Original Paper

Shock Wave Therapy Promotes Cardiomyocyte Autophagy and Survival during Hypoxia

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Key Words

Ischemia/hypoxia • Autophagy • Shock wave • AMPK/mTOR

Abstract

Background: Autophagy plays an important role in cardiovascular disease. Controversy still exists regarding the effect of autophagy on ischemic/hypoxic myocardium. Cardiac shock wave therapy (CSWT) is an effective alternative treatment for refractory ischemic heart disease. Whether CSWT can regulate cardiomyocyte autophagy under hypoxic conditions is not clear. We established a myocardial hypoxia model using the H9c2 cell line and performed shock waves (SWs) treatment to evaluate the effect of SW on autophagy. **Methods:** The H9c2 cells were incubated under hypoxic conditions, and SW treatment was then performed at energies of 0.02, 0.05, or 0.10 mJ/mm². The cell viability and intracellular ATP level were examined. Western blot analysis was used to assess the expression of LC3B, AMPK, mTOR, Beclin-1, Sirt1, and HIF-1 α . Autophagic vacuoles were visualized by monodansylcadaverine staining. **Results:** After the 24-hour hypoxic period, cardiomyocyte viability and ATP levels were decreased and autophagy was significantly increased in H9c2 cells. SW treatment with an energy of 0.05 mJ/mm² significantly increased the cellular viability, ATP level, LC3B-II/I, and number of autophagic vacuoles. In addition, phosphorylated AMPK and Sirt1 were increased and phosphorylated mTOR and HIF-1 α were decreased after SW treatment. **Conclusion:** SW treatment can potentially promote cardiomyocyte autophagy during hypoxia and protect cardiomyocyte function by regulating the AMPK/mTOR pathway.

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Introduction

Cardiovascular diseases are the leading cause of death worldwide. Despite robust successes of drug, percutaneous coronary intervention (PCI), and surgical therapies, the morbidity and mortality of coronary heart disease (CHD) are still high. Indeed, PCI and coronary artery bypass grafting (CABG) treatments are not suitable for some patients with

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severe and complicated coronary lesions, and some patients still suffer from angina pectoris even after PCI and/or CABG treatment [1]. Stenosis or occlusion of the coronary arteries can cause cardiomyocyte ischemic/hypoxic injury, leading to cardiomyocyte loss and heart function depression. Chronic ischemia/hypoxia may cause repeated episodes of angina pectoris, myocardial infarction, or heart failure and seriously affect a patient's quality of life. The prognosis of patients with ischemic cardiomyopathy is still poor because medication treatment is the only option [2]. Therefore, the development of a new therapeutic strategy for the treatment of refractory and symptomatic CHD is urgently needed.

Extracorporeal shock waves (SWs) were first introduced for clinical use for urolithiasis more than 20 years ago. Low-energy shock waves have been applied as an important therapeutic method for several orthopedic diseases and soft tissue injuries because of their anti-inflammatory effects [3]. Recently, an increasing number of studies have indicated that cardiac shock wave therapy (CSWT) is an efficient method for treating ischemic heart disease in animal models [4-6] and human patients [7, 8]. According to those studies, the acoustical energy can be transmitted and focused on the ischemic areas and the border zone around the infarcted myocardium, improving symptoms and myocardial perfusion, ameliorating ventricular remodeling, and promoting the restoration of left ventricular ejection fraction (LVEF). The mechanisms of heart protection after CSWT are still unclear but may include promotion of the recruitment of endothelial progenitor cells [9], stimulation of neovascularization [10], and reduction of oxidative stress, inflammation [5], and fibrosis [6]. Our recent *in vitro* [11] and *in vivo* studies (unpublished data) demonstrated that CSWT could inhibit apoptosis in ischemic/hypoxic myocardium and consequently enhance cell vitality and heart function. However, the mechanism of CSWT appears to be multifactorial and needs to be further elucidated.

Autophagy is an evolutionarily conserved energy-dependent process that occurs during a series of pathological stresses (nutrient limitation, oxidative stress, protein damage, or organelle accumulation) and plays an essential role in diseases such as cancer, infection, neurodegeneration, diabetes, and cardiac diseases [12]. It is a unique biological process used to cope with stress and maintain cellular metabolism, homeostasis, and survival. By mediating protein degradation, organelle turnover, and the recycling of cytoplasmic components after fusion with lysosomes, autophagy helps cells adapt to the changes in nutritional and energy demands. It has been well established for several years that autophagy is a key regulator of the cardiovascular system and is essential for normal maintenance, repair, and adaptation of the heart over the course of a lifetime [13, 14]. Several studies have demonstrated that autophagy is obviously induced during myocardial ischemia, but the functional role of autophagy is still unclear and is currently under intense investigation [15-17]. Despite the key beneficial effect of autophagic activity, excessive or insufficient autophagy may contribute to cell death. Furthermore, whether SW treatment can protect cardiac myocytes against hypoxic injury by influencing the process of autophagy is still unknown.

In the present study, we established an ischemia/hypoxia model *in vitro* using H9c2 myoblasts to determine whether SWs can protect cardiomyocyte function by regulating cell autophagy and its possible signal transduction pathways.

Materials and Methods

Cell resuscitation and cultivation

The rat cardiomyocyte cell line H9c2, which was derived from embryonic heart tissue of the BD1X rat, was purchased from the American Type Culture Collection (ATCC, USA). The main components of the culture medium, including Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin solution, fetal bovine serum (FBS), and 0.25% trypsin, were purchased from HyClone. All of the cell culture vessels were purchased from Corning. The H9c2 cells were frozen in 10% DMSO, 40% FBS, and 50% DMEM at -80°C or liquid nitrogen. When resuscitated, the cell freezing medium was rapidly melted in the 37°C water. After centrifugation at 800 rpm for 6 min, the cells were re-suspended in high-glucose DMEM supplemented

with 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin. Then, they were cultured under an atmosphere of 5% CO₂ and 95% air (v/v) at 37°C. The passages were performed when the confluence reached approximately 90%-95% (1:4 to 1:6).

Hypoxia culture

The culture medium of H9c2 cells with a confluence of 60%-70% was replaced by high-glucose DMEM containing 1% FBS. Then, the cells were cultured for approximately 12 h to inhibit cell proliferation (synchronization) before hypoxia induction. Subsequently, the cells were cultured in an anoxia chamber (HERAccl VIOS 160i, Thermo, USA), saturated with 92% N₂, 5% CO₂, and 3% O₂ (v/v/v) at 37°C for 0, 6, 12, 24, 36, 48, and 72 h. The appropriate hypoxia time was defined as the time necessary to cause relatively moderate hypoxic injury and obvious autophagy. Then, the subsequent experiments included the following four groups: normal control (NC), NC+SW, ischemia/hypoxia, and ischemia/hypoxia+SW. For the control groups, the cells were cultured in a normal incubation chamber for identical periods before SW treatment.

Shock wave treatment

The cells were digested with trypsin, centrifuged after being subjected to hypoxia for a certain period, and re-suspended to a density of 5×10⁶ cells/mL. Every centrifuge tube containing 1 mL of the cell suspension was treated with the SW device (Duolith VET, Storz Medical, Switzerland). Both the ultrasonic probe and the tube were submerged in the water bath at 37°C to avoid the attenuation of ultrasonic energy. To ensure cell survival after SW treatment, the process was performed as quickly as possible. Two hundred shocks with energies of 0.02, 0.05, or 0.10 mJ/mm² were administered to different tubes. In this manner, the optimal SW energy with the best therapeutic effect was chosen. After SW treatment, the cells were cultured in the incubator with 5% CO₂ and 95% air (v/v) for another 24 h before being harvested [11].

Assay of viable cells

To determine the number of viable cells, the Cell Titer 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega, USA) containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and an electron coupling reagent, phenazineethosulfate (PES), was used. The H9c2 cells were seeded into 96-well plates, and each well contained 100 µL of medium with approximately 5000 cells in quintuplet. After incubation for 24 h, 20 µL of the detection reagent was added to each well. Two hours later, the optical density (OD) was measured at 490 nm and is presented as a percentage of that of the untreated control group.

Determination of intracellular ATP

Intracellular ATP was extracted from the H9c2 cells and measured with an ATP bioluminescent assay kit (Beyotime Biotech S0027, Jiangsu, China). The cells were cultured in 6-well plates. After lysis, the lysates were centrifuged at 12,000 rpm at 4°C for 5 min (Heraeus Fresco 21 Centrifuge, Thermo, USA). The luminescence (RLU) of the supernatant was detected by a luminometer (GloMax®, Promega, USA).

Autophagic vacuole assay with monodansylcadaverine (MDC) dyeing

Monodansylcadaverine (MDC) dyeing was a specific method used to detect autophagic vacuoles during the process of autophagy. The MDC kit was purchased from KeyGEN BioTECH, Jiangsu, China (KGATG001). When the adherent H9c2 cells reached the appropriate confluence (50-60%) in 24-well plates, the medium was discarded, and the cells were washed twice with 300 µL of 1× washing buffer. Then, 100 µL of working solution (1× washing buffer: MDC = 9:1) was added to the cells, which were kept in a dark place at room temperature for 20 min. The cells were washed with 1× washing buffer 3 times to remove redundant dye. Finally, intracellular autophagic vacuoles were visualized at 200× magnification using a fluorescence microscope (Olympus BX51, USA) with an excitation wavelength of 355 nm and an emission filter of 512 nm. The fluorescence was quantified using ImageJ software (NIH). At least 100 cells were counted in 3-5 independent experiments.

Western blotting

The protein was extracted from the H9c2 cells by adding 1× cell lysis buffer (Cell Signaling Technology, CST). Equivalent proteins (10-20 µg) were separated by SDS-polyacrylamide gels (12%) and electrically

transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA) [11]. The membranes were probed with rabbit primary antibodies in a 1:1000 dilution, including anti-LC3B (Sigma), anti-GAPDH (internal reference, CST), anti-Bec1 (CST), anti-Phospho-AMPK (Thr172, CST), anti-AMPK (CST), anti-Phospho-mTOR (Ser2448, CST), anti-mTOR (CST), anti-Sirt1 (CST), and anti-HIF-1 α (Abcam). After incubation with goat anti-rabbit secondary antibody (1:5000 dilution, CST), the membranes were visualized with ECL (Millipore, USA). Densitometric analysis was performed using ImageJ software. Three to five independent experiments were performed, and the average densitometry values were calculated.

Statistical analysis

All of the numerical variables were expressed as the means $\pm$ SEM and were analyzed using the statistical software GraphPad Prism 6.0 (GraphPad Software, CA). Student's unpaired t-test was used to analyze the difference between two groups, and a one-way ANOVA with Bonferroni correction was used for multiple comparisons. $P < 0.05$ was considered statistically significant. Every experiment was repeated at least three times.

Results

Ischemia/hypoxia reduced H9c2 cell viability and ATP levels and promoted cell autophagy

The viability of H9c2 cells was detected by the MTS method after incubation in low-oxygen conditions for 0, 6, 12, 24, and 36 h. It was found that with the increase in hypoxic time, the number of viable cells gradually decreased (Fig. 1A). At 12, 24, and 36 h, the cellular viability decreased by 30.31%, 34.90%, and 49.63%, respectively (Table 1). To determine the energetic state of cardiomyocytes under hypoxia, we quantified the intracellular ATP levels. Hypoxia significantly decreased the intracellular ATP levels in a time-dependent manner (Fig. 1B). ATP level was reduced by 19.26% in H9c2 cells after hypoxic incubation for 24 h (Table 1).

Fig. 1. Hypoxia reduced the H9c2 cell viability and ATP level in a time-dependent manner. The average MTS OD measured at 490 nm (A) and intracellular ATP levels detected by the luminometer (B) after incubation in a hypoxic chamber for 0, 6, 12, 24, or 36 h. Ratios of the values in the hypoxic groups to those in the NC group are presented. Results are expressed as the means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ vs. NC.

Table 1. Inhibition rate of cell viability (%) and reduction rate of ATP (%) calculated from the OD and RLU values

Hypoxic period	0 h	6 h	12 h	24 h	36 h
Inhibition rate of cell viability (%)	0	18.46	30.31	34.90	49.63
Reduction rate of ATP level (%)	0	2.24	13.26	19.26	25.97

Fig. 2. Ischemia/hypoxia promoted the autophagy of H9c2 cells. Western blot results (A) and average LC3B-I and LC3B-II expression (B) in H9c2 cells after 0, 6, 12, 24, 36, 48, or 72 h of hypoxia to analyze autophagy activation. Values are presented as percentages relative to the NC group. Results are expressed as the means $\pm$ SEM. * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$ vs. NC.

Fig. 3. SWs with an energy of 0.05 mJ/mm² improved cell viability and autophagy during ischemia/hypoxia. The average MTS data for cell viability are presented under normal (A) and hypoxic (B) conditions following treatment with SWs at energies of 0.02, 0.05, and 0.10 mJ/mm². Western blots and the average LC3B-I and LC3B-II expression under normal (C, E) and hypoxic (D, F) conditions following treatment with SWs at energies of 0.02, 0.05, and 0.10 mJ/mm² are shown from C to F. Values are presented as percentages relative to the NC group. Results are expressed as the means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$ vs. NC.

During the process of autophagy in mammalian cells, microtubule-associated protein 1 light chain 3 (LC3) is converted from the cytosolic form (LC3-I) into the membrane-bound form (LC3-II), promoting the formation of autophagosomes. Therefore, the increase in LC3-II expression (or LC3-II/LC3-I ratio) is widely used as a specific and classical marker to indicate the activation of autophagy. In this study, we performed Western blot analysis to investigate the expression of LC3B-II and LC3B-I (Fig. 2A) with extended anoxic times (6, 12, 24, 36, 48,

Fig. 4. SWs increased the number of autophagic vacuoles and intracellular ATP levels during ischemia/hypoxia. (A) The morphology of H9c2 cells viewed from an inverted microscope in the NC, NC+SW, 24 h hypoxia (H24), and H24+SW groups. The black arrow indicates the floating, clustered, and dead cells. The rulers in the photographs represent 100 μm . (B) Autophagic vacuoles were stained as green fluorescent dots around the nucleus (200 $\times$). The rulers in the photographs represent 100 μm . (C) The average data on the fluorescence intensity of the autophagic vacuoles in the four groups measured by ImageJ software. (D) The average luminescence of the ATP levels before and after SW treatment. Values are presented as percentages relative to the NC group. Results are expressed as the means $\pm$ SEM. **P<0.01 vs. NC, #P<0.05 vs. H24.

and 72 h). It was found that LC3B-II/I increased over time and that the ratio reached a peak at 24 h of hypoxia (Fig. 2B), whereas it exhibited a decreasing trend from 36 to 72 h.

SW treatment with an energy of 0.05 mJ/mm² improved cell viability and autophagy during ischemia/hypoxia

After incubation in a normal or hypoxic chamber for 24 h (NC vs. ischemia/hypoxia condition), H9c2 cells were digested, re-suspended, and prepared for SW treatment. Compared with the Modulith SLC system with a complex water sac that was used in our previous study [11], the Duolith VET system used in the present study was designed with an independent probe, and the cells could be directly treated in the water bath. The attenuation of ultrasonic energy was markedly avoided, and relatively lower energies (0.02, 0.05, and 0.10 mJ/mm²) were chosen to evaluate the effect of SW treatment on hypoxic H9c2 cells. The number of viable cells was significantly increased, especially those treated with an energy of 0.05 mJ/mm² in the hypoxic group (Fig. 3B). However, none of the three energies of SW treatment affected cell viability in the NC group (Fig. 3A).

Furthermore, the effect of SW treatment on cell autophagy was assessed by Western blot analysis. The expression of LC3B-II was significantly increased following treated with 0.05 mJ/mm² SWs after hypoxia (24 h), whereas no significant changes were found following SW treatment at the milder (0.02 mJ/mm²) or higher (0.10 mJ/mm²) energy (Fig. 3D and F). Similarly, there was no obvious autophagy level change in the NC group following SW treatment at different energies (Fig. 3C and E).

Fig. 5. SWs attenuate ischemia/hypoxia-induced cardiomyocyte injury by regulating the AMPK/mTOR pathway. (A) Western blot results of AMPK, mTOR, Beclin-1, Sirt-1, and HIF-1α in the NC, NC+SW, 24-hour hypoxia (H24), and H24+SW groups. (B) The average data on signal pathway protein expression in the four groups. Values are presented as percentages relative to the NC group. Results are expressed as the means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. NC. # $P < 0.05$, ## $P < 0.01$ vs. H24.

SW treatment increased the number of autophagic vacuoles and the ATP level during ischemia/hypoxia

Under normal conditions, H9c2 cells presented in long fusiform form and proliferated adherently. Following 24 h of hypoxia, the morphology of the cells changed. More fragile, triangular, or even round cells could be seen, as well as floating and clustered dead cells. After the SW treatment, the morphological changes in the cells were alleviated, and the number of dead cells was remarkably reduced (Fig. 4A).

During the process of autophagy, the formation of autophagic vacuoles includes two consecutive stages that involve autophagosomes and autolysosomes. The former ones are closed vacuoles containing damaged proteins and organelles with double membranes, and the latter are defined as the fusion of autophagosomes with lysosomes. Consequently, mature vacuoles are acidic compartments with lysosomal hydrolases and can be detected by pH-sensitive reporter molecules [18]. MDC is an acidophilic autofluorescent compound that aggregates in vacuoles containing the lysosome enzymes acid phosphatase and mature cathepsin D. Autophagic vacuoles can be directly detected by fluorescence microscopy and serve as another specific marker of autophagy activation. To further confirm the occurrence of autophagy, we used MDC staining to visualize autophagic vacuoles in the H9c2 cells and found that the number of autophagic vacuoles significantly increased after hypoxia. Furthermore, the intensity was enhanced by SW treatment (Fig. 4B). The quantification of fluorescence was consistent with the results of LC3B-II expression determined by Western blot analysis (Fig. 4C). Simultaneously, intracellular ATP was detected before and after the SW treatment. It was found that the ATP level was significantly increased by SWs after hypoxia induction (Fig. 4D).

SW treatment improves ischemia/hypoxia-induced cardiomyocyte autophagy by regulating the AMPK/mTOR pathway

It is known that mammalian target of rapamycin (mTOR) is a key regulator of cell growth, proliferation, and many other cellular processes. As a sensor of the cellular nutrient status, mTOR is inhibited during energy insufficiency and has been identified as a negative regulator of autophagy in mammalian cells [16, 19, 20]. AMP-activated protein kinase (AMPK) is a protein kinase member that can be activated by a reduction in the intracellular ATP/AMP ratio [21]. In mammals, ATP depletion inhibits mTOR phosphorylation by activating AMPK and subsequently phosphorylating tuberous sclerosis complex 2 (TSC2) [22]. The AMPK/mTOR pathway is considered a crucial regulator of autophagy under starvation conditions [23]. Moreover, Beclin 1 is another rate-limiting factor of autophagosome formation from the available LC3-II in cardiac myocytes [16]. However, whether these pathways are involved in promotion of hypoxia-induced autophagy by SW treatment has not yet been investigated. The results of our study showed that hypoxia significantly attenuated mTOR phosphorylation (Ser2448) but increased the phosphorylation of AMPK (Thr172) and the expression of Beclin-1 in H9c2 cells (Fig. 5). The SW treatment further weakened mTOR phosphorylation and enhanced AMPK phosphorylation. However, SWs did not significantly influence the expression of Beclin-1. Therefore, SW treatment promoted hypoxia-induced autophagy through the AMPK/mTOR pathway.

To further explore the mechanism of the protective effects of SW treatment on ischemic/hypoxic H9c2 cells, cell-survival-related signaling pathways were also determined in the present study. Sirt1, known as an anti-aging protein, is a member of the sirtuin family and is involved in most complicated physiological processes, including endothelial dysfunction, atherothrombosis, obesity, diabetes, liver steatosis, and myocardial infarction. Recently, several studies have demonstrated the effect of Sirt1 in protecting against oxidative stress, apoptosis, and inflammation under the condition of ischemia/hypoxia [24]. Along with Sirt1, hypoxia-inducible factor (HIF)-1 α is another key regulator in ischemic/hypoxic conditions. We found that the expression of Sirt-1 was markedly decreased during hypoxia but that SW treatment increased its expression (Fig. 5). Conversely, the upregulation of HIF-1 α after hypoxia was significantly attenuated by SW treatment.

Discussion

To the best of our knowledge, this is the first report evaluating the effect of SWs on cardiomyocyte autophagy under hypoxic conditions. We found that hypoxia attenuated cell viability, reduced the intracellular ATP level, and increased autophagy in H9c2 cells. SWs further enhanced cell autophagy through the AMPK/mTOR signaling pathway. In addition, SW treatment significantly improved cell viability and ATP levels.

Neonatal rat cardiomyocytes (NRCMs) and H9c2 cells are often used in cardiomyocyte *in vitro* studies. Theoretically, the characteristics of NRCMs are more similar to those of adult cardiomyocytes. However, large numbers of neonatal animals and strict requirements are necessary to ensure NRCMs survival and proliferation *in vitro*. The H9c2 cell line is derived from embryonic BDIX rat heart tissue and shows many characteristics similar to those of NRCMs [25]. Previous studies have used H9c2 cells instead of cardiomyocytes to investigate the occurrence of autophagy under hypoxia and have reached conclusions consistent with those of *in vivo* studies [26, 27]. Our *in vitro* study using the hypoxic model of H9c2 [11] demonstrated that CSWT could inhibit hypoxia-induced apoptosis, which was further verified in an animal study (unpublished). Thus, the model used in this study may be reliable, but further *in vivo* studies must be conducted in the future.

Under normal conditions with a sufficient nutrient supply, autophagy is kept at a low level, and it may be induced under circumstances of nutrient limitation and other cellular stresses. Recently, more clinical and basic studies have indicated that autophagy is a prominent feature in cardiac diseases such as chronic ischemia [28], hypertrophy, heart

failure [29], atherosclerosis [30], and ischemia-reperfusion injury [15, 16]. Although the role of autophagy in cardiomyocytes is still controversial, its protective effect under mild and moderate ischemia/hypoxia has been clarified in some studies. Zhang H et al. found that oxygen deprivation causes an abrupt cessation of oxidative phosphorylation and a decline in intracellular ATP during ischemia, resulting in a state reminiscent of starvation [31]. Autophagy was adaptively induced to meet the needs of cellular metabolism, eliminating damaged mitochondria and thus inhibiting damaging reactive oxygen species (ROS) release and alleviating apoptosis. In the study by Valentim L, cardiomyocyte death was increased by pharmacologically inhibiting autophagy under ischemic conditions, suggesting that autophagy is a pro-survival process [32]. Consistent with these results, our study demonstrated that when the hypoxic time was less than 24 h (0, 6, 12, and 24 h) in the culture of H9c2 cells, the cellular viability and ATP level decreased gradually in a time-dependent manner. Simultaneously, autophagy activation was gradually increased to maintain the mitochondrial membrane potential and cellular homeostasis as well as to replenish nutrients. However, when the hypoxic incubation period was more than 24 h, the cellular viability and ATP level continued decreasing, but the occurrence of autophagy (rate of LC3B-II/I) began to decrease. Loos B reported that mild ischemia causes the induction of autophagy and apoptosis, while moderate or severe ischemia induces both apoptotic and necrotic death without increasing autophagy [33]. Under severe hypoxic conditions, apoptosis and necrosis (instead of autophagic activity) played a critical role. Moreover, it was speculated that when the hypoxic period was less than 24 h, autophagy may be protective to enable cells to cope with the stimulation of nutrient insufficiency. We concluded that the hypoxic stimulation was relatively moderate and that the protective effect of autophagy was stronger at 24 h. Therefore, this period was chosen as the appropriate mode of ischemia/hypoxia *in vitro* in the following experiments.

Extracorporeal low-energy SW treatment is a non-invasive physiotherapy focusing on a specific target with an energy range of 0.003-0.890 mJ/mm² [34], and its effectiveness and safety have been demonstrated in animal models of chronic myocardial ischemia [10] and in patients with angina pectoris. The functional mechanisms of SWs are complicated and still not completely understood. Recently, partial mechanisms for improving ischemic organ function have been investigated, including the increase in angiogenesis via VEGF regulation, enhancement of progenitor cell homing, augmentation of nitric oxide production, and reduction of oxidative stress, apoptosis, and inflammation. Despite the possible above-mentioned effects, there are still additional aspects to investigate regarding the protective function of SWs. It remains to be elucidated whether this new therapeutic method influences myocardial autophagy during ischemia/hypoxia.

In the present study, SW treatment with an energy of 0.05 mJ/mm² was found to promote cell autophagy after hypoxia based on an analysis of the ratio of LC3B-II/I and number of autophagic vacuoles dyed by MDC. It was also found that the SW treatment alleviated the morphological change in cells and improved the vitality and intracellular ATP level. It is well established that optimal autophagic activation is critical to the maintenance of cellular homeostasis and function, whereas excessive autophagy may be maladaptive and promote cell death [35]. Because the effect of SW is subtle, we hypothesized that SWs may upregulate myocardial autophagy within a safe range and protect the heart from anoxic injury. In addition, SW treatment influenced cell autophagy in normal control cells, which indicated the safety of this therapy as mentioned in our previous study [11, 36].

Previous investigations have suggested that mTOR is inhibited during energy insufficiency, which subsequently stimulates autophagy. During myocardial ischemia, a small decline in cytosolic ATP or increase in AMP may lead to AMPK activation, which has made AMPK a key regulator of autophagy. AMPK activates autophagy by directly binding to and phosphorylating Ulk1, which is the upstream component of the core autophagy machinery [19]. In addition, Beclin 1 plays a central role in autophagosome formation in cardiac myocytes. Our study revealed that strong activation of AMPK and Beclin 1 and inactivation of mTOR were induced by hypoxia, which were well correlated with the induction of autophagy in H9c2

cells. Moreover, SW treatment significantly enhanced AMPK/mTOR pathway-dependent autophagy and protected against hypoxia-induced cell death. By stimulating autophagy, SWs accelerated the recycling of dysfunctional proteins and organelles, supplemented nutrients, and promoted the survival of cardiac myocytes. Consequently, cardiac energetics were preserved and intracellular ATP was restored. According to the study by Troncoso R, during mild ischemic stress, the activation of autophagy is cardioprotective and depends on the AMPK-mediated inhibition of mTOR [37]. The AMPK-induced autophagy may contribute to ATP production and the promotion of cell survival in the ischemic myocardium. SW treatment improved cell viability, further enhanced the occurrence of autophagy through AMPK activation, and showed protective effects under ischemic/hypoxic conditions.

Regarding other possible signaling pathways participating in the protective effect of SW treatment, Sirt1 and HIF-1 α were also analyzed in this study. We found that Sirt1 was decreased after 24 h of ischemia/hypoxia, whereas SW significantly increased Sirt1 expression and enhanced cell viability. Therefore, Sirt1 may be another main target of SW treatment during hypoxia in cardiomyocytes. The biological interactions between Sirt1 and HIF-1 α are complex and inconsistent. As shown in our study, ischemia/hypoxia promoted HIF-1 α expression, which was in accordance with previous research [38]. However, after the SW treatment, its expression decreased while the expression of Sirt1 increased. The study by Lim demonstrated that Sirt1 inactivates HIF-1 α signaling by deacetylating HIF-1 α at Lys674; thus, during hypoxia, when Sirt1 was downregulated due to the decrease in NAD (+), the activation of HIF-1 α was further promoted [39]. Consequently, when the expression of Sirt1 is enhanced by SW, HIF-1 α is simultaneously down-regulated.

In summary, our findings demonstrated that ischemia/hypoxia induces cardiomyocyte death and function loss. SW treatment potentially promoted cell autophagy through the AMPK/mTOR pathway and improved cell viability and function during hypoxia. SW treatment may serve as an effective alternative therapeutic option for ischemic heart disease.

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Disclosure Statement

The authors declare no conflict of interest.

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