a prospective intervention for ischemic heart disease.

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