ARALOSIDE A METHYL ESTER FROM PANAX BIPINNATIFIDUS SEEM PROTECTS H9C2 CARDIOMYOCYTES AGAINST CHEMICAL HYPOXIA/REOXYGENATION-INDUCED INJURY BY PRESERVING MITOCHONDRIAL FUNCTION

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ABSTRACT

Panax bipinnatifidus Seem (PB) is a precious herb used in many traditional remedies in Vietnam. Previous studies have proven that the saponin-rich extract of this herb has a protective effect on cardiomyocytes in hypoxia-reoxygenation injury (HR). However, which compound in PB plays a major role in this effect has not been elucidated. Therefore, this study was conducted to evaluate the effects of saponin extracted from PB extract, Araloside A Methyl Ester (AAME) on H9C2 cardiomyocytes in HR injury. Cells were grown in normal conditions (control) and HR-$CoCl_2$ conditions, and AAME was added to the culture medium during reoxygenation. Cell viability and some mitochondrial indices (cardiolipin content, mitochondrial membrane potential, ROS) were determined by suitable kits. The results showed that AAME at concentrations of 10 μM significantly reduced the cell death rate by limiting mitochondrial dysfunction (preserving cardiolipin content and mitochondrial membrane potential, reducing mitochondrial ROS) of H9C2 cells in HR-$CoCl_2$ injury ($p<0,05$). This result demonstrated the potential of AAME to protect cardiomyocytes from HR injury.

Keywords: *Araloside A Methyl Ester, mitochondria, H9C2.*

1. INTRODUCTION

Hypoxia-reoxygenation (HR) injury is damage caused by reperfusion /reoxygenation to tissue or cells that were ischemic/hypoxic previously. HR is a key mechanism responsible for cellular and tissue harm in various pathological scenarios, notably encompassing ischemic heart disease (IHD) [3].

As a powerhouse, mitochondria have been shown to play an important role in the mechanism of this disease [4]. Limiting mitochondrial dysfunction to minimize HR-induced injury has been the target of many studies [7]. In recent decades, research on screening and searching for natural active ingredients with cardioprotective effects has achieved many achievements, but clinical applications are still limited. Therefore, this research direction still needs to be further expanded.

Ginseng is a precious medicinal herb widely used in traditional medicine in many countries and Viet Nam. For ischemic diseases, the efficacy of ginseng and its ginsenosides on stroke outcomes with the anti-oxidant property has been described [6]. Also, ginseng extracts have been shown to possess beneficial effects in hypoxia/reoxygenation injury [1, 14]. In which, Panax bipinnatifidus Seem. has anti-platelet activity with its major saponin component, araloside A methyl ester [10, 11]. In our previous study, the total saponin extract of Panax bipinnatifidus Seem. in hab the ability to protect H9C2 cells in HR injury by modulating mitochondrial function during the hypoxia phase. However, which compound in PB plays a major role in this effect has not been elucidated. Therefore, this study was conducted to evaluate the effects of saponin extracted from PB extract, Araloside A Methyl Ester (AAME) on H9C2 cardiomyocytes in HR injury.

2. METHODOLOGY

2.1. Samples

The H9C2 cell line (ATCC©-USA) was provided by the Cardiovascular and Metabolic Disease Center, Inje University, Korea. AAME was provided by Phenikaa University. The research was conducted at the Animal cell biotechnology laboratory, Life science research center, Faculty of Biology, VNU University of Science.

2.2. Materials

Dulbecco's Modified Eagle Medium 4,5g/l glucose (DMEM, Gibco, USA); Phosphate buffered saline (PBS, Gibco, USA); Fetal bovine serum (FBS, Gibco, USA); Cell Counting Kit-8 (CCK-8, Dojindo, Japan); 2′,7′-dichlorodihydrofluorescein-diacetate (CM-H_2DCFDA; ex/em 485/525 nm, Invitrogen, USA); MitoSox Red (ex/em: 510/580 nm, Invitrogen, USA); Penicillin-Streptomycin (PS, Gibco, USA); Dimethyl Sulfoxide (DMSO, Sigma, USA); Cobalt chloride (CoCl2, Sigma, USA); Inverted microscopy Axiovert (S100, Carl Zeiss, Germany); Culture dishes 90x20, 60x15, 35x15 mm (SPL, Korea); 96-well black, glass bottom plates (CAT. 33196, SPL), confocal dishes (CAT. 100350, SPL); CO2 Incubator (Shellab, USA); ApoTome.2 Fluorescence Microscope (Zeiss, Germany); Microreader plate (Tristar2, USA).

2.3. Cell Culture

H9C2 cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS and 100 U/mL penicillin, 100 µg/mL streptomycin at 37 °C, 5% CO_2. The culture medium was changed every 2-3 days.

2.4. Inducing hypoxia-reoxygenation by $CoCl_2$ and treatment

H9C2 cells were grown in a 96-well plate at a density of 5.10^3 cells/well at 37°C, 5% CO_2 for 24 h. Then, the cells were divided into 2 groups: In the normal control group, the H9C2 cells were continuously cultured in normal media (DMEM, 10% FBS, 1% PS, 37°C, and 5% CO_2) for 48 h. In the HR-$CoCl_2$ group, the cells were subjected to $CoCl_2$ 300 µM for 24 h. Then, the medium containing $CoCl_2$ was removed, and the cells were continued to be grown for 18-24 h in normal media containing NEC 10 µM or AAME at the range of concentrations (1-100 µM). At the end of the experiment, the values of cell viability, mitochondrial membrane potential, cardiolipin content, and mitochondrial stress were determined indirectly through the analysis of CCK-8, fluorescent indicators. Experiments were performed in triplicate.

2.5. Measurement of cell viability

After treatments, cell viability was assessed by using CCK-8 (Dojindo) as previously described [13]. For each group, H9C2 cells were further incubated for 1 h with CCK-8. The absorbance value indicating cell viability was measured at 450 nm using a microplate reader. The number of alive cells in each well was expressed as a value relative to the normal control. Experiments were repeated in triplicates.

2.6. Measurement of mitochondrial cardiolipin and mitochondrial membrane potential

H9C2 cells were seeded at a density of 5.10^3 cells/well in 96-well black, glass bottom plates or 3.10^5 cells/well in a 60 mm dish and subjected to $CoCl_2$ treatments. After treatment, the cells were stained with either NAO 0.1 µM (ex/em: 495/519 nm, Invitrogen, USA) or TMRE 0.1 µM (ex/em: 535/570 nm) for 30 min at room temperature [13]. The cells were washed twice with PBS before measuring fluorescence intensity using a microplate reader or flow cytometry. The NAO or TMRE intensity in each well was expressed as a percentage value relative to the normal control. NAO or TMRE-stained cells were captured using an Axio observer

combined with the ApoTome.2. Experiments were repeated 3 times.

2.7. Measurement of oxidative stress

After being treated with different conditions, H9C2 cells were stained with either 5 µM CM-H2DCFDA (ex/em: 485/525 nm) or 5 µM MitoSox Red (ex/em: 510/580 nm) at 37°C for 30 min at room temperature to detect changes in H_2O_2 or O_2^- levels [12]. After washing twice with PBS 1X, the different fluorescence intensities of the dyes were measured using the microplate reader or flow cytometry. The total intensity in each well was expressed as a percentage value relative to the normal control. CM-H_2DCFDA and MitoSox Red-stained cells were captured using an Axio observer combined with the ApoTome.2. Experiments were performed in triplicate.

2.8. Statistical Analysis

Data are presented as means ± standard error (SD) by using Excel 2016, Origin 8.5 software. Differences between the two groups were evaluated by ANOVA and Tukey test; a p-value ≤ 0,05 was considered significant.

3. RESULTS AND DISCUSSION

3.1. The cytotoxicity of AAME on H9C2 cells in normal condition

The effect of AAME on the viability of H9C2 cells under normal conditions was assessed by the CCK-8 kit. The results are shown in Figure 1.

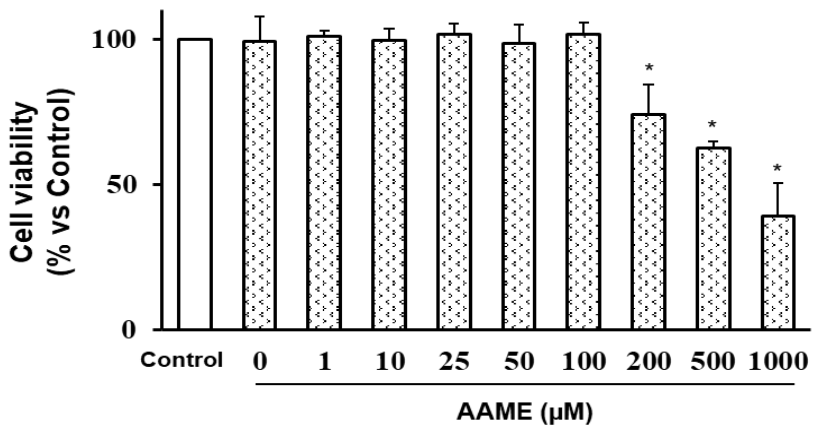

Figure 1. The viability of H9C2 cells in normal condition

**p<0,05 vs control, n=3.*

The results in Figure 1 show that, the group of cells incubated with AAME at concentrations from 0 to 100 µM had no difference compared with the control group (p>0.05). The cell viability started to decrease in the AAME 200 µM group with the cell survival rate of 81.35±7.06% compared with the control group (p<0.05). When the concentration of AAME increased to 200, 500, and 1000 µM, the percentage of viable cells decreased sharply to 62.71±2.28%, 39.25±11.15%, and 13.64±2.07, respectively, (p<0.05 compared with the control). Thus, AAME at concentrations of ≤ 100 µM did not show cytotoxicity to H9C2 cells and was selected to be used for further analyses.

3.2. AAME enhances the cell viability in HR-$CoCl_2$ condition

The effect of AAME on the viability of H9C2 cells under HR-$CoCl_2$ conditions was shown in Figure 2.

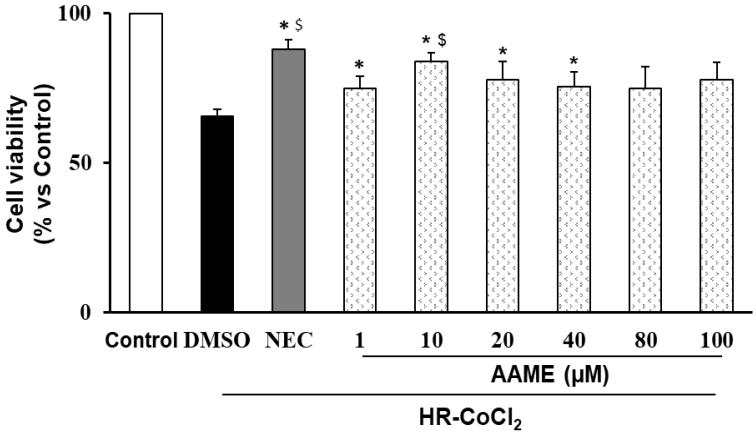

Figure 2. The viability of H9C2 cells in HR-$CoCl_2$ condition

**p<0,05 vs HR, $p<0,05 vs HR-$CoCl_2$+AAME 1 μM; n=3.*

The results shown in Figure 2 showed that the HR-$CoCl_2$+DMSO group had a sharp decrease in the percentage of viable cells to 65.68±2.35% (compared to 100% of the control, p<0.05). Compared with the HR-$CoCl_2$+DMSO group, this ratio increased significantly in the groups of cells supplemented with NEC or AAME (at concentrations of 1, 10, 20, and 40 μM, p < 0.05). In which, the percentage of viable cells was highest in the group supplemented with AAME at the concentration of 10 μM. On this basis, a concentration of 10 μM of AAME was selected for further studies.

3.3. AAME limited mitochondrial dysfunction in HR-$CoCl_2$ condition

3.3.1. AAME preserved mitochondrial cardiolipin content and membrane potential

In this study, the change in mitochondrial inner membrane cardiolipin (CL) content of H9C2 cells was determined indirectly through the blue fluorescence signal of NAO.

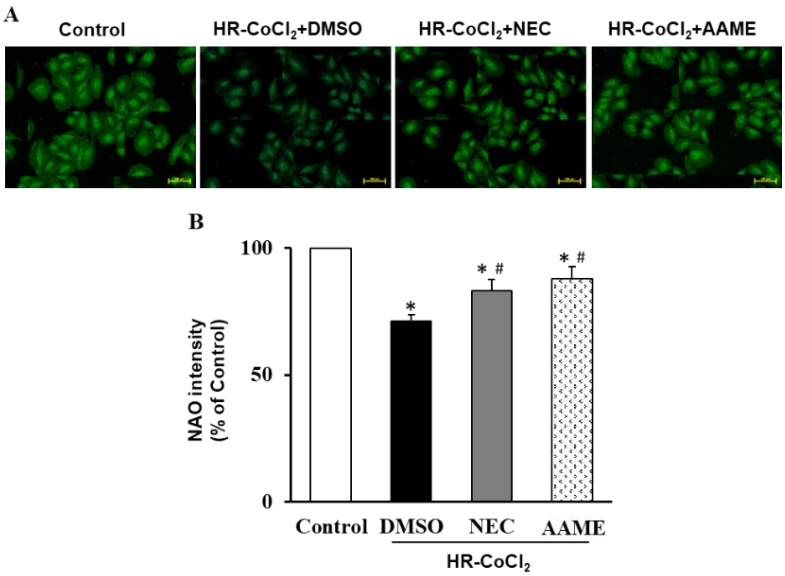

Figure 3. The NAO intensity in different conditioned-H9C2 cells

A. NAO fluorescence images were taken by the Axio observer system combined with Apo Tome.2; B. The graph of NAO fluorescence intensity values (%) was analyzed by a

*fluorescence microplate reader; Objective lens: 20X, Scale bar: 100 µm; *p<0,05 vs Control, #p<0,05 vs HR-$CoCl_2$+DMSO; n=3.*

The results in Figure 3 show that NAO fluorescence density in the HR-$CoCl_2$+DMSO group had a strongly reduced ratio of NAO fluorescence density and only reached about 71.06±2.80% (compared to 100% of control, p<0.05). This percentage in NEC-supplemented and AAME cell groups increased significantly with values of 83.25±4.36%, and 87.93±4.62%, respectively, (p<0.05 compared with HR-$CoCl_2$+DMSO group, Figure 3). This suggests that like NEC, AAME has the ability to preserve CL content, limit mitochondrial structural abnormalities, and stabilize electron transport chain activity [8], thereby limiting the rate of cell death under hR conditions.

Besides, to investigate the effect of AAME on mitochondrial function, the mitochondrial membrane potential was also determined indirectly through TMRE fluorescence kit. The results are shown in Figure 4.

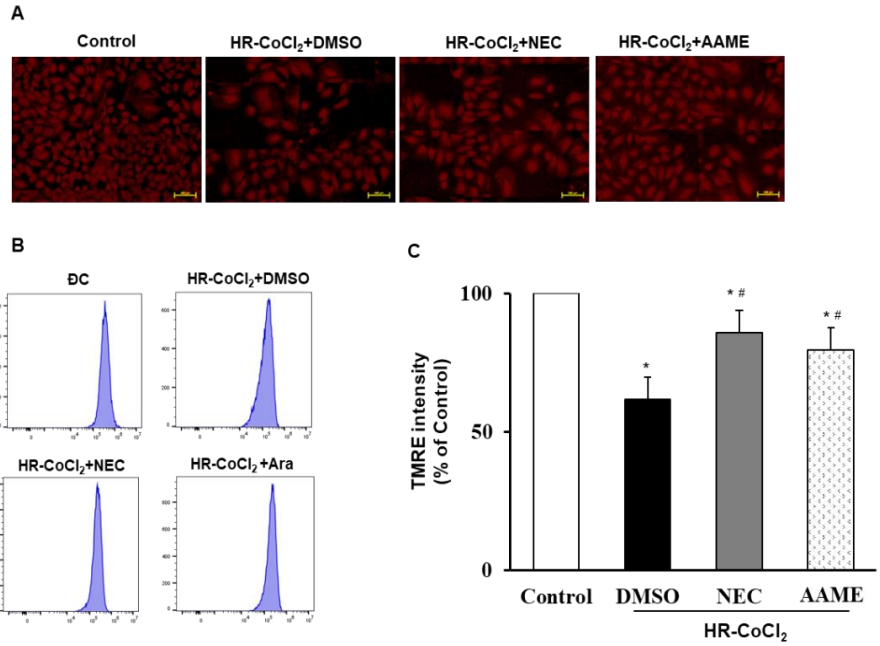

Figure 4. The TMRE intensity in different conditioned-H9C2 cells

*A. TMRE fluorescence images were taken by the Axio observer system combined with Apo Tome.2; B, C. Histograms, and the graph of TMRE fluorescence intensity (%) values analyzed by flow cytometer; Objective lens: 20X, Scale bar: 100 µm; *p<0,05 vs control; #p<0,05 vs HR-$CoCl_2$+DMSO; n=3.*

The results in Figure 4 show the HR-$CoCl_2$+DMSO group had the TMRE fluorescence density ratio reduced to 61.77±3.49% (compared to 100% of the control, Figure 4). This percentage in the group of cells subjected to the disease model supplemented with NEC, and AAME during the reoxygenation phase increased significantly, with values of 85.99±5.49%, and 79.63±7.17% respectively (p<0.05 compared with HR-$CoCl_2$+DMSO group). This result is consistent with the results of CL content and the rate of viable cells above. In particular, the preservation of CL content contributed to stabilizing membrane potential, limiting mitochondrial dysfunction, and increasing cell viability

under HR conditions. The effect of AAME on mitochondrial cadiolipin and membrane potential is quite similar to the effect of Panax bipinnatifidus Seem [14] extract and saponin MR2 extracted from Panax Vietnamese (Ngoc Linh) ginseng in our previous studies [13]. This suggests that AAME may protect H9C2 cell mitochondria in HR injury induced by $CoCl_2$. And to further explore the role of this active ingredient, the effect of AAME on the ros amount of cells in the disease model was evaluated.

3.3.2. AAME inhibited mitochondrial stress

In HR pathological conditions, mitochondria are both the major source and target of ROS [9]. Mitochondrial oxidative stress is expressed in the excessive proliferation of H_2O_2 and O_2^-. In this study, the H_2O_2 and O_2^- contents were assessed indirectly through the change in the blue fluorescence signal of CM-H_2DCFDA and the red color of MitoSOX Red. The results are shown in Figures 5 and 6.

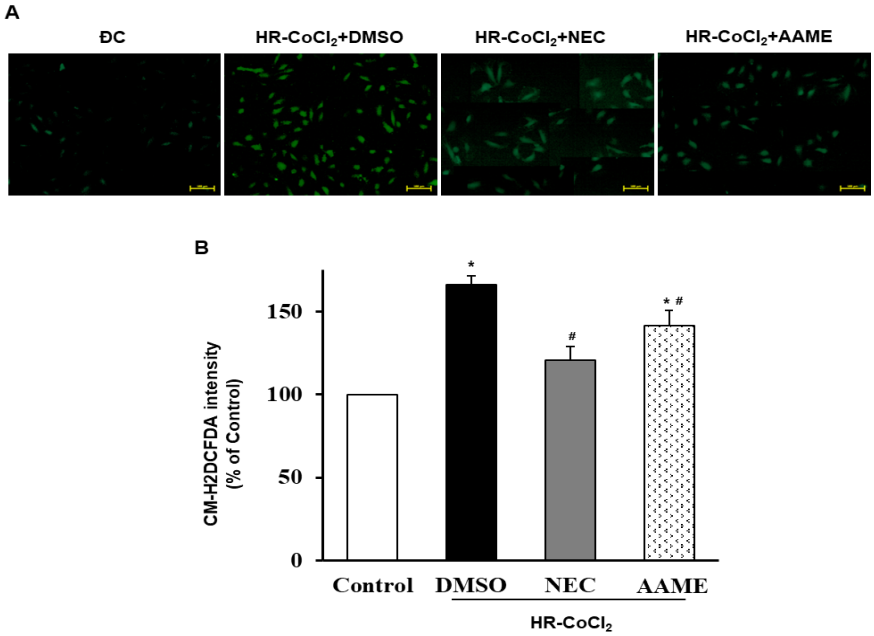

Figure 5. The CM-H_2DCFDA intensity in different conditioned-H9C2 cells

*A. CM-H_2DCFDA fluorescence images were taken by the Axio observer system combined with Apo Tome.2; B. The graph of CM-H_2DCFDA fluorescence intensity values analyzed by a fluorescence microplate reader; Objective lens: 20X, scale bar: 100 µm; *p<0,05 vs control, #p<0,05 vs HR-$CoCl_2$+DMSO; n=3.*

Figure 5A shows that HR-$CoCl_2$+DMSO cell groups have a significant increase in CM-H_2DCFDA fluorescence signal compared to the control group, shown in bright blue color and a greater number of cells carrying green color. The group of cells supplemented with AAME at the dose of 10 µM at the time of reoxygenation had a decrease in CM-H_2DCFDA fluorescence signal (greener green, fewer green-bearing cells) compared to those in HR-$CoCl_2$+DMSO cell group. Corresponding to the fluorescence image, an increase in CM-H_2DCFDA fluorescence density is clearly shown in Figure 5B. In which, HR condition highly increased the CM-H_2DCFDA fluorescence density of H9C2 cells to 166,03±5,49% (HR-$CoCl_2$+DMSO group) compared to 100% of the control group (p<0,05). This result showed that $CoCl_2$ 300 µM causes

overproduction of H_2O_2, thereby causing cell damage and death, which was consistent with a previous report [15]. However, supplementation of AAME 10 µM to the reoxygenation phase, the CM-H_2DCFDA fluorescence density of the AAME cell group was strongly decreased to 141,65±8,82% compared to the control, which was lower by about 20% than the HR group ($p<0.05$).

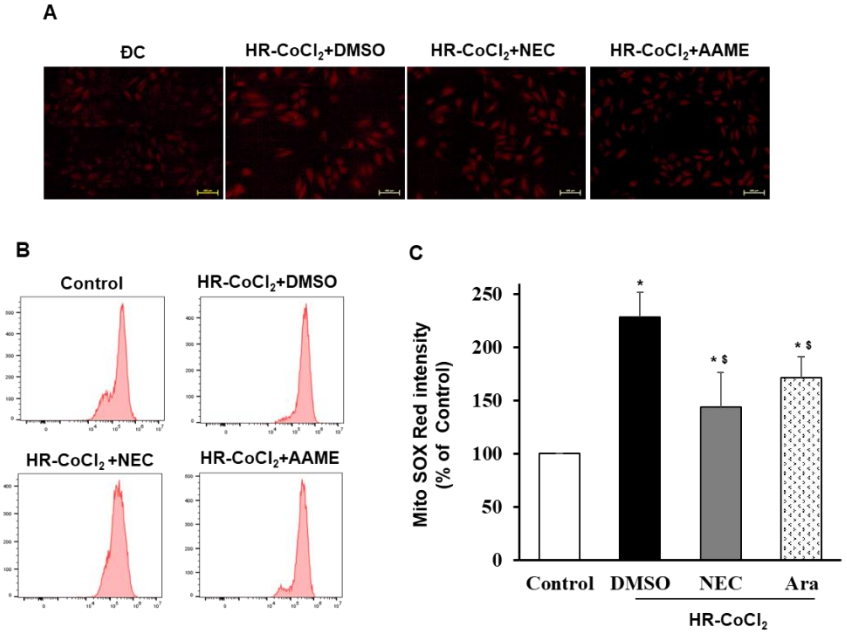

Figure 6. The MitoSOX Red intensity in different conditioned-H9C2 cells

*A. MitoSOX Red fluorescence images were taken by the Axio observer system combined with Apo Tome.2; B, C. Histograms, and the graph of MitoSOX Red fluorescence intensity (%) values analyzed by flow cytometer; Objective lens: 20X, Scale bar: 100 µm; *p<0,05 vs control, $p<0,05 vs HR-$CoCl_2$+DMSO, n=3.*

As mentioned above, the superoxide radical is a byproduct of a deficient transferring the electrons from mitochondrial respiratory chain complexes I–IV to oxygen. In fact, complexes I and III are reported as the central sites to form mitochondrial superoxide radicals [5]. Superoxide is unstable and dismutation of O_2^- to H_2O_2 can be occured spontaneously or through a reaction catalyzed by superoxide dismutase [2]. Thus, to find out the relationship between the above alterations in H_2O_2 level with O_2^-, we determined the change in O_2^- level. The results in Figures 6 A, B, and C show that MitoSOX Red fluorescence signal and density of the cells in the HR-$CoCl_2$+DMSO group increased to 228.55±23.55% compared with the control ($p<0.05$). The addition of NEC or AAME 10 µM during the reoxygenation phase reduced this ratio to 144.25±31.98% or 171.47±20.18%, respectively. Compared with HR-$CoCl_2$+DMSO, the value of MitoSOX Red fluorescence density in the remaining model groups were significantly different with $p<0.05$.

4. CONCLUSION

Taken together, the present study documents the ability of AAME in protecting cardiomyocytes in $CoCl_2$-induced HR injury *in vitro* via preserving mitochondrial function.

ACKNOWLEDGMENTS

This work was supported by QG.22.03. We thank student Vu Viet

Tung, Assoc. Prof. Nguyen Huu Tung, Professor Han Jin for help and support.

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