Eriodictyol protects H9c2 cardiomyocytes against the injury induced by hypoxia/reoxygenation by improving the dysfunction of mitochondria

YANLI XIE1, RONGWEI JI2 and MINGHUI HAN3

1Department of Cardiothoracic Surgery, Wuhan Women and Children Medical Care Center, Wuhan, Hubei 430000; 2Department of Pathology, Xi’an XD Group Hospital, Xi’an, Shaanxi 710077; 3Department of Thoracic Surgery, The First People's Hospital of Qingdao Economic and Technological Development Zone, Qingdao, Shandong 266555, P.R. China

Received December 5, 2016; Accepted June 29, 2017

DOI: 10.3892/etm.2018.6918

Abstract. Myocardial infarction is a leading cause of mortality worldwide, while myocardial ischemia and timely reperfusion contribute to myocardial injury. The mitochondria are involved in the injury and mediate the apoptosis of cardiomyocytes. In order to develop novel therapeutic approaches for myocardial infarction, the present study evaluated the myocardial protective effects of eriodictyol and investigated relevant mechanisms in H9c2 cardiomyocytes. As a result, eriodictyol was observed to improve the H9c2 cardiomyocyte viability and block the leakage of cytosolic lactate dehydrogenase under hypoxia/reoxygenation. In addition, the dysfunction of mitochondria induced by hypoxia/reoxygenation was ameliorated by eriodictyol through suppressing the overload of intracellular Ca2+, preventing overproduction of reactive oxygen species, blocking mitochondrial permeability transition pore opening, increasing mitochondrial membrane potential level and decreasing ATP depletion. Finally, the apoptosis of H9c2 cardiomyocyte induced by hypoxia/reoxygenation was prevented by eriodictyol through upregulation of the expression of B-cell lymphoma-2 (Bcl-2) and downregulation of the expression levels of Bcl-2-associated X protein and caspase-3. These results provided evidence for further investigation on myocardial protection and the treatment of myocardial infarction using eriodictyol.

Introduction

Myocardial infarction is a leading cause of mortality in humans worldwide, which results from myocardial ischemia (1,2).

Keywords: eriodictyol, H9c2 cardiomyocyte, hypoxia/reoxygenation, mitochondrial dysfunction, myocardial protection

Materials and methods

Correspondence to: Dr Minghui Han, Department of Thoracic Surgery, The First People's Hospital of Qingdao Economic and Technological Development Zone, 9 Huangpu River Road, Qingdao, Shandong 266555, P.R. China
E-mail: medhanmh@126.com

Keywords: eriodictyol, H9c2 cardiomyocyte, hypoxia/reoxygenation, mitochondrial dysfunction, myocardial protection

Eriodictyol (Fig. 1) is a flavonoid identified in numerous medicinal plants, such as Bauhinia ungulata (10), Arctophyllum thymifolium (11), Elsholtzia bodinieri (12) and Clinopodium chinense (13). Pharmacological investigations have revealed that eriodictyol possesses several bioactivities, including neuroprotection (14-16), renoprotection (17) and lung protection (18), exerted via anti-inflammation and anti-oxidation. In addition, its anti-inflammatory and anti-oxidative capacities have drawn attention to its therapeutic potential (19-23).

With the aim to investigate bioactive phytochemicals for the treatment of myocardial infarction, the protective effects of eriodictyol on H9c2 cardiomyocyte injury induced by hypoxia/reoxygenation are investigated in the present study. The protective effect of eriodictyol is reported in vitro.
Jiancheng Bioengineering Institute (Nanjing, China). JC-1, Flu-o-3 AM, ATP detection kit and caspase-3 assay kit, as well as cleaved caspase-3 (cat. no. AC033), B-cell lymphoma-2 (Bcl-2; cat. no. AB112), Bcl-2-associated X protein (Bax; cat. no. AB026) and actin (cat. no. AA128) antibodies (all 1:1,000) were purchased from Beyotime Institute of Biotechnology (Nantong, China).

Cell culture and treatment. The H9c2 cardiomyocytes, a rat embryonic cardiac myoblast line, were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured as previously described (9). Briefly, H9c2 cells were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin under humid condition with 5% CO₂ and 95% air at 37°C. Next, cells at the logarithmic phase were incubated in 96-well plates at a density of 1x10⁵/ml. The cells were then divided into the control group (CG), model group (MG) and three eriodictyol groups, which were pretreated with 1, 10 and 50 µM eriodictyol in DMSO for 4 h. In order to establish the hypoxia/reoxygenation model, H9c2 cells in the MG and eriodictyol groups were subjected to an atmosphere with 95% N₂ and 5% O₂ at 37°C for 4 h, and then cultured under a condition of 95% air and 5% O₂ at 37°C for a further 4 h. The CG cells were cultured under normoxic conditions.

Cell viability assay. To evaluate the protective effect of eriodictyol on the H9c2 cardiomyocyte injury induced by hypoxia/reoxygenation, an MTT assay was conducted. Following the aforementioned treatments, the cells were incubated with 0.2 ml MTT for 4 h at 37°C. Subsequently, 200 µl DMSO was added into each well to dissolve the formazan crystals, and the absorbance was recorded on an iMark microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 490 nm. The experiments were repeated three times.

Determination of LDH activity. The extracellular LDH activity was determined using the LDH assay kit, according to the manufacturer's instructions. Following treatment and incubation, the H9c2 cardiomyocyte culture medium was centrifuged at 400 x g and room temperature for 5 min, and then 20 µl supernatant was mixed with 20 µl 2,4-dinitrophenylhydrazin. The mixture was incubated at 37°C for 15 min. Next, 250 µl NaOH (0.4 M) was added into the reaction system and incubated for a further 15 min at 37°C. Subsequent to keeping at room temperature for 5 min, the absorbance was recorded on a microplate reader at 450 nm. The activity of LDH was calculated based on the absorbance as previously reported (24) and is expressed as U/l.

Detection of intracellular Ca²⁺. In order to monitor the cytosolic Ca²⁺ content in H9c2 cardiomyocytes, the Flu-o-3 AM molecular fluorescence probe was employed. Following treatment as described earlier, H9c2 cardiomyocytes were incubated with Flu-o-3 AM at 37°C for 30 min and washed twice with phosphate-buffered saline (PBS) to remove any extracellular dye. Subsequent to incubation for a further 30 min, the fluorescence intensity was measured on a SpectraMax M5 microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) at an excitation wavelength of 488 nm and emission wavelength of 525 nm.

Measurement of ROS generation. The production of intracellular ROS was detected by a fluorescence method (25) with the ROS assay kit, according to the manufacturer's protocol. Following treatment, the medium was replaced and the cells were loaded with 10 µM 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA). After incubation at 37°C for 30 min, the cells were rinsed with PBS and the fluorescence intensity was recorded on a fluorescence microplate reader at an excitation wavelength of 480 nm and emission wavelength of 525 nm.

Assessment of mitochondrial membrane potential (MMP). The fluorescent probe JC-1 was used to detect the MMP in H9c2 cardiomyocytes. In normal mitochondria, the JC-1 monomer aggregates in the matrix, whereas JC-1 maintains its monomeric form in mitochondria with reduced MMP. The fluorescence of JC-1 aggregates is measured at an excitation wavelength of 530 nm and emission wavelength of 590 nm. In the current investigation, cardiomyocytes were loaded with JC-1 (100 µM) at 37°C for 20 min and then washed with PBS. The fluorescence intensity of the JC-1 aggregate was recorded on a fluorescence microplate reader, and the MMP was determined as the ratio of the JC-1 fluorescence intensity to that of the control group.

Opening of MPTP. The opening of MPTP was evaluated by determining the release of mitochondrial green calcein, as previously described (26). Briefly, cardiomyocytes were incubated with 2 µM calcein-AM and 1 mM CoCl₂ at room temperature for 30 min. Next, free calcein-AM and CoCl₂ were washed away with Hank's balanced salt solution, and cells were incubated with CoCl₂ for a further 20 min at 37°C in order to quench the fluorescence of free cytosolic calcein. The fluorescence of mitochondrial green calcein in the cardiomyocytes was recorded on a fluorescence microplate reader at 490 nm for excitation and 515 nm for emission. The quenching of fluorescence in H9c2 cardiomyocytes indicated the opening of MPTP.

Level of intracellular ATP. The intercellular ATP in H9c2 cardiomyocytes was determined by the firefly luciferase method (25) with the ATP detection kit according to the manufacturer's protocol. Luciferin generates fluorescence under the catalysis of firefly luciferase, and the process consumes ATP quantitatively. The treated H9c2 cardiomyocytes were lysed on ice with 200 µl lysis reagent from the assay kit. The lysed cells were then centrifuged at 12,000 x g for 4 min at 4°C, and 100 µl supernatant was mixed with 100 µl ATP monitoring reagent. Subsequently, the luminescence was detected on a microplate reader, and the level of intracellular ATP was derived from the standard curve.

Figure 1. Chemical structure of eriodictyol.
Caspase-3 activity. The activity of caspase-3 was quantified through a colorimetric detection kit, following the manufacturer's instructions. Briefly, subsequent to the aforementioned treatments, the H9c2 cardiomyocytes were lysed and centrifuged at 16,000 x g for 10 min at 4°C. The supernatant was then incubated with the substrate Ac-DEVD-pNA at 37°C for 2 h, and the absorbance was measured on a microplate reader at 405 nm. The relative caspase-3 activity was expressed as a percentage of the control group, as previously described (27).

Western blot analysis. The protein expression levels of caspase-3, Bcl-2 and Bax in pretreated H9c2 cardiomyocytes were analyzed by western blot analysis. In brief, the cells were lysed with lysis reagent containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride on ice for 30 min. Next, the lysate was centrifuged at 12,000 x g for 15 min at 4°C, and the supernatant was collected for the analysis of cleaved caspase-3, Bcl-2 and Bax levels. The total protein concentration in the samples was determined by BCA assay kit. Subsequently, the protein was separated by electrophoresis on a 15% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes. Following blocking with 5% skimmed milk at room temperature for 1 h, the membranes were incubated overnight at 4°C with primary antibodies against cleaved caspase-3, Bcl-2, Bax and actin. The membranes were was with TBST three times, treated with the respective secondary antibodies conjugated to horseradish peroxidase (cat. no. LDA0310; 1:1,000; Shanghai Lengton Bioscience Co., Ltd., Shanghai, China) at room temperature for 1 h and detected by an Enhanced ECL Chemiluminescent Substrate kit (cat. no. 36222E560; Shanghai Yeasen Biotechnology Co., Ltd., Shanghai, China). Actin was used as the internal control.

Statistical analysis. All results are expressed as the means ± standard deviation. GraphPad Prism (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA) was employed to analyze the results. Statistical differences between different groups were compared by one-way analysis of variance followed by Dunnett's test for multiple comparisons and Student's t-test for single comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of eriodictyol on H9c2 cardiomyocyte viability. As shown in Fig. 2, the MTT assay demonstrated that the viability of H9c2 cardiomyocytes decreased when subjected to the hypoxia/reoxygenation (P<0.001). Upon treatment with different dosages of eriodictyol, the survival of H9c2 cardiomyocytes was significantly improved and the cells viability was 77.75±7.06% of the cell viability of CG when cells were treated with 50 µM eriodictyol (P<0.001). The viability in H9c2 cardiomyocytes treated with 50 µM eriodictyol (77.75±7.06%) was significantly higher than the cells treated with 10 µM eriodictyol (63.56±2.75%; P<0.001) and the latter was significantly increased compared with the group treated with 1 µM eriodictyol (58.16±3.17%; P<0.05). These results indicate the potential cardioprotective effect of eriodictyol on H9c2 cells in a dose-dependent manner at the range of 1-50 µM eriodictyol.

Effect of eriodictyol on LDH activity. The leakage of LDH from the cytoplasm is associated with cell death. In this investigation, the activity of LDH in the culture medium of the MG (301.01±28.81%) was significantly higher as compared with that in the CG (P<0.001), which further demonstrated that the viability of H9c2 cells was affected by hypoxia/reoxygenation. When pretreated with eriodictyol, the activity of LDH in the 10 µM group with was reduced to 256.81±13.75%, which was significantly higher than the 50 µM group (191.56±13.75%; P<0.001) and lower than the 1 µM group (272.94±8.44%; P<0.05). The results indicated the release of LDH from the cytosol of H9c2 cardiomyocytes was evidently decreased in a dosage-dependent manner (Fig. 3).

Effect of eriodictyol on intracellular Ca²⁺. To assess the intracellular Ca²⁺ content, the fluorescence probe Fluo-3 AM...
was used. Following the induction of hypoxia/reoxygenation, the level of intracellular Ca\(^{2+}\) was markedly elevated to 155.28±6.13% as compared with the CG (P<0.001; Fig. 4). However, in comparison with the model group, eriodictyol reduced the overload of intracellular Ca\(^{2+}\) to 139.25±8.82%, 122.41±7.64% and 102.39±8.17% upon pretreatment with 1, 10 and 50 µM, respectively (Fig. 4). These results provided evidence that eriodictyol was able to reduce the overload of intracellular Ca\(^{2+}\) in the hypoxia/reoxygenation cell model.

**Effect of eriodictyol on ROS generation.** The intracellular ROS generation was determined through the fluorescence intensity of DCFH-DA. The results indicated that the relative fluorescence intensity in the MG (148.84±5.16%) was significantly higher compared with that in the CG (P<0.001). By contrast, when cells were treated with eriodictyol, the fluorescence intensity was significantly decreased to 139.25±8.68% (P<0.05), 111.89±9.10 and 101.33±4.57% (both P<0.001) compared with the MG group (Fig. 5). The fluorescence intensity of 10 µM (111.89±9.10%) was lower compared with 1 µM (139.25±8.68%; P<0.05) and higher compared with 50 µM (101.33±4.57%; P<0.05). This indicated that eriodictyol was able to downregulate the generation of intracellular ROS.

**Effect of eriodictyol on MMP.** In order to determine the MMP in H9c2 cardiomyocytes, the fluorescent probe JC-1 was used. Compared with the CG, the fluorescence intensity in the MG (41.89±3.75%) was significantly decreased (P<0.001), which indicated the collapse of MMP in H9c2 cardiomyocytes following hypoxia/reoxygenation. However, when pretreated with 1, 10 and 50 µM eriodictyol, the fluorescence intensity was significantly elevated to 48.00±4.74% (P<0.05), 62.92±8.56 and 79.89±6.24% (both P<0.001), respectively, compared with the MG group (Fig. 6). Meanwhile, among these groups treated with eriodictyol, the 10 µM group was significantly higher than the 1 µM group lower than the 50 µM (both P<0.05). These findings revealed that the collapse of MMP in hypoxia/reoxygenation-treated H9c2 cardiomyocytes was attenuated by eriodictyol.

**Effect of eriodictyol on the opening of MPTP.** The MPTP opening was evaluated through the fluorescence intensity of free calcein in mitochondria. As shown in Fig. 7, under hypoxia/reoxygenation, the fluorescence intensity of mitochondrial calcein was approximately half that of the CG (50.11±6.00%; P<0.001), which demonstrated that the MPTP opened. Upon treatment with 1, 10 and 50 µM eriodictyol, the fluorescence intensity increased significantly to 58.83±6.84% (P<0.05), 58.83±6.84% (P<0.01) and 58.83±6.84% (P<0.001), respectively, compared with the MG group (Fig. 7). Compared with the CG group (100±11.83%), the ATP level in the MG group (52.62±5.87%) was significantly decreased (P<0.001). Eriodictyol (1, 10 and 50 µM) pre-treatment increased ATP levels to 59.69±4.95% (P<0.05), 69.23±3.27% (P<0.01) and 80.52±9.52% (P<0.001), respectively, compared with the MG group. These results implied that eriodictyol inhibited the MPTP opening.

**Effect of eriodictyol on ATP depletion.** The level of intracellular ATP represents the function of mitochondria. To detect

![Figure 4](image)

**Figure 4.** Effect of eriodictyol treatment (1, 10 and 50 µM) on intracellular Ca\(^{2+}\). n=6. ###P<0.001 vs. the CG; *P<0.05 and ***P<0.001 vs. the MG; &P<0.05 vs. the 1 µM eriodictyol; @P<0.05 vs. the 10 µM eriodictyol. CG, control group; MG, model group.

![Figure 5](image)

**Figure 5.** Effect of eriodictyol treatment (1, 10 and 50 µM) on ROS production. n=6. ###P<0.001 vs. the CG; *P<0.05 and ***P<0.001 vs. the MG; &P<0.05 vs. the 1 µM eriodictyol; @P<0.05 vs. the 10 µM eriodictyol. ROS, reactive oxygen species; CG, control group; MG, model group.

![Figure 6](image)

**Figure 6.** Effect of eriodictyol treatment (1, 10 and 50 µM) on the MMP in H9c2 cardiomyocytes. n=6. ###P<0.001 vs. the CG; *P<0.05 and ***P<0.001 vs. the MG; &P<0.05 vs. the 1 µM eriodictyol; @P<0.05 vs. the 10 µM eriodictyol. MMP, mitochondrial membrane potential; CG, control group; MG, model group.
the depletion of intracellular ATP, the firefly luciferase method was used. Hypoxia/reoxygenation in H9c2 cardiomyocytes resulted in the decline of the intracellular ATP level, whereas treatment with eriodictyol enhanced the ATP level. The ATP level in the 10 µM eriodictyol group was significantly higher than that in the 1 µM group as well as lower than 50 µM group (both P<0.05; Fig. 8). Compared with the control group (100±11.83%), the ATP level in the MG group (52.62±5.87%) was significantly decreased (P<0.001). Pretreated with eriodictyol (1, 10 and 50 µM), compared with the MG group, the ATP levels were increased to 59.69±4.95% (P<0.05), 69.23±3.27% (P<0.001) and 80.52±9.52% (P<0.001), respectively. These results indicated that eriodictyol treatment was able to improve the depletion of intracellular ATP in the cardiomyocytes.

**Effect of eriodictyol on caspase-3 activity and expression levels of caspase-3, Bcl-2 and Bax.** As a member of the cysteinyl aspartate specific protease family, caspase-3 serves a pivotal role in apoptosis through hydrolyzed cleavage (28). Western blot analysis revealed that hypoxia/reoxygenation promoted the expression of caspase-3 in H9c2 cardiomyocytes, while eriodictyol reduced this expression by different extents (Fig. 9A). In addition, colorimetric detection further confirmed the activity of caspase-3 quantitatively. Compared with the control group, the caspase-3 activity was markedly elevated in H9c2 cardiomyocytes treated by hypoxia/reoxygenation. However, in the presence of eriodictyol, the increased activity of caspase-3 was inhibited accordingly. Similarly, the activity of caspase-3 in the 10 µM eriodictyol group was significantly lower than that in 1 µM group as well as higher than 50 µM group (both P<0.05; Fig. 9B). The activity of caspase-3 in the MG group (253.38±17.80%) was significantly decreased compared with the CG group (100.00±24.74%; P<0.001). In contrast to the MG group, caspase-3 activity in the 1, 10 and 50 µM eriodictyol-treated groups was significantly decreased to 230.80±15.03% (P<0.05), 179.40±18.85% (P<0.001) and 147.09±17.41% (P<0.001), respectively.

Bcl-2 and Bax are the major members of the Bcl-2 protein family, which are involved in mitochondrion-mediated apoptosis. The former demonstrates an anti-apoptotic effect, whereas the latter exhibits a pro-apoptotic effect (29). In the present study, the expression of Bcl-2 was downregulated by hypoxia/reoxygenation in contrast to that in the control group. However, treatment with eriodictyol was observed to upregulate Bcl-2 expression. Accordingly, hypoxia/reoxygenation upregulated the expression of Bax, while eriodictyol treatment suppressed this increased expression (Fig. 9B).
Discussion

Myocardial ischemia and subsequent reperfusion is the major cause of myocardial infarction. The oxygen deprivation will lead to the breakdown of redox homeostasis and ROS overproduction. As the major site of ROS production, mitochondria serve an important role in the injury of myocardial ischemia and reperfusion (30). In addition to the overproduction of ROS, overload of intracellular Ca\(^{2+}\) also affects the function of the mitochondria (31). As the key determinant of mitochondrial dysfunction, the MPTP will open (32). The electrochemical gradient across the inner mitochondrial membrane (MMP) is necessary for mitochondrial function (33). Following the opening of MPTP, free solutes and proteins can be distributed across the inner mitochondrial membrane and result in the collapse of the MMP (34). The dysfunction of mitochondria also leads to the depletion of ATP due to MPTP opening (35), and finally results in the cardiomyocyte apoptosis (36).

Caspases are cysteiny1 aspartate specific proteases with a central role in apoptosis, and their activation occurs through cleavage at specific sites (37). As an effector enzyme, caspase-3 is the key mediator responsible for promoting cell apoptosis (38). In addition, Bcl-2 and Bax are members of the Bcl-2 protein family that participate in mitochondrion-mediated apoptosis. Bcl-2 prevents apoptosis and blocks the activation of caspase-3, while Bax promotes cell apoptosis (39).

In the present study, mitochondrial dysfunction and cell injury induced by hypoxia/reoxygenation were observed. Pretreatment with eriodictyol increased the cell survival and blocked the leakage of LDH from the cytosol, which indicates the potential cardioprotective effect of eriodictyol. Further experiments revealed that eriodictyol improved the dysfunction of mitochondria through suppressing the overload of intracellular Ca\(^{2+}\), preventing the overproduction of ROS, blocking the opening of MPTP, increasing the MMP level and decreasing ATP depletion. As important intracellular signaling molecules, there is interplay between Ca\(^{2+}\) and ROS production. Ca\(^{2+}\) may increase ROS production by enhancing metabolism and ROS regulates Ca\(^{2+}\) homeostasis through reciprocal redox (40). Meanwhile, the interplay between ROS and Ca\(^{2+}\) triggers the opening of MPTP opening, which leads to the collapse of MMP (41). In addition, as the main source of ATP, mitochondria may fail to synthesize enough ATP to maintain cellular function due to the dysfunction of MMP (42).

Furthermore, eriodictyol inhibited the apoptosis of H9c2 cardiomyocytes through upregulating the expression of Bcl-2 and downregulating the expression levels of Bax and caspase-3, as well as reducing the activity of caspase-3. As a bioactive flavonoid, eriodictyol showed many protective effects through upregulating the expression of Bcl-2, downregulating of Bax and reducing the activity of caspase-3. As a bioactive flavonoid, eriodictyol showed many protective effects through upregulating the expression of Bcl-2, downregulating of Bax and reducing the activity of caspase-3. As a bioactive flavonoid, eriodictyol showed many protective effects through upregulating the expression of Bcl-2, downregulating of Bax and reducing the activity of caspase-3. As a bioactive flavonoid, eriodictyol showed many protective effects through upregulating the expression of Bcl-2, downregulating of Bax and reducing the activity of caspase-3.

In conclusion, the results of the present study demonstrated the myocardial protective effects of eriodictyol and relevant mechanisms in vitro. Eriodictyol can enhance the survival of H9c2 cardiomyocytes injured by hypoxia/reoxygenation. The mechanisms involve the improvement of mitochondrial dysfunction and inhibition of apoptosis via the mitochondria-mediated signaling pathway, including the upregulation of Bcl-2, downregulation of Bax and inhibition of caspase-3. These results provided evidence for further evaluations in vivo for the development of novel therapeutic approaches for myocardial infarction.

References


21. Habtemariam S and Dagne E: Comparative antioxidant, proxi-


26. Wang M, Sun GB, Zhan JY, Luo Y, Yu YL, Xu XD, Meng XB, Zhang MD, Lin WB and Sun XB: Elatoside C protects the heart from ischaemia/reperfusion injury through the modula-
tion of oxidative stress and intracellular Ca\textsuperscript{2+} homeostasis. Int J Cardiol 185: 167-176, 2015.


30. Madungwe NB, Zilberstein NF, Feng Y and Bopassa JC: Critical role of mitochondrial ROS is dependent on their site of produc-


32. Weiss JN, Korge P, Honda HM and Ping P: Role of the mitochon-


36. Whelan RS, Kaplinsky V and Kitiss RN: Cell death in the patho-


