



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 ( $1.54 \pm 0.06$  and  $1.74 \pm 0.38$ ,  $p < 0.05$ ). HR also strongly induced the elevation of cytosolic  $Ca^{2+}$  and mitochondrial  $Ca^{2+}$  of H9C2 cardiomyocytes with the values were  $1.96 \pm 0.05\%$  and  $1.62 \pm 0.33$  (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 ( $1.00 \pm 0.10$  and  $1.29 \pm 0.03$ ,  $p < 0.05$ ) and the better modulation of cytosolic and mitochondrial  $Ca^{2+}$  homeostasis ( $1.94 \pm 0.05$  and  $1.25 \pm 0.01$ ) 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

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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 (PPAR $\gamma$ ) 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 PPAR $\gamma$  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<sup>®</sup>-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<sub>2</sub>DCFDA; ex/em 485/525 nm, Invitrogen, USA); MitoSOX Red (ex/em: 510/580 nm, Invitrogen, USA); Rhod-2 AM (5  $\mu$ M, ex/em: 533/576 nm, Invitrogen, USA), Fluo-4 AM (5  $\mu$ M, ex/em: 488/525 nm, Invitrogen, USA), MeOH, Culture dishes 90x20 mm (SPL, Korea), 96-well black, glass bottom plates (CAT. 33196, SPL), CO<sub>2</sub> 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, 11.6 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% CO<sub>2</sub>) 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 5.10<sup>3</sup> cells/well at 37 °C, 5% CO<sub>2</sub>. 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% N<sub>2</sub>, 5% CO<sub>2</sub>, and 2% O<sub>2</sub> 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 divided 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 Ca<sup>2+</sup> levels were tested by the suitable fluorescence kits.

#### 2.2.3. Measurement of Reactive Oxygen Species and Ca<sup>2+</sup> Levels

Mitochondrial hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and superoxide (O<sub>2</sub><sup>-</sup>), mitochondrial Ca<sup>2+</sup> and cytosolic Ca<sup>2+</sup> 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-H<sub>2</sub>DCFDA (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 H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>-</sup> levels; or Ca<sup>2+</sup> and cytosolic Ca<sup>2+</sup> 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 were characterized with white crystalline; mp. 252-254 °C; UV (MeOH) λ<sub>max</sub>: 284.326 nm; IR (KBr) ν<sub>max</sub> cm<sup>-1</sup>: 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]<sup>-</sup> (C<sub>28</sub>H<sub>33</sub>O<sub>15</sub>); <sup>1</sup>H-NMR (500 MHz, DMSO-*d*<sub>6</sub>) δ<sub>H</sub>: 5.50 (1H, dd, *J* = 3.0, 12.5 Hz, H-2), 2.77 (1H, dd, *J* = 3.0, 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''');  $^{13}\text{C}$ -NMR (125 MHz, DMSO- $d_6$ )  $\delta_{\text{C}}$ : 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 (OCH<sub>3</sub>), 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.

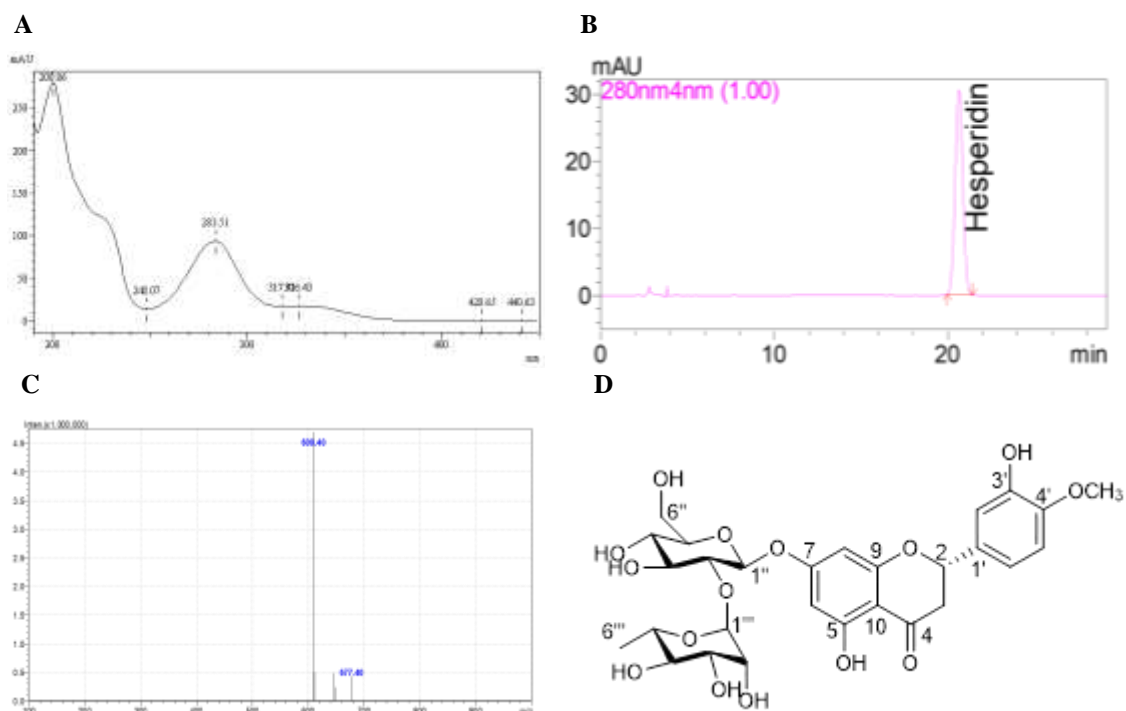


Figure 1. Hesperidin (CF-2) extracted from *Citrus Reticulata* Blanco.

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 PPAR $\gamma$  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  $H_2O_2$  and  $O_2^-$  levels in H9C2 cells to  $1.54 \pm 0.06$  and  $1.74 \pm 0.38$  (ratio to control,  $p < 0.05$ ). Post-hypoxic treatment of RuR and Hes effectively decreased  $H_2O_2$  and  $O_2^-$  overproduction with the ratio values (to control) were about  $1.00 \pm 0.10$  and  $1.29 \pm 0.03$ , respectively (Figure 2). Particularly,  $H_2O_2$  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  $O_2^-$  levels between RuR and Hes could be a result of the rapid conversion of  $O_2^-$  to  $H_2O_2$

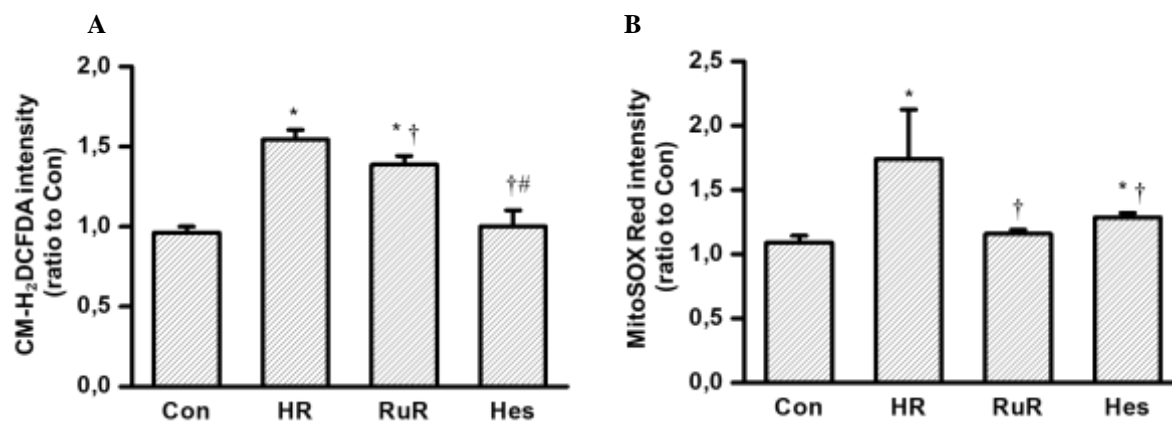


Figure 2. Reactive oxygen species production in H9C2 cells under different conditions.

A: The CM-H<sub>2</sub> DCFDA 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;  $n = 3 \div 6$ .

### 3.3. Hes Ameliorates $Ca^{2+}$ Homeostasis Dysregulation in H9C2 Cardiomyocytes Against HR Injury

Dysregulation of  $Ca^{2+}$  homeostasis is one of mitochondrial malfunction indexes. During reoxygenation, mitochondria encounter harsh environmental changes with mitochondrial  $Ca^{2+}$  accumulation and overload [25].  $Ca^{2+}$  influx from cytosolic to mitochondria during reoxygenation is dependent on the MCU [26]. In this study, the effects of Hes on mitochondrial and cytosolic  $Ca^{2+}$  levels of H9C2 cells under different conditions were

(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  $Ca^{2+}$  homeostasis dysregulation [4]. Thus, the present results suggested that Hes exerts antioxidant properties in limiting mitochondrial oxidative stress against HR injury.

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  $Ca^{2+}$  levels. The increase in  $Ca^{2+}$  was more pronounced in the HR group without any treatment. HR-induced  $Ca^{2+}$  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  $\text{Ca}^{2+}$  in H9C2 cells (Figure 3A,  $p > 0.05$ ). The Fluo-4 AM fluorescent intensities (ratio to control) in HR and Hes were  $1.96 \pm 0.50$  and  $1.94 \pm 0.05$ , respectively. In contrast to cytosolic  $\text{Ca}^{2+}$ , mitochondrial  $\text{Ca}^{2+}$  level was markedly decreased in Hes-treated cell group. Additionally, RuR group had a lower mitochondrial  $\text{Ca}^{2+}$  content compared with the Hes group. Although the influx of  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  level in Hes-treated group (Figure 3A). Previous study had shown that the high mitochondrial  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  overload (Figure 3A), subsequently attenuating cardiac cell death as proved in a recent report.

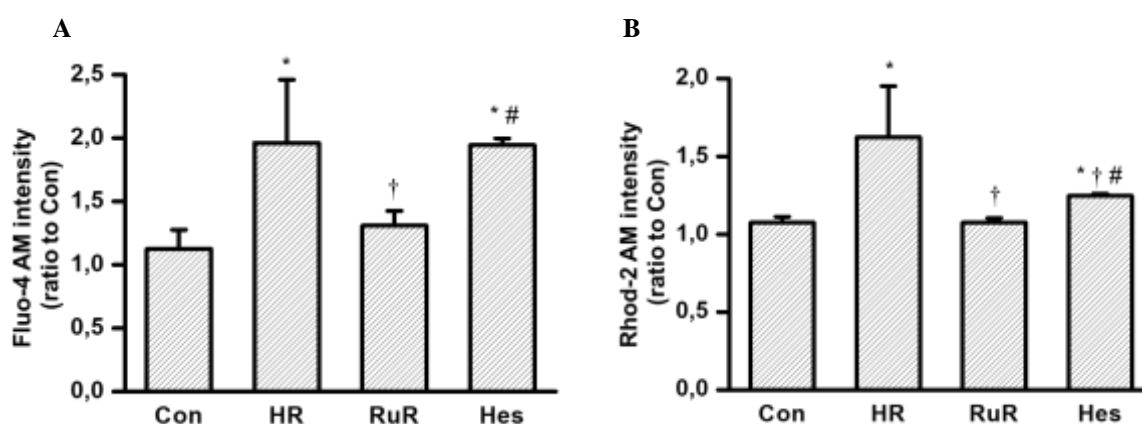


Figure 3. Cytosolic and mitochondrial  $\text{Ca}^{2+}$  levels in H9C2 under different conditions.

A: The Fluo-4 Am intensity in different conditioned-H9C2 cells; B: the Rhod-2 Am intensity in different conditioned-H9C2 cells. Con: H9C2 cells were cultured in normal condition (normoxia); 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;  $n = 4-6$ .

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  $\text{Ca}^{2+}$  homeostasis regulation was weaker than RuR (Figure 3). Hes has the ability to protect H9C2 rat cardiomyocytes through targeting mitochondrial oxidative stress and mitochondrial  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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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