Original Article

Hesperidin Extracted from *Citrus reticulata* Blanco Protects Cardiac Mitochondria Against Hypoxia/Reoxygenation Injury

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Abstract: This study was conducted to evaluate the protective effect of Hesperidin (Hes) extracted from *Citrus reticulata* Blanco on cardiac mitochondria in hypoxia/reoxygenation (HR) injury in vitro. H9C2 cardiomyocytes were cultured under normal (control), HR, and treatment conditions. The reactive oxygen species and calcium levels in experimental groups were analyzed by using suitable fluorescence kits. The obtained results showed that the addition of Hes at dose of 0.01562 mg/mL sharply decreased the mitochondrial oxidative stress of H9C2 cells under HR conditions. In particular, Hes showed the remarkable efficiency in maintaining cellular calcium levels. In HR-exposed H9C2 cell group, the hydrogen peroxide and superoxide levels were highly increased compared to those in control group (1.54±0.06 and 1.74±0.38, p<0.05). HR also strongly induced the elevation of cytosolic Ca²⁺ and mitochondrial Ca²⁺ of H9C2 cardiomyocytes with the values were 1.96±0.05% and 1.62±0.33 (ratio to control, p<0.05), respectively. Interestingly, post-hypoxic supplementation of Hes effectively abolished the negative increment of these indicators with the lower levels of hydrogen peroxide and superoxide levels (1.00±0.10 and 1.29±0.03, p<0.05) and the better modulation of cytosolic and mitochondrial Ca²⁺ homeostasis (1.94±0.05 and 1.25±0.01) compared to those in HR-treated cells. The present results are pilot data on the effects of Hes in protecting cardiac mitochondria against HR injury.

Keywords: Hesperidin, Mitochondria, Hypoxia/reoxygenation, Calcium.

1. Introduction

Heart attack or ischemic heart disease is characterized by reduced blood supply to the heart tissue [1, 2]. Ischemic heart disease is normally unpredictable and rescuing the patient depends on revascularization time and on the drugs administered during reperfusion. Effective intervention for rescuing the patient depends on myocardial ischemia duration and revascularization time [3]. However, reperfusion with restoration of normal oxygen level to ischemic myocardium can also result in severe or irreversible injury to heart, so called ischemia/reperfusion (IR) or hypoxia/reoxygenation (HR) injury [4-7]. At
the cellular levels, phenomena of the malfunctions include an excessive generation of reactive oxygen species (ROS) [8], an overload of mitochondrial calcium, and cell death [4, 9]. As mitochondria play important role in physiological and pathological processes, many reports have focused on the assessment of anti-ischemic drugs based on mitochondrial functional analysis [10, 11].

In recent decades, there has been great progress in screening and identifying natural compounds to develop new drugs, which can be used to preserve mitochondrial function and subsequently to improve cardiac function. Though Vietnamese plant compounds have been demonstrated to have the ability to reduce oxidative stress, inflammation and apoptosis [12-15], the functions of these promised candidates on treatment of ischemic heart disease are not fully understood yet.

Of those, Hesperidin (Hes) is a flavanone glycoside with a wide range of biological effects found primarily in the peels of citrus fruits (genus Citrus) [16-18]. Previous research demonstrated that Hes possesses the lipid peroxidation and antioxidant activities [18]. Hes reduces oxidative stress, apoptosis and improves cardiac function via the peroxisome proliferator-activated receptor gamma (PPARγ) pathway in isoproterenol-induced myocardial dysfunction in rat diabetes [17]. The preventive effect of Hes modulated the inflammatory response and antioxidant status following acute myocardial infarction through downregulation of the expression of PPARγ and B-cell lymphoma 2 (Bcl2) in the model animal. Moreover, pretreatment with Hes protects against myocardial IR injury by suppressing myocardial apoptosis, the inflammatory response and oxidative stress [19]. A recent study had demonstrated that Hes could be a potential active compound in protecting H9C2 against HR injury targeting mitochondria [20]. Post-hypoxic treatment of Hes reduced H9C2 cardiomyocyte death and preserved mitochondrial cardiolipin content [20]. However, the mechanism underlying the protective effects of Hes against malfunction of cardiac mitochondria remains poorly defined. Therefore, in this study, we isolated Hes from Citrus reticulata Blanco and then evaluated the protective effects of Hes on H9C2 cells by examining ROS and calcium levels.

2. Materials and Methods

2.1. Materials

The main materials and equipments used in this study were fruit peels of Citrus reticulata Blanco (Hanoi, Vietnam), H9C2 cell line (ATCC®-USA), Dulbecco’s Modified Eagle Medium 4.5g/L glucose (DMEM, Gibco, USA), Fetal bovine serum (FBS, Gibco, USA), Penicillin-Streptomycin (PS, Gibco, USA), Phosphate buffered saline (PBS, Gibco, USA), Dimethyl Sulfoxide (DMSO, Sigma, USA), phosphate buffer saline, MeOH, 2,7'-dichlorodihydrofluorescein-diacetate (CM-H_2DCFDA; ex/em 485/525 nm, Invitrogen, USA); MitoSOX Red (ex/em: 510/580 nm, Invitrogen, USA); Rhod-2 AM (5 μM, ex/em: 533/576 nm, Invitrogen, USA), Fluo-4 AM (5 μM, ex/em: 488/525 nm, Invitrogen, USA), MeOH, Culture dishes 90x20 mm (SPL, Korea), 96-well black, glass bottom plates (CAT. 33196, SPL), CO_2 Incubator (Shellab, USA); and Microplate reader (Tristar, USA), liquid chromatography-mass spectrometry (LCMS-8045, Shimadzu, Japan).

2.2. Methods

2.2.1. Hesperidin Preparation

Sample preparation

The peels of citrus fruits (Citrus reticulata Blanco) were sliced (3-4 cm long and 0.5-1 cm wide) and then dried in an oven at 60 °C until the moisture content less than 10%.

Extraction and purification of hesperidin

The dried sample (1 kg) was powdered and extracted with methanol (MeOH) under reflux three times (each 10 L). After filtration, the combined MeOH extract was evaporated to about one-half its original volume, and then partitioned with n-hexane to remove impurities. The remaining MeOH extract was concentrated
to remove the solvent, then cooled below 10 °C; then a crude precipitate (CF-2, 116 g) was obtained.

The crude CF-2 (4.7 g) was refluxed with MeOH (1 L) for 60 min. The solution was then filtered and allowed to stand at 5 °C for 48 h in order to crystallize. The crystals of CF-2 were filtered off and dried at 60 °C for 2 h. After that, the white crystalline CF-2 (3.75 g) was collected. The yield of purification was 79.8%.

2.2.2. Cell Culture and Hypoxia-Reoxygenation In Vitro Model

H9C2 cells were maintained in normal condition (DMEM, 10% FBS, and 1% PS at 37 °C with 5% CO₂) and subjected to HR model and treatment as previously described [20]. For HR in vitro model, H9C2 cells were further transferred to 96-well black, glass bottom plates at density of 5.10³ cells/well at 37 °C, 5% CO₂. After 24 h, the cells were then subjected to hypoxic condition and treatments. The experimental cells were divided into different groups. For control group, H9C2 cells were continuously cultured under normal condition for 48 h. For HR groups, H9C2 cells were cultured in serum-free low-glucose DMEM at 37 °C, 95% N₂, 5% CO₂, and 2% O₂ for 6 h. Then, the old medium was removed. The H9C2 cells were then transferred to normal condition for reoxygenation for 24 h. The HR groups were further devised to sub-groups based on post-hypoxic treatments:

i) HR group: the reoxygenation stage normal culture condition;

ii) RuR group: the reoxygenation stage medium contained DMEM, 10% FBS, 1% PS, and Ruthenium Red (RuR) at doses of 5 μM. RuR was used as positive control of mitochondrial calcium uniporter (MCU) inhibitor.

iii) Hes group: the reoxygenation stage medium contained DMEM, 10% FBS, 1% PS, and Hes at doses of 0.01562 mg/mL as previous study [20].

RuR and Hes stocks were prepared in DMSO and the final concentration of DMSO in cultured medium was about 0.1%. At the end of the experiment period, ROS and Ca²⁺ levels were tested by the suitable fluorescence kits.

2.2.3. Measurement of Reactive Oxygen Species and Ca²⁺ Levels

Mitochondrial hydrogen peroxide (H₂O₂) and superoxide (O₂⁻), mitochondrial Ca²⁺ and cytosolic Ca²⁺ levels were indirectly assessed as following a previously described [4, 21]. H9C2 cells were seeded in 96-well black, glass bottom plates and subjected to HR model and treatments. After being subjected to different conditions, cells were double stained with CM-H₂DCFDA (5 μM) and MitoSOX Red (5 μM); or with Rhod-2 AM (5 μM) and Fluo-4 AM (5 μM) to detect changes in mitochondrial H₂O₂, O₂⁻ levels; or Ca²⁺ and cytosolic Ca²⁺ levels, respectively. After washing twice with phosphate buffer saline, samples were analyzed using a microplate reader. The total fluorescence intensities were expressed as ratio relatives to normal control. Experiments were performed 3-6 times.

2.2.4. Statistical Analysis

Origin 8.0 software was chosen to analyze data. Data are presented as means ± Standard error of the mean (SEM). Differences between the two groups were evaluated by ANOVA and Turkey test. A p-value ≤ 0.05 was considered to be significant.

3. Results and Discussion

3.1. Hesperidin (CF-2) Extracted From Citrus Reticulata Blanco

The obtained compound CF-2 was characterized with white crystalline; mp: 252-254 °C; UV (MeOH) λmax: 284.326 nm; IR (KBr) νmax cm⁻¹: 3439 (phenolic OH), 2983, 2934 (C–H), 1648 (C=O), 1607, 1520, 1447 (aromatic C=C), 1280, 1205, 1132, 1072 (C-O); ESI-MS: m/z 609.4 [M-H] (C₂₈H₁₃O₁₅); ¹H-NMR (500 MHz, DMSO-d₆) δH: 5.50 (1H, dd, J = 3.0, 12.5 Hz, H-2), 3.27 (1H, dd, J = 12.5, 17.5 Hz, H-3), 3.27 (1H, dd, J = 12.5, 17.5 Hz, H-3), 6.14 (1H, d, J = 2.0 Hz, H-6), 6.12 (1H, d, J = 2.0 Hz, H-8), 6.94 (3H, m, H-2', H-5', H-6'), 9.08 (1H, s, 3'-OH), 12.01 (1H, s, 5'-OH), Glc:
4.97 (1H, d, J = 7.5 Hz, H-1′″), Rha: 4.52 (1H, s, H-1‴), 1.08 (3H, d, J = 6.0 Hz, H-6‴); 13C-NMR (125 MHz, DMSO-d6) δ: 78.4 (C-2), 42.0 (C-3), 197.0 (C-4), 163.0 (C-5), 96.4 (C-6), 165.1 (C-7), 95.5 (C-8), 162.5 (C-9), 103.3 (C-10), 130.9 (C-1′), 114.1 (C-2′), 146.6 (C-3′), 148.0 (C-4′), 112.0 (C-5′), 117.9 (C-6′), 55.7 (OCH3), Glc: 100.6 (C-1″), 73.0 (C-2″), 76.3 (C-3″), 69.6 (C-4″), 75.5 (C-5″), 66.0 (C-6″), Rha: 99.5 (C-1‴), 70.3 (C-2‴), 70.7 (C-3‴), 72.1 (C-4‴), 68.3 (C-5‴), 17.8 (C-6‴).

The spectral data of CF-2 was completely identical with those of published Hesperidin [22]. In this study, the natural purified CF-2 compound was determined to be hesperidin as shown in Figure 1.

A

B

C

D

Figure 1. Hesperidin (CF-2) extracted from Citrus Reticulata Blanco.
A: The absorption spectrum; B: HPLC chromatogram; C: Mass spectrum; D: The structure of Hesperidin.

3.2. Hesperidin Decreased Oxidative Stress in HR Injury

H9C2 cells were cultured in normal condition or subjected to HR conditions. With RuR and Hes groups, the HR-subjected cells were supplied with RuR and Hes at the selected dose to culture media during reoxygenation period. The effects on the levels of reactive oxygen species in H9C2 cells were shown in Figure 2.

Previous researches had demonstrated that Hes exerts cardioprotective and anti-diabetic properties in in vivo rat model by reducing oxidative stress and apoptosis and improving cardiac function [17, 19]. The study showed that treatment of Hes decreased the down-regulated PPARγ and Bcl2 apoptosis regulator expressions in myocardial infarcted diabetic hearts [17]. Short-term pretreatment with Hes protected against myocardial IR injury by suppressing myocardial apoptosis, the inflammatory response and oxidative stress via phosphoinositide 3-kinases/protein kinase B pathway activation and high mobility group box 1 protein inhibition [19]. Recently study had demonstrated that Hes also protected H9C2 cells against HR damage by decreasing cell death and preserving mitochondrial cardiolipin content [20]. Consistent with these researches,
our data demonstrated that HR significantly increased the H₂O₂ and O₂⁻ levels in H9C2 cells to 1.54±0.06 and 1.74±0.38 (ratio to control, p<0.05). Post-hypoxic treatment of RuR and Hes effectively decreased H₂O₂ and O₂⁻ overproduction with the ratio values (to control) were about 1.00±0.10 and 1.29±0.03, respectively (Figure 2). Particularly, H₂O₂ levels in Hes-treated cells was significant lower than in RuR-treated cells (Figure 2A, p<0.05), suggesting the ability of Hes in limiting oxidative stress under HR injury was stronger than RuR. Also, the insignificant O₂⁻ levels between RuR and Hes could be a result of the rapid conversion of O₂⁻ to H₂O₂ (Figure 2B). The obtained results were consisted with the last study [23], mitochondrial calcium uniporter (MCU) is involved in oxidative stress-induced cell death, representing therapeutic targets for oxidative stress related diseases [23]. Ruthenium red, a well-known MCU inhibitor, delayed the onset of cell death during oxidative stress of rat hepatocytes [24]. Moreover, NecroX-5, a novel MCU inhibitor, protected myocytes and myocardium against HR damage induced by oxidative stress and Ca²⁺ homeostasis dysregulation [4]. Thus, the present results suggested that Hes exerts antioxidant properties in limiting mitochondrial oxidative stress against HR injury.

![Figure 2](image)

Figure 2. Reactive oxygen species production in H9C2 cells under different conditions. 
A: The CM-H₂DCFDA intensity in different conditioned-H9C2 cells; B: the MitoSOX Red intensity in different conditioned-H9C2 cells. Con: H9C2 cells were cultured in normal condition; HR: H9C2 cells were cultured in HR condition; RuR: H9C2 cells were cultured in conditions of post-hypoxic treatment with Ruthenium Red; Hes: H9C2 cells were cultured in conditions of post-hypoxic treatment with Hesperidin; *p<0.05 vs. Con, †p<0.05 vs. HR, *p<0.05 vs. RuR; n=3±6.

3.3. Hes Ameliorates Ca²⁺ Homeostasis Dysregulation in H9C2 Cardiomyocytes Against HR Injury

Dysregulation of Ca²⁺ homeostasis is one of mitochondrial malfunction indexes. During reoxygenation, mitochondria encounter harsh environmental changes with mitochondrial Ca²⁺ accumulation and overload [25]. Ca²⁺ influx from cytosolic to mitochondria during reoxygenation is dependent on the MCU [26]. In this study, the effects of Hes on mitochondrial and cytosolic Ca²⁺ levels of H9C2 cells under different conditions were evaluated via checking Fluo-4 AM fluorescence intensity and Rhod-2 AM fluorescence intensity. The total intensities of these fluorescence dyes in different cell groups were presented in Figure 3.

The obtained data showed that HR conditions induced the increase in both cytosolic and mitochondrial Ca²⁺ levels. The increase in Ca²⁺ was more pronounced in the HR group without any treatment. HR-induced Ca²⁺ overload was strongly attenuated in the RuR-treated cell group compared with the HR group (Figure 3). Post-hypoxic treatment of Hes
has no effect on cytosolic Ca²⁺ in H9C2 cells (Figure 3A, p>0.05). The Fluo-4 AM fluorescent intensities (ratio to control) in HR and Hes were 1.96±0.50 and 1.94±0.05, respectively. In contrast to cytosolic Ca²⁺, mitochondrial Ca²⁺ level was markedly decreased in Hes-treated cell group. Additionally, RuR group had a lower mitochondrial Ca²⁺ content compared with the Hes group. Although the influx of Ca²⁺ into the mitochondria during reoxygenation was strongly inhibited in the RuR group than those in Hes, the results suggested that Hes may be targeting to MCU. It could be explained by the high level of cytosolic Ca²⁺ level in Hes-treated group (Figure 3A). Previous study had shown that the high mitochondrial Ca²⁺ level in the HR group finally led to hypercontracture and cardiac cell death [4].

Similar to RuR (the positive control), Hes post-hypoxic treatment may show its ability to prevent HR-induced Ca²⁺ overload (Figure 3A), subsequently attenuating cardiac cell death as proved in a recent report.

The results show that Hes showed the stronger antioxidant effect on mitochondrial oxidative stress than RuR (Figure 2). In contrast, the effect of Hes on Ca²⁺ homeostasis regulation was weaker than RuR (Figure 3). Hes has the ability to protect H9C2 rat cardiomyocytes through targeting mitochondrial oxidative stress and mitochondrial Ca²⁺ regulation [20]. However, the detail mechanism of Hes on HR-related molecules is still remained and needed to be examined in further study.

4. Conclusion

The study demonstrated that post-hypoxic treatment with Hes significantly decreased the mitochondrial oxidative stress and mitochondrial Ca²⁺ overload in HR-subjected H9C2 cardiomyocytes. Hes may be a promising compound for the attenuation of myocardial damage resulting from HR damage.

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