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Evaluating the protective effects of *Panax bipinnatifidus* Seem. extracts on hypoxia/reoxygenation-subjected cardiomyocytes

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Abstract:

The search for new products to treat ischemic heart diseases has interested scientists for years as it is the main cause of cardiovascular disease mortality. In this study, we evaluated the protective effects of *Panax bipinnatifidus* Seem. extracts (PSE) on hypoxia/reoxygenation (HR)-treated rat H9C2 cardiomyocytes. Rat H9C2 cardiomyocytes were either grown under normal condition or subjected to HR conditions. NecroX-5 (10 µM) was used as positive control for cardiac protective effect. The PSE (5-500 µg/ml) was employed to culture media at the onset of reoxygenation. Cell viability and mitochondrial function were tested using a Cell Counting Kit-8 and suitable fluorescence kits. The obtained data shows that HR conditions dramatically induced H9C2 cell death ($p<0.05$). Treatment of PSE (5-62.5 µg/ml) effectively reduced HR injuries in a dose-dependent manner. Similar to the NecroX-5 group, ginseng extract-treated cells had higher viability levels compared to that of the HR-subjected cells ($p<0.05$). In addition, supplementing PSE at the onset of reoxygenation strongly prevented the collapse of mitochondrial membrane potential and reduced mitochondrial oxidative stress ($p<0.05$). This study suggested the total saponin extracts from *Panax bipinnatifidus* Seem. exerts cardioprotective properties against HR damage.

Keywords: **hypoxia/reoxygenation, H9C2, NecroX-5, oxidative stress.**

Classification numbers: **3.3, 3.5**

Introduction

Myocardial infarction is the main cause of death from cardiovascular disease [1]. The timing of revascularization in combination with anti-oxidants and anti-platelet agents are decisive factors for an effective treatment of patients [2]. The search for natural products to develop new anti-ischemic drugs has been of intense interest of scientists due to ischemic heart disease being the main cause of cardiovascular disease mortality [3, 4]. Experimental data have shown that drugs used in the treatment of myocardial infarction have mechanisms related to mitochondrial preservation [5-8].

Ginseng is a precious medicinal herb that is widely used in traditional medicine and pharmaceutical industries in many countries. Many naturally growing ginseng species such as Vietnamese Ginseng, *Panax bipinnatifidus* Seem., Radix Angelicae Sinensis, and Salvia miltiorrhiza Bunge have been recognized and widely used in traditional remedies for anti-fibrotic treatment, anti-infarction, increasing nerve function, reducing the risk of cancer, and maintaining a healthy immune system or blood sugar stability [9-11]. For ischemic diseases, the efficacy of the antioxidant property of ginseng and its ginsenosides on stroke outcomes has been described [12-14]. Also, ginseng extracts have been shown to possess beneficial effects in the treatment of myocardial infarction [15-17]. Of these extracts, *Panax bipinnatifidus* Seem. has anti-platelet activity with its major saponin components like stipuleanoside R2 and araloside A methyl ester [10, 18, 19]. However, experimental analysis of the role of total saponin extract of *Panax bipinnatifidus* Seem. in modulating cardiomyocytes and mitochondrial function during the revascularization phase is still lacking. Therefore, this study was carried out to evaluate the potential role of *Panax bipinnatifidus* Seem. extract in an ischemic heart model using H9C2 cardiomyocytes.

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Materials and methods

Samples

H9C2 cardiomyocytes (ATCC©-USA) were provided by the Cardiovascular and Metabolic Disease Centre, Inje University and *Panax bipinnatifidus* Seem. extract [10, 18] was provided by Phenikaa University. The study was performed at Life Science Research Centre, Faculty of Biology, University of Science, Vietnam National University, Hanoi.

Materials

Dulbecco's Modified Eagle Medium 4.5 g/l glucose (DMEM, Gibco, USA); Penicillin-Streptomycin (PS, Gibco, USA); Fetal Bovine Serum (FBS, Gibco, USA); Phosphate Buffered Saline (PBS, Gibco, USA); Cobalt Chloride ($CoCl_2$, Sigma, USA); Dimethyl Sulfoxide (DMSO, Sigma, USA); Cell Counting Kit-8 (CCK-8, Dojindo, Japan); 2′,7′-Dichlorodihydrofluorescein-diacetate (CM-H_2DCFDA, Invitrogen, USA); Tetramethyl Rhodamine Ethyl Ester (TMRE, Invitrogen, USA); Inverted microscopy Axiovert (S100, Carl Zeiss, Germany); Culture dishes, 96-well black, glass bottom plates, CO_2 incubator (Shellab, USA); Microreader plate (Tristar, USA).

Methods

Cell culture, hypoxia-reoxygenation (HR) in vitro model and treatments:

H9C2 cells were maintained in DMEM supplemented with 100 µg/ml of PS and 10% FBS at 37°C and 5% CO_2. The cells were grown in a 96-well black plate with glass bottom at a density of 5×10^3 cells/well. Cells were cultured under normal conditions and supplied with *Panax bipinnatifidus* Seem. extract (0-1000 µg/ml) to evaluate the toxicity as well as to determine the optimal dose of PSE. For a $CoCl_2$-stimulated HR model, after 24 h, the cells were subjected to $CoCl_2$ (300 µM) at 37°C and 5% CO_2 for 24 h (simulating the hypoxia stage) as described in the previous publication [20]. Then, the medium containing $CoCl_2$ was removed and the cells were continuously grown for 24 h in new media containing either DMSO (HR, HR-$CoCl_2$+DMSO) or DMSO plus PSE (PSE, HR-$CoCl_2$+DMSO+ PSE). Cells were cultured under normal conditions (DMEM, 10% FBS, 1% PS, 37°C and 5% CO_2) for 54 h without any treatment, which served as a control group. The optimal dose of PSE was chosen to assess its cardioprotective effects against HR via cell viability and mitochondrial indexes. Experiments were performed at least in triplicate.

The HR model was also created by alternating the O_2 levels of the culture conditions. H9C2 cells were cultured in normal media at 37°C with 5% CO_2 for 18 h. For hypoxic cultures, the cells were then cultured in serum-free low-glucose DMEM at 37°C, 95% N_2, 5% CO_2, and 2% O_2. For reoxygenated culture, cells were grown under normal conditions following the hypoxic condition. After incubating under hypoxic condition for 6 h, H9C2 cells were then transferred to reoxygenation for 24 h. At the time of reoxygenation, H9C2 cells were separately treated with either PSE or NecroX-5 (10 µM, serving as positive drug control). The stocks of NecroX-5 and PSE were prepared in DMSO. The final concentration of DMSO in cultured medium was 0.1%. The difference was expressed as a percentage value relative to the HR group.

Cell viability assay:

Cell viability was assessed by using a CCK-8 kit as described in the last study [21]. H9C2 cells were seeded in triplicate into 96-well culture plates at a density of 5×10^3 cells per well. At the end of the experiment, the cell groups were further incubated with CCK-8 solution for 1-4 h. The absorbance value, indicating cell viability, was measured at 450 nm using the microplate reader. The number of alive cells was expressed as a value relative to the normal control or HR. The cell images were also captured by using an Axiovert inverted microscope. Experiments were repeated at least 3 times.

Measurement of mitochondrial membrane potential:

At the end of experiment, the cell groups were stained with 0.1 µM TMRE (ex/em: 535/570 nm) for 30 min at room temperature. After washing twice with PBS, fluorescence intensities were measured using the microplate reader. The TMRE intensity was expressed as a percentage value relative to the HR. Experiments were performed in triplicate.

Measurement of hydroperoxide production:

At the end of experiment, the cell groups were stained with 5 µM CM-H_2DCFDA (ex/em: 485/525 nm) at 37°C for 30 min to detect changes in hydroperoxide

(H_2O_2) levels. After washing, the fluorescence intensity was measured using the microplate reader. The total fluorescence intensity in each well was expressed as a percentage value relative to the control or HR or NecroX-5. Experiments were repeated in triplicate.

Statistical analysis:

Data are presented as mean ± standard error of the mean (SEM) using Origin 8.5. Differences between the two groups were evaluated by one-way analysis of variance (ANOVA) and Tukey's test. Differences with a $p \leq 0.05$ were considered significant.

Results and discussion

PSE reduced H9C2 cell death under $CoCl_2$-stimulated HR injury in a dose-dependent manner

H9C2 cells were cultured in DMEM supplemented with 10% FBS, 100 µg/ml of PS, and 5% CO_2 at 37°C for 24 h and were then treated with PSE (0-1000 µg/ml) for the next 48 h. The cell viability of PSE was determined using the CCK-8 kit as described in the previous study [21]. The 48-h EC_{50} of PSE was determined to be 199.41 (µg/ml) using Origin 8.5 (Fig 1). The results indicate that PSE showed less toxicity to the H9C2 cells under normal conditions.

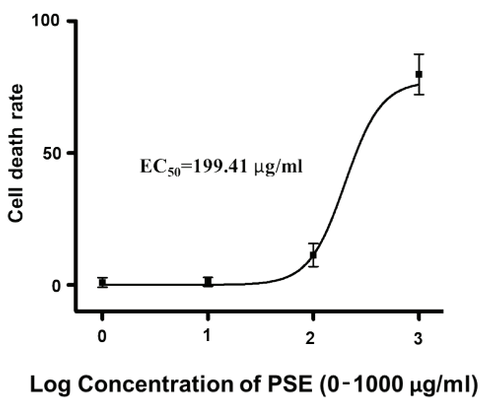

Fig. 1. EC_{50} value of *Panax bipinnatifidus* Seem. extract.

Consistent with the previous study [20], the results showed that viabilities were dramatically reduced in the $CoCl_2$-exposed H9C2 cells compared to normal cells (Fig. 2, $p<0.05$). PSE at doses of 5, 10, 31.25, and 62.5 µg/ml strongly prevented cell death from HR. In contrast, PSE at doses of 125, 250, and 500 µg/ml showed toxicity to cardiomyocytes under HR conditions.

Interestingly, among the $CoCl_2$-exposed groups, the high percentages of H9C2 viability were presented in PSE treatments at doses of 31.25 and 62.5 µg/ml. Treatment of PSE at doses of 31.25 µg/ml and 62.5 µg/ml dramatically increased the cell viability up to 81.63±2.01% and 81.88±0.96%, respectively (where 100% is for the normal control). Though PSE showed less toxicity to H9C2 cells under normal conditions (Fig. 1), the viability of the PSE-treated groups with the higher doses (125, 250, and 500 µg/ml) was lower than that of the $CoCl_2$-stimulated HR. The results indicated that PSE exerted cardioprotective effects on cardiomyocytes under $CoCl_2$-stimulated HR were in a dose-dependent manner. Cell morphology and cell count are shown in Fig. 2C, which portrays less alive cells with PSE doses at 125, 250, and 500 µg/ml. It was reported that PSE has anti-platelet activity with its major saponin component, araloside A methyl ester [10, 18, 19]. Another research had screened the bioactive role of crude PSE and reported its weak inhibitory effect on markedly nitric oxide production in lipopolysaccharide-treated RAW 264.7 cells [22]. The current results further document the bioactivity of PSE with different effects on cell viability that depend on HR treatments. These findings showed that the effective doses of PSE against HR damage were about 31.25 and 62.5 µg/ml. In this study, a PSE post-hypoxic treatment at a dose of 31.25 µg/ml was chosen for further evaluation.

PSE protected cardiomyocytes via preserving mitochondria function against HR damage

The data showed the cardioprotective effects of PSE against HR injury via elevating cell viability (Fig. 3B), enhancing mitochondrial membrane potential ($\Delta\Psi m$), and reducing oxidative stress levels (Fig. 4). The experiment designed for HR model is depicted in Fig. 3A. In this model, NecroX-5, the positive control, showed its efficacy in protecting the cells against HR as mentioned in the previous study [7]. As described, H9C2 cells were subjected to different conditions and the cell survival rates were measured using the CCK-8 kit (Fig. 3). Post-hypoxic treatment of either NecroX-5 or PSE significantly increased the cell viability compared to HR condition ($p<0.05$). Among HR-exposed groups, the highest H9C2 viability was shown in the PSE-treated

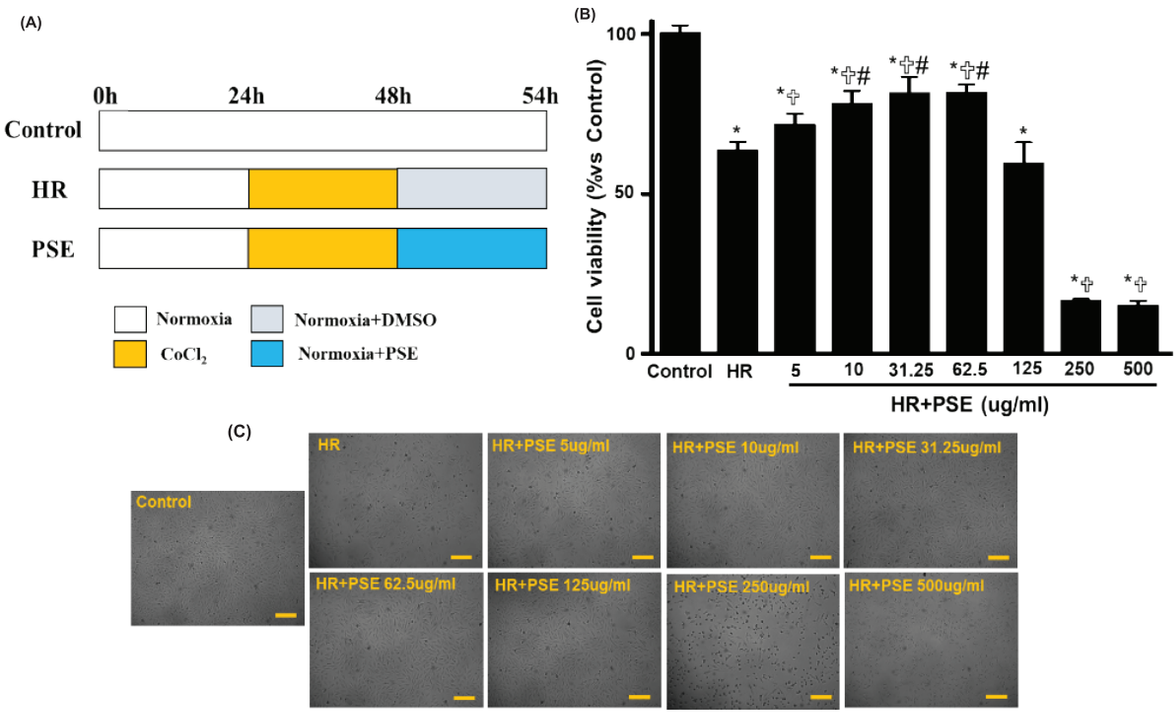

Fig. 2. **Experimental design and cell viability assay**. **(A)** $CoCl_2$-stimulated HR model and *Panax bipinnatifidus* Seem. extract treatment. **(B)** The graph indicates H9C2 cell viability under different conditions and treatments. **(C)** H9C2 cell images captured under different conditions and treatments. HR: $CoCl_2$-stimulated condition; PSE: HR+PSE; *p<0.05 vs. Control; +p<0.05 vs. HR; #p<0.05 vs. PSE at dose of 5 µg/ml; scale bar=100 µm; 10x of magnification; n=3-4 for each group.

cells (231.34±12.38, % vs. HR). Though the cell viability of the PSE-treated group (at dose of 31.25 µg/ml) was higher than another PSE-treated group (i.e., at dose of 62.5 µg/ml, 213.14±10.98, % vs. HR), there was no significant difference between these two groups. Moreover, the use of DMSO (0.1%) showed no significant effect on the life of H9C2 cells under HR conditions as mentioned in a previous study [23]. These results further confirmed the effective role of PSE in protecting H9C2 cardiomyocytes against HR damage (Fig. 2) and the protective effects of the other products of ginseng [16]. The data suggests that different protective levels could be dependent on the concentration and components of the tested extracts.

In this study, TMRE was used to evaluate the role of PSE on the mitochondrial function according to the manufacturer's instructions. The values of TMRE intensity in HR, DMSO, NecroX-5 and PSE groups were 61.24±0.05%, 61.23±0.13%, 81.30±1.37%, and 69.10±0.26%, respectively (100% of normal control, Fig. 4A). Thus, consistent with a previous report [16], PSE significantly protected mitochondrial function against HR. Post-hypoxic treatment of NecroX-5 and PSE significantly increased the ΔΨm compared to non-treated HR condition (Fig. 4A, p<0.05). There was no significant difference in TMRE intensity between NecroX-5 and PSE groups (p>0.05). Thus, the results indicate that intervention methods could be varied by changing ginseng species and/or saponin components. Moreover, the loss of ΔΨm during HR was prevented by pre-treating

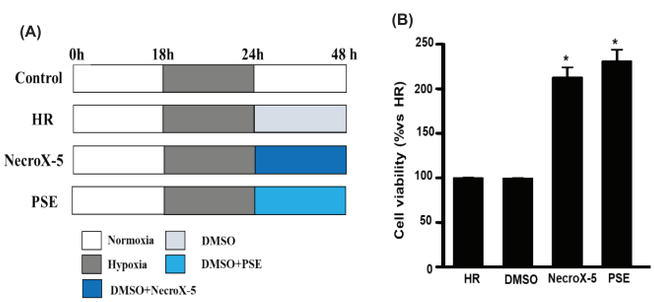

Fig 3. Experimental design and the H9C2 cell viability under conditions. **(A)** Hypoxia/reoxygenation (HR) model and treatments. **(B)** The graph indicates H9C2 cell viability under different treatments. NecroX-5: HR+NecroX-5 (10 µM); PSE: HR+PSE (*Panax bipinnatifidus* Seem. extract, 31.25 µg/ml); *p<0.05 vs. HR; n=3 for each group.

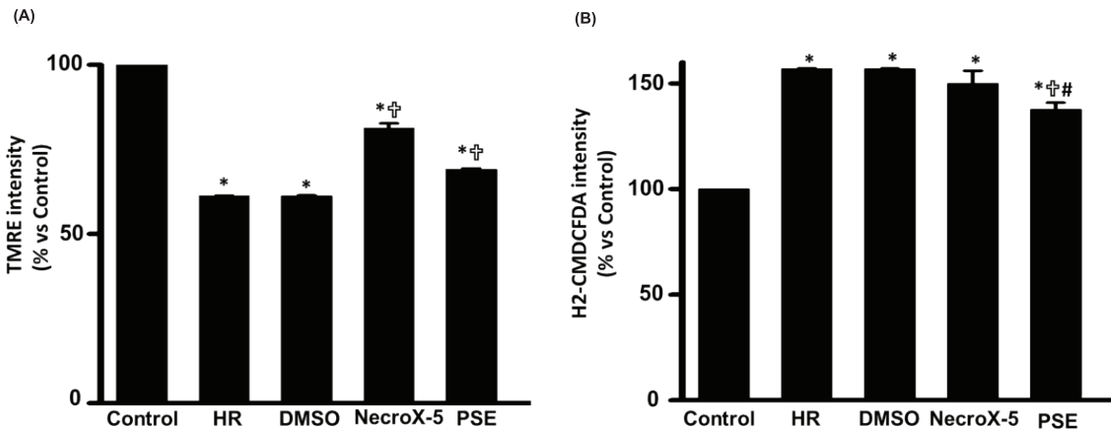

Fig. 4. Mitochondrial function. The graphs present mitochondrial alterations in H9C2 cells subjected to different treatments. **(A)** Mitochondrial membrane potential (TMRE intensity) and **(B)** H_2O_2 production (CM-H_2DCFDA intensity). HR: hypoxia/reoxygenation; NecroX-5: HR + NecroX-5 (10 µM); PSE: HR+PSE (31.25 µg/ml); *p<0.05 vs. Control; †p<0.05 vs. HR; #p<0.05 vs. NecroX-5; n=3 for each group.

with the triterpenoid saponin clematichinenoside [24] or post-hypoxic treatment of majonoside-R2 [16].

Besides, the ability of PSE to preserve mitochondrial function can be further demonstrated by H_2O_2 production. Indeed, the H_2O_2 levels in H9C2 determined by using CM-H_2DCFDA were minimized in the NecroX-5- and PSE-treated groups as compared to the non-treated HR group (Fig. 4B). CM-H_2DCFDA intensity values evaluated in HR, DMSO, NecroX-5, and PSE were 157.01±0.16%, 156.83±0.24%, 149.98±6.01%, and 137.64±3.21%, respectively (where 100% is the normal control). Interestingly, there was a significant difference between NecroX-5 and PSE groups ($p<0.05$). This finding provides more evidence for the antioxidant properties of ginseng products under HR damage [16, 25]. Most likely this phenomenon is a result of the saponin components [26] and extraction solvents [27]. The efficacy in maintaining mitochondrial function of ginseng products against HR damage has been previously described [17, 25, 28]. However, more studies should be done to clarify the exact mechanism action of PSE on cardiomyocytes under HR injuries.

Conclusions

To the best of our knowledge, this is the pilot study investigating the protective effects of *Panax bipinnatifidus* Seem. extract against HR damage via protecting cardiomyocytes as well as preserving mitochondrial function.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

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