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FGF1 inhibits H_2O_2-induced mitochondrion-dependent apoptosis in H9c2 cells

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The goal of this research was to reveal the protective effect and mechanism of fibroblast growth factor-1 (FGF1) on oxidative stress injury of H9c2 cells induced by hydrogen peroxide (H_2O_2). The effects of various concentrations of H_2O_2 and FGF1 on the activity of H9c2 cells were analyzed by Real Time Cell Analysis (RTCA). The content of ROS, calcium ion, mitochondrial membrane potential and apoptosis were detected by fluorescence probe, the mRNA expression of Bcl-2, Bax and Caspase-3 were detected by real-time PCR to evaluate whether FGF1 has ability to resist the apoptosis of cardiomyocytes caused by oxidative damage. The results showed that the proliferation of H9c2 cells could be inhibited after being treated with 200 μM H_2O_2 for 12 h, and 100 μg/ml FGF1 could increase the proliferation rate of H9c2 cells, mitochondrial membrane potential and the mRNA expression of Bcl-2, and reduce the ROS accumulation, the level of apoptosis, the content of intracellular calcium and the mRNA expression of Bax and Caspase-3 caused by H_2O_2. The results showed that FGF1 could regulate oxidative stress by improving mitochondrial function and inhibit the H_2O_2-induced apoptosis in H9c2 cells.

1. Introduction

Cardiovascular disease (CVD) seriously endangers human health, about 17 million people die of CVD every year in the world, accounting for more than 30% of the total deaths, the pathogenesis of CVD is complex, and cardiomyocyte injury caused by cardiomyocyte apoptosis is the common pathophysiological mechanism of many CVD (Collaborators 2017). The dysfunction of mitochondrial function is one of the key links of cardiomyocyte apoptosis, the underlying causes are calcium overload and excessive reactive oxygen species (ROS) production (Santulli et al. 2015). Oxidative stress and mitochondrial dysfunction are closely related to the pathogenesis of CVD (Paneni et al. 2017). They interact with other pathophysiology and directly or indirectly participate in the occurrence and development of heart failure, hypertension, myocardial injury, arrhythmia, cardiac hypertrophy and other diseases (Madamanchi and Runge 2013).

Fibroblast growth factor 1 (FGF1), often called acidic fibroblast growth factor (aFGF), is a member of a large growth factor family, which plays a pivotal role in the process of embryonic development, vascular growth, wound healing, etc. (Mellers et al. 2018). The biological characteristics of FGF affect wound healing through mitotic effect and non-mitogenic effects, but the role of FGF1 in H_2O_2-induced cardiomyocyte injury is not clear. The purpose of this study was to explore whether FGF1 can prevent cardiomyocyte injury by regulating the apoptosis of cardiomyocytes mediated by oxidative stress and mitochondrial dysfunction, to provide new research ideas for the treatment of cardiomyopathy.

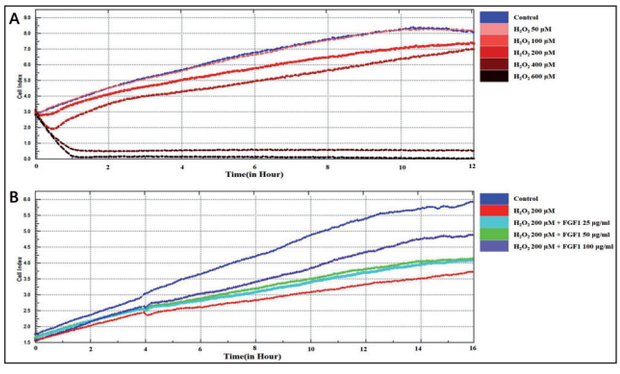

Fig. 1: Detection of H9c2 cell proliferation by RTCA system. (A) Effect of different concentrations of H_2O_2 on viability of H9c2 cell. (B) Effects of different concentrations of FGF1 pretreatment on 200 μM H_2O_2-induced cell viability damage.

2. Investigations and results

2.1. Effects of FGF1 on H_2O_2-induced oxidative damage in H9c2 cells detected by RTCA

To exploit the inhibitory effects of H_2O_2 on cell viability, H9c2 cells were treated with different concentrations of H_2O_2, then cell proliferation was monitored using the RTCA system every 3 min for 12 h. As shown in Fig. 1A, after 12 h of H_2O_2 treatment on H9c2 cells, compared with the control group, the CI value of H9c2 cells decreased gradually with the increase of H_2O_2 concentration in the treatment group, indicating that H9c2 cells proliferated more and more slowly, the mortality rate increased gradually, showing a certain dose-dependency. While the oxidative damage of H9c2 cells was not obvious when the concentration of H_2O_2 was 50 μM, concentrations of of 100 and 200 μM

H_2O_2 could significantly affect the proliferation of H9c2 cells. When the concentration of H_2O_2 exceeded 400 μM, the degree of cell damage was too serious. Therefore, 200 μM H_2O_2 was selected for the follow-up model treatment group. As shown in Fig. 1B, after 4 h of pretreatment with different concentrations of FGF1, cells were treated with 200 μM H_2O_2 for 12 h. The results showed that 100 μg/ml FGF1 could significantly reduce the effect of H_2O_2 on the activity of H9c2 cells. Therefore, 100 μg/ml FGF1 was selected as the dose concentration of the follow-up experimental intervention treatment group.

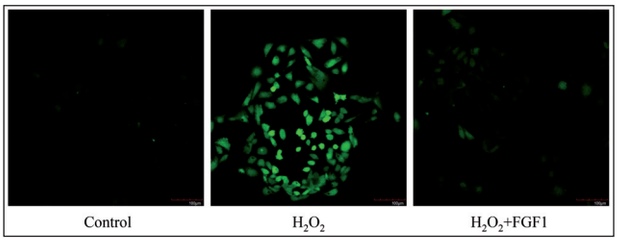

Fig. 2: Effects of FGF1 on the level of ROS in H9c2 cells induced by H_2O_2. Production of ROS was observed using a DCFH-DA fluorescent probe under a laser confocal microscope (magnification x100). Scale bar, 100 μm.

2.2. Effects of FGF1 on the level of ROS in H9c2 cells induced by H_2O_2

When the degree of oxidation exceeds the antioxidant capacity of cells to remove the oxide, the oxidation system and antioxidant system are out of balance, resulting in tissue damage. In order to further study the effect of FGF1 on the content of reactive oxygen species in H9c2 cells induced by H_2O_2, we used DCFH-DA as a fluorescent probe to label H9c2 cells. As shown in Fig. 2, the results of laser confocal microscopy showed that there was almost no fluorescence in the normal control group, and an obvious green fluorescence could be observed in the H_2O_2 treatment group. The fluorescence in the FGF1 treatment group was significantly weaker than that in the H_2O_2 treatment group, indicating that FGF1 can reduce the level of active oxygen produced in H9c2 cells induced by H_2O_2.

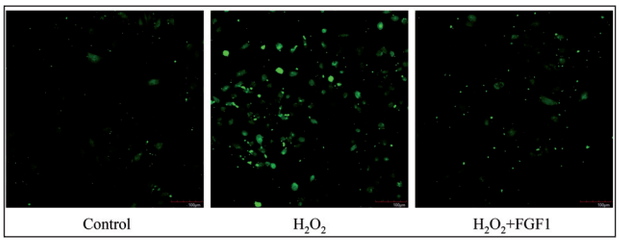

Fig. 3: Effects of FGF1 on the calcium content in H9c2 cells induced by H_2O_2. Production of calcium was observed using a Fluo-3 AM fluorescent probe under a laser confocal microscope (magnification x100). Scale bar, 100 μm.

2.3. Effects of FGF1 on the calcium content in H9c2 cells induced by H_2O_2

Oxidative stress and calcium overload also have a synergistic effect on each other. The increased level of reactive oxygen species will increase the release of intracellular calcium and further induce mitochondrial swelling. In order to determine whether FGF1 can affect the intracellular calcium content of H9c2 cells induced by H_2O_2, we used fluo-3AM fluorescence probe to detect the intracellular calcium concentration. As shown in Fig. 3, the results of laser confocal microscopy showed that a green fluorescence could be observed in the H_2O_2 treated group, indicating the high concentration of intracellular calcium. FGF1 can significantly reduce the intracellular calcium concentration of H9c2 cells induced by H_2O_2.

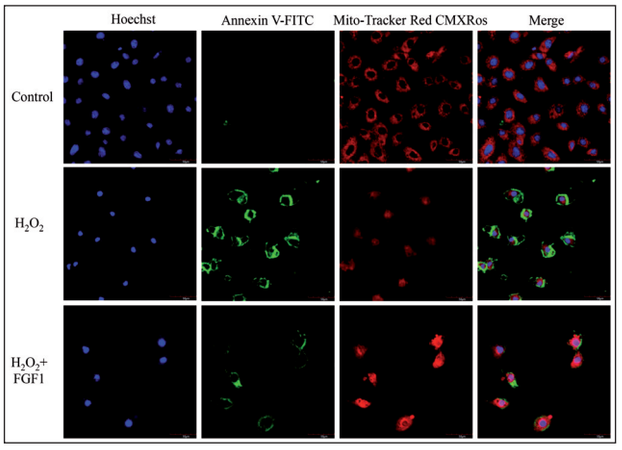

Fig. 4: Effects of FGF1 on the mitochondrial membrane potential and apoptosis in H9c2 cells induced by H_2O_2. The level of mitochondrial membrane potential and apoptosis were observed using Mito-Tracker Red CMXRos and Annexin V-FITC fluorescent probe under a laser confocal microscope (magnification x200). Scale bar, 50 μm.

2.4. Effects of FGF1 on the mitochondrial membrane potential and apoptosis in H9c2 cells induced by H_2O_2

The change of mitochondrial permeability is the key factor in the early stage of apoptosis and plays a decisive role in apoptosis. The mitochondrial membrane potential and apoptosis kit with Mito tracker red cmxros and annexin V-FITC were used to detect the level of mitochondrial membrane potential and apoptosis. Red fluorescent Mito tracker red cmxros labeled living cells that maintain mitochondrial membrane potential, while green fluorescent annexin V-FITC labeled cells that have apoptosis or necrosis. As shown in Fig. 4, the results of laser confocal microscopy showed that the integrity of H9c2 cell membrane in H_2O_2 treatment group was destroyed, the membrane potential was lost, and a great quantity of apoptotic cells were formed. FGF1 can inhibit the apoptosis induced by H_2O_2 to some extent.

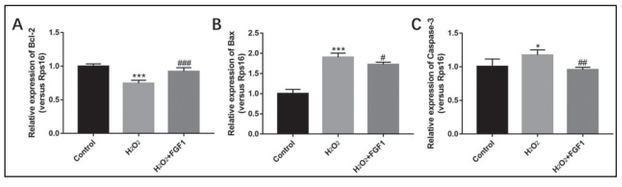

Fig. 5: FGF1 interference reduced mRNA expression levels of apoptosis-associated in H_2O_2-induced H9c2 cells. (A-C) RT-qPCR analysis of Bcl-2, Bax and Caspase-3 mRNA levels. Data represent mean±SD of at least three independent experiments. * $P < 0.05$ vs. Control group; *** $P < 0.001$ vs. Control group; $^{\#}P < 0.05$ vs. H_2O_2 group; $^{\#\#}P < 0.01$ vs. H_2O_2 group; $^{\#\#\#}P < 0.001$ vs. H_2O_2 group.

2.5. Effects of FGF1 on mRNA expression levels of apoptosis-associated in H9c2 cells induced by H_2O_2

In order to investigate the effect of FGF1 on the expression of apoptosis related marker mRNA in H9c2 cells induced by H_2O_2. As shown in Fig. 5, RT-qPCR showed that Bax and Caspase-3 mRNA expression increased in H9c2 cells treated with H_2O_2 and decreased ($P < 0.05$) bcl-2 mRNA expression significantly ($P < 0.001$) compared with the control group. Compared with the H_2O_2 group, the expression of Bax and Caspase-3 mRNA decreased ($P < 0.05$) in FGF1 group, and the expression of Bcl-2 increased significantly ($P < 0.001$). These results showed that FGF1 significantly reduced the mRNA expression level of apoptosis related markers and inhibited the apoptosis of H9c2 cells induced by H_2O_2.

3. Discussion

The occurrence and development of CVD is a complex process in which many factors participate and interact with each other. Mitochondrial apoptosis is one of the main ways of cardiomyocyte apoptosis, and the excessive production of mitochondrial ROS is the root cause of cardiomyocyte apoptosis (You et al. 2019). In this study, we found that FGF1 significantly reduced the apoptosis of cardiomyocytes induced by H_2O_2, which was closely related to its inhibition of H_2O_2-induced mitochondrial damage.

More and more study results show that ROS plays a crucial catalytic role in the occurrence and development of CVD (Senoner and Dichtl 2019). *In vivo*, H_2O_2 is involved in many important cell processes such as gene expression regulation, cell proliferation and apoptosis. Excessive ROS can directly play a cytotoxic role in cell damage. ROS can cause lipid peroxidation, change membrane permeability, and promote more calcium ion influx. Calcium overload can aggravate the generation of oxygen free radicals. Calcium overload can cause damage to cell membranes, mitochondria and the sarcoplasmic reticulum (Giorgi et al. 2015). Too high calcium ion concentrations in the cytoplasm can also activate calcium dependent enzymes, increase the membrane permeability of cardiomyocytes, DNA cleavage, and eventually lead to myocardial dysfunction. Mitochondria account for 30–40% of the total volume of cardiomyocytes (Barjaktarovic et al. 2011). They are the main organelles for the energy supply of cardiomyocytes, and the main source of active free radicals in the myocardium. When the antioxidative effect of antioxidant enzymes in mitochondria of cardiomyocytes is weakened or the accumulation of active free radicals in mitochondria is increased, the permeability of mitochondrial membranes will be changed and the mitochondrial membrane potential will be decreased, while the maintenance of mitochondrial membrane potential is necessary to maintain the mitochondrial function (Wang et al. 2019). Therefore, when the membrane potential is reduced or has disappeared, caspase apoptosis in the cytoplasm will be activated and cell apoptosis is induced. Therefore, too much ROS cannot only directly attack the mitochondria and destroy the integrity of the membrane, but also cause irreversible damage to the mitochondria through calcium overload, resulting in the decline of myocardial contractility, cardiac dysfunction and cardiovascular and cerebrovascular diseases (Ellulu et al. 2016). In this study, H_2O_2 was used to establish the model of myocardial oxidative damage *in vitro*. H_2O_2 has a dose-dependent effect on myocardial cell damage, resulting in the decline of cell viability. Under the effect of H_2O_2, H9c2 mitochondria were damaged, intracellular calcium and apoptosis increased significantly. FGF is involved in the process of tissue repair and response to injury. It is a kind of bioactive protein that has a significant regulatory effect on wound healing. FGF1 is a member of FGFs family and a mitogenic factor. Previous studies focused on the regulation of FGF1 on glucose and lipid metabolism (Gasser et al. 2017). This study found that FGF1 can significantly improve the injury of H_2O_2 on cardiomyocytes, reduce the content of ROS and calcium ions in cells, and maintain the integrity of mitochondria. Meanwhile, FGF1 can alleviate the oxidative damage of H9c2 cells caused by H_2O_2 by regulating the expression of Bcl-2, Bax and Caspase-3. Bcl-2 is an important intracellular component, which can stabilize membrane permeability, protect the integrity of mitochondria and inhibit cell apoptosis by contacting the outer membrane of mitochondria. Bax, as a sensor of cell injury and stimulation, can lead to the decrease of mitochondrial membrane potential and activate Caspase-3-mediated mitochondrial apoptosis pathway after cell injury and stimulation.

To sum up, the results of this study showed that FGF1 could improve mitochondrial function by regulating oxidative stress and then inhibit the apoptosis of H9c2 cardiomyocytes induced by H_2O_2. This provides a new strategy for the prevention and treatment of CVD and other diseases caused by oxidative stress.

4. Experimental

4.1. Reagents and materials

Recombinant Human FGF1 was purchased from PeproTech, Inc (Oak Park, CA, USA). H_2O_2 was purchased from Tianjin Tianli Chemical Reagent Co. (Tianjin, China). Fetal bovine serum (FBS) was obtained from PAN (Aidenbach, Germany). DMEM/F12 medium was obtained from HyClone (Logan, Utah, USA). Reactive Oxygen Species Assay Kit, calcium probe Fluo-3/AM and Mitochondrial Membrane Potential and Apoptosis Detection Kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). All of primers were designed and synthesized by Sangon Biotech (Shanghai, China).

4.2. Cell culture

Rat cardiomyoblasts cell line H9c2 were obtained from iCell Bioscience Inc (Shanghai, China). H9c2 cells were grown in DMEM/F12 medium containing 10% FBS, 100 units/mL of penicillin and 100 μg/mL of streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The medium was replaced every other day.

4.3. Cell viability assays

To determine the effects of H_2O_2 on H9c2 cells, the cells harvested at about 80% confluence were seeded into E-plate 16 for 0.1 mL (1×10^4 cells/well). When the cells entered the stationary phase and then treated with various concentrations of H_2O_2 (50 μM, 100 μM, 200 μM, 400 μM, 600 μM) for 12 h. In the drug treatment experiments, H9c2 cells were pretreated with FGF1 (25 μg/ml, 50 μg/ml, 100 μg/ml) for 4 h and were maintained under 200 μM H_2O_2 for 12 h. Cell viability was monitored in the Real-Time Cell Analysis (RTCA) xCELLigence system (ACEA Biosciences, San Diego, CA, USA). Cell index (CI) is directly related to the rate and degree of cell proliferation.

4.4. ROS, calcium content, mitochondrial membrane potential and apoptosis measurement

H9c2 cells were seeded into 6-well culture plate at the density of 2×10^5/well and maintained overnight at 37 °C. Cells were pretreated with 100 μg/ml FGF1 for 4 h and were maintained under 200 μM H_2O_2 for 12 h. DCFH-DA, Fluo-3 AM, Mito-Tracker Red CMXRos and Annexin V-FITC fluorescent probes were used to detect the level of intercellular ROS, calcium content, mitochondrial membrane potential and apoptosis. All assays were performed strictly according to the manufacturer's protocol. Subsequently, the cells were washed again three times with PBS and then observed using confocal laser scanning microscope (Olympus, Japan).

4.5. RNA extraction and reverse transcription-quantitative PCR (RT-qPCR) analysis

Total RNA in H9c2 cells were extracted using HP Total RNA Kit (OMEGA, China) according to the manufacturer's instructions. Additionally, 1 μg total RNA was reverse-transcribed using the First-strand cDNA synthesis kit (Roche Diagnostics) according to the manufacturer's protocol. Relative gene expression level was measured by RT-qPCR using the StepOne Real-time PCR system (Applied Biosystems). The sequences of the primers used were as follows:
Rps16, forward 5′-AAGTCTTCGGACGCAAGAAA -3′, reverse 5'-TGCCCAGAAGCAGAACAG -3';
Bcl-2, forward 5′-AGCGTCAACAGGGAGATGTC-3, reverse 5′- TATGCACCCA-GAGTGATGCA -3′;
Bax, forward 5'-AGACACCTGAGCTGACCTTGGAG-3', reverse 5'-GTTGAAGTTGCCATCAGCAAACA-3′;
and Caspase-3, forward 5′-GGAGCTTGGAACGCGAAGA-3′, reverse 5′-ACACAAGCCCATTTCAGGGT -3′. mRNA expression levels were quantified by the $2^{-\Delta\Delta Ct}$ method and normalized to the internal control Rps16 (Livak and Schmittgen 2001).

4.6. Statistical analysis

All data reported was in the form of mean±standard deviation (SD) and statistically analyzed using a one-way ANOVA analysis of variance with Tukey's multiple comparison test using GraphPad Prism 7.0 software (GraphPad Software, CA, USA). Differences were considered significant at $P < 0.05$.

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Conflicts of interest: The authors declare no conflict of interest.

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