

Protective effects of paeoniflorin on acrolein-induced apoptosis in H9c2 cardiomyocytes

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Abstract: Acrolein is a highly toxic unsaturated aldehyde which is abundant in many circumstances. People exposed to acrolein may have significant clinical relevance in human cardiotoxicity situations. Paeoniflorin (PEF) is a bioactive glucoside isolated from the roots of *Paeonia lactiflora* Pall. It is reported that PEF performs a beneficial role in cardiovascular system. The aim of the current research was to evaluate the potential protective effect of PEF against acrolein-induced apoptotic damage in H9c2 cardiomyocytes. This study revealed that PEF exerted a protective effect on acrolein-induced cardiomyocyte apoptosis. Furthermore, treatment with acrolein could markedly increase the levels of reactive oxygen species (ROS) and expression of cleavage of caspase-9 and caspase-3 in H9c2 cardiomyocytes, which were significantly reversed by co-treatment with PEF (100uM). These results demonstrated that PEF protects H9c2 cardiomyocytes against acrolein-induced cardiomyocyte injury via decreasing ROS production and down regulating caspases cascade reaction, indicating that PEF is a potential therapeutic agent for cardiac toxic environmental pollutant injury.

Keywords: Paeoniflorin, acrolein, cardiomyocytes, apoptosis, caspase, reactive oxygen species.

INTRODUCTION

As a highly toxic α , β -unsaturated aldehyde, acrolein is a widespread global environmental pollutant. It is abundant in over-heated organic materials, food and cigarette smoke. Except for from outside, acrolein is also an endogenous toxic byproduct of cellular lipid per oxidation (Shah *et al.*, 2015). Acrolein is one of the most reactive biological aldehydes, which reacts with various biomolecules including DNA, proteins and phospholipids, resulting in the formation of stable Michael adducts of Schiff bases (Pizzimenti *et al.*, 2013). Thereby, acrolein adducts potentially impair protein structure and function, which may induce cytotoxicity, lead to mutations, alter gene transcription and regulate apoptosis (Thompson *et al.*, 2017). Epidemiological data revealed that people who exposed to acrolein may have a higher risk of cardiovascular disease and death because the exposure triggers adverse cardiovascular events (Ismahil *et al.*, 2011). Moreover, exposure to acrolein directly causes myofilament dysfunction and systemic toxicity, which was related to the accumulation of acrolein-protein adducts and protein carbonyls involved in myocardial contraction and energy metabolism (Shi *et al.*, 2015). The acrolein-mediated heart dysfunction in pathologies was characterized by high-oxidative stress, which ultimately led to cardiomyocyte cytotoxicity and apoptosis (Wang *et al.*, 2011).

Paeoniflorin (PEF) is a monoterpene glucoside extracted from *Paeonia lactiflora* Pall, which is used in traditional Chinese herbal medicine for thousands of years. Previous pharmacological studies have demonstrated that PEF

affords various biological activities, such as anti-arrhythmic, antioxidative, anti-inflammatory and anti-fibrosis effects and so on (Chu *et al.*, 2011; Hu *et al.*, 2017; Lu *et al.*, 2014; Wang *et al.*, 2011). Our preceding studies revealed that PEF plays a potential protective role in cardiovascular system. PEF has been shown to protect against doxorubicin-induced myocardial cell apoptosis through inhibiting NADPH oxidase and reducing ROS production (Li *et al.*, 2016; Li *et al.*, 2012). Moreover, PEF could improve cardiac function and decrease adverse postinfarction left ventricular remodeling in a rat model of acute myocardial infarction (Chen *et al.*, 2018). However, it is still not clear whether PEF can arrest the development of acrolein-induced cardiac damage.

In the current research, H9c2 cardiomyocytes were incubated with 10 μ M acrolein to set up a chemotherapy-induced cardiac toxicity model. Our aim was to unravel the influence of PEF on acrolein-induced cardiomyocyte apoptosis and to investigate the potential molecular mechanisms in H9c2 cardiomyocytes. Our study may provide evidence supporting the use of PEF for the prevention of toxic environmental pollutant.

MATERIALS AND METHODS

The following reagents were used: H9c2 cell line (Academia Sinica, Shanghai, China); Paeoniflorin (Yangling Dongke Maidisen Pharmaceutical Co., Ltd., Xi'an, China); Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA); 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT), 4',6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO, USA). The following kits were used: lactate dehydrogenase (LDH)

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assay kits, reactive oxygen species (ROS) (Nanjing Jiancheng Bioengineering Institute, Nanjing, China); RNA isolation kit and M-MLV reverse transcriptase kit (Promega Corporation, Madison, WI, USA); The following antibodies were used: anti-caspase-9, anti-caspase-3 and GAPDH (Cell Signaling Technology, USA).

Cell culture and treatments

H9c2 cardiomyocytes were cultured in DMEM containing 10% FBS, 1mM glutamine, 100µg/ml streptomycin and 100IU/ml penicillin. The cells were grown at 37°C under an atmosphere of 5% (v/v) CO₂. In order to investigate the effects of PEF following acrolein-induced cell injury, acrolein-treated H9c2 cells were maintained in complete medium with 100uM PEF. The control cells were cultured in normal condition. H9c2 cardiomyocytes were stochastically divided into the following groups: Group I (Control), cells treated without PEF or acrolein; group II, cells treated with 10µM acrolein for 14h; group III, cells pretreated with 100uM PEF for 2h and then treated with 10µM acrolein for 14h; group IV, cells treated with 100uM PEF for 16h.

Cell viability assay

The cell viability was evaluated by MTT assay. H9c2 cardiomyocytes cultured in 96-well plates were received different treatment. At the end of each treatment, cells were cultured with 5mg/ml MTT at 37°C for 4h. The reaction was brought to an end by the addition of 150µl DMSO. The OD values were read on a scanning multi-well spectrophotometer at 570nm. Cell viability was presented as the relative percentage of the OD value of the control group.

LDH assay

To further confirm the protective effects of PEF, the LDH activity was determined by an LDH assay kit following the manufacturer's protocol. After treatment, the culture medium was centrifuged at 400×g for 5min. The supernatants were collected for analysis using an LDH assay kit. The OD values were recorded at 450nm on a microplate reader. And the activity of LDH was presented as the relative percentage of the OD value of the control group.

Nuclear staining for assessment of apoptosis

Nuclear morphological change was evaluated by DAPI staining, which illustrated the characteristic of apoptotic cell. Briefly, the treated cells were rinsed once and stained with DAPI solution (1µg/ml) at 37°C for 15min, then rinsed with methanol. After rinsing again with PBS, cells were observed and photographed by inverted fluorescence microscopy. The data are presented as a percentage of apoptotic cells to total cells.

Measurement of intracellular ROS level

Intracellular ROS level in H9c2 cardiomyocytes was

determined employing 2', 7'-dichlorofluoresceindiacetate (DCFDA) according to the manufacturer's protocol. Subsequent to treatment, cells were resuspended in PBS. Then the cells were loaded with 10µM DCFDA for 30min at 37°C followed by rinsing again with PBS to remove the excessive dye. ROS levels were monitored with a fluorospectrophotometer (Shimadzu Corporation, Kyoto, Japan) at Ex 488nm/ Em 525nm.

Quantitative real-time PCR

For real-time PCR, H9c2 cells were rinsed twice with PBS. The total RNA was isolated employing the RNA isolation kit and reverse-transcribed to cDNA employing the M-MLV reverse transcriptase kit. The gene primer sequences are as follows: 5'-ACTGCCTCATCAT CAACA-3'/5'-GTTCT TCACCTCCACCAT-3' for caspase-9, 5'-CAAGTCGATGGACTCTGGAA-3'/5'-GTACCATTGCGAGCTGACAT-3' for caspase-3, 5'-TGGCCTCCAAGGAGTAAGAAAC-3'/5'-GGCCTCTCTCTTGCTCT CAGTATC-3' for GAPDH. Quantitative analysis of mRNA expression was accomplished employing the ABI 7300 real-time PCR system with the Power SYBR-Green PCR Master Mix kit. Reaction conditions were as follows: 95°C for 10min, followed by 40 cycles at 95°C (15sec) and 60°C (30sec). All amplification reactions were implemented three times for each sample. Gene expression changes were standardized to endogenous reference GAPDH using the 2^{-ΔΔC_q} method (Livak and Schmittgen, 2001).

Western blot analysis

Subsequent to different treatment, cells (1×10⁶ per sample) for lysate preparation were resuspended in ice-cold cell lysis buffer. After centrifugation (400g, 15min) at 4°C, the protein concentration was determined using Bradford protein assay and then solubilised in Laemmli sample buffer. Fifty micrograms of protein was separated by 12% SDS-PAGE, followed by transfer to PVDF membranes. After transferring the protein samples, the membranes were blocked with 5% milk powder for 1h in TBST and then incubated with the appropriate antibodies diluted 1:1000: anti-caspase 3, anti-caspase 9 and anti-GAPDH. Subsequently, the blots were incubated with HRP-conjugated anti-rabbit (1:5000) secondary antibody for 1h at room temperature. Detection was realized with an ECL kit following the manufacturer's directions. GAPDH was regularly used as loading control.

STATISTICAL ANALYSIS

SPSS statistical software was used for statistical analysis (version 21.0). Data were expressed as means ± standard deviation of the mean. All data were analyzed by using one-way analysis of variance (ANOVA) and the Newman-Keuls Student's t-test. Differences were considered significant when P<0.05.

RESULTS

Effect of PEF on acrolein-induced cell viability and the leakages of intracellular LDH

Cell viability was evaluated by MTT assay to exactly quantify the cell survival. Following treatment, cell viability of the acrolein group was significantly reduced relative to that of the control group ($P < 0.01$), as shown in fig. 1A. PEF-containing serum could ameliorate the decreased cell viability caused by acrolein ($P < 0.01$). LDH, reflecting cellular injury and membrane permeability, is regarded as diagnostic marker enzymes. LDH release is a sign of cellular injury. As shown in fig. 1B, the levels of LDH was obviously increased in the acrolein group relative to that of the control group ($P < 0.001$). PEF treatment significantly reduced the releases of LDH in H9c2 cardiomyocytes ($P < 0.01$). Together, these results indicated that PEF exhibited protective effects on H9c2 cardiomyocytes injured by acrolein.

Effect of PEF on acrolein-induced cell morphology change

We further observed the influence of PEF on acrolein-induced apoptosis in H9c2 cardiomyocytes. As shown in fig. 2, in the control group, the cells displayed coincidentally intact cell membrane and dispersed chromatin, which were different from the stress cells. However, we found that stressed cells in the acrolein group had lost normal cell morphology and displayed apoptosis features, including chromatin condensation, nucleic fragmentation and apoptotic body formation ($P < 0.001$). When cells were first pretreated with PEF, the number of apoptotic cells was significantly reduced ($P < 0.01$). PEF alone did not affect cell morphology and the percentage of apoptotic cells.

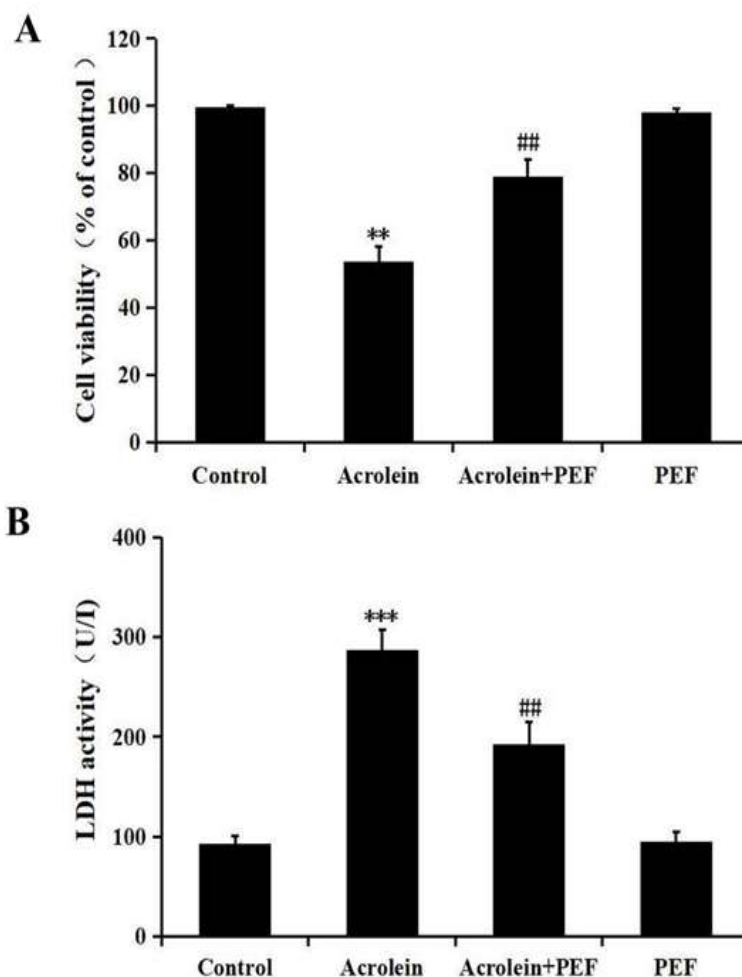


Fig. 1: Effect of PEF on acrolein-induced cell viability and the leakages of intracellular LDH in H9c2 cardiomyocyte. (A) Cell viability (expressed as the percentage of the control). (B) LDH activity. Each result shown is representative of three independent experiments. All data are expressed as mean $\pm$ SD. ** $P < 0.01$, *** $P < 0.001$ vs. the control group; ## $P < 0.01$ vs. the acrolein group. PEF, paeoniflorin; LDH, lactate dehydrogenase.

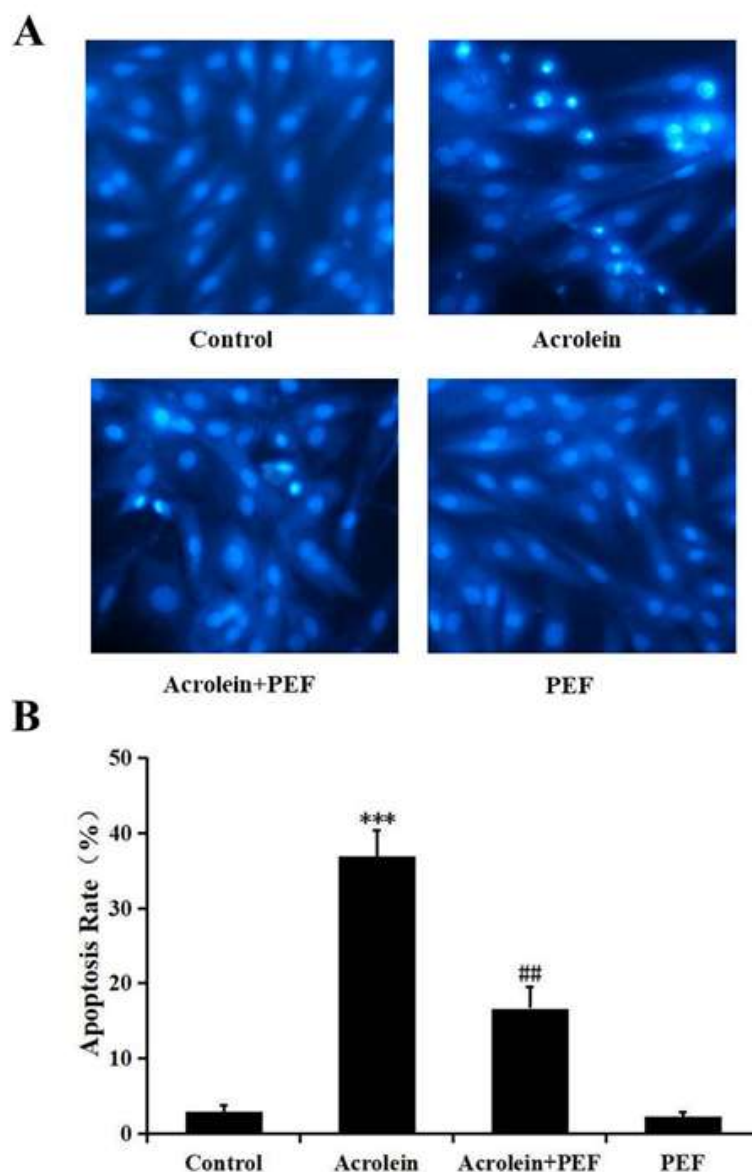


Fig. 2: Effect of PEF on acrolein-induced cell morphology change in H9c2 cardiomyocytes induced. (A) DAPI staining followed by fluorescence imaging to observe cell apoptosis. (B) The apoptotic rate was analyzed with a cell counter. Each result shown is representative of three independent experiments. All data are expressed as mean $\pm$ SD. *** P <0.001 vs. the control group; ## P <0.01 vs. the acrolein group. PEF, paeoniflorin.

Effect of PEF on acrolein-induced ROS generation

Cardiovascular injury is associated with the elevated ROS levels, which subsequently cause cell apoptosis. Studies demonstrated that oxidative stress serves an important role in acrolein-induced cardiotoxicity (Xu *et al.*, 2018). Therefore, we evaluated the influence of PEF on acrolein-induced intracellular ROS levels in H9c2 cardiomyocytes. As shown in fig. 3, the results of our current experiments indicated that exposures of acrolein facilitated the production of ROS (P <0.01) and these effects were significantly inhibited by PEF (P <0.01), which further demonstrating its antioxidant effects.

Effect of PEF on cell apoptosis-regulating genes and protein expression

As shown in fig. 4A, the results of the real-time PCR revealed that treatment with acrolein obviously increased the mRNA expressions of caspase-9 and caspase-3, when compared to the control group (P <0.01). However, pretreatment with PEF attenuated the expressions of caspase-9 and caspase-3, when compared to the acrolein group (P <0.05 or P <0.01, respectively).

The effects of PEF on protein expression levels in H9c2 cardiomyocytes were examined by western blot. As

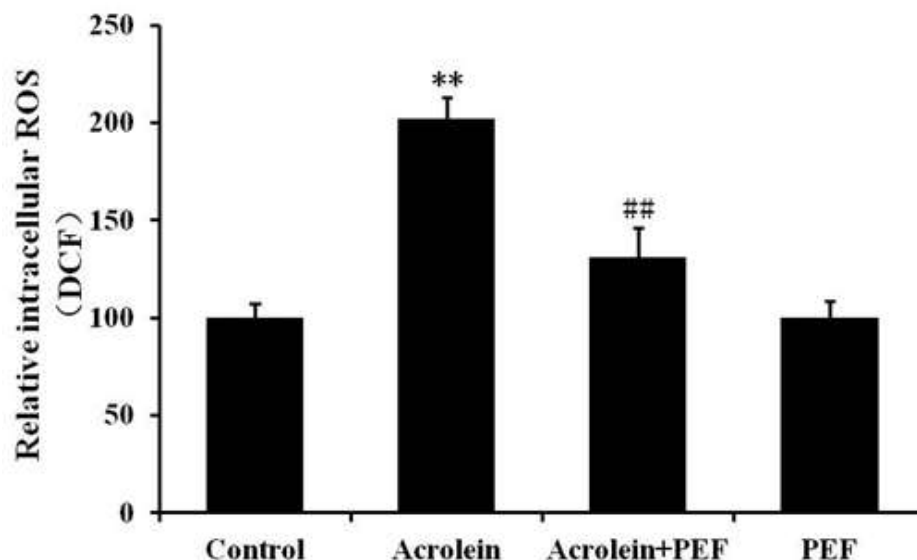


Fig. 3: Effect of PEF on acrolein-induced ROS generation in H9c2 cardiomyocytes. The intracellular ROS production under different experimental conditions were detected by fluorescence intensity of 2',7'-dichlorofluorescein. Each result shown is representative of three independent experiments. All data are expressed as mean $\pm$ SD. ** P <0.01 vs. the control group; ## P <0.01 vs. the acrolein group. PEF, paeoniflorin.

shown in fig.4B and C, the western blot analysis indicated that acrolein stimulated an increase protein expressions of cleaved caspase-9 and caspase-3 relative to that of the control group (P <0.01). On the contrary, PEF markedly decreased the expressions of cleaved caspase-9 and caspase-3 relative to that of the acrolein group (P <0.05 or P <0.01, respectively). PEF alone did not influence the basal expressions of caspases cascade reaction. These data indicated that PEF inhibit apoptosis-associated genes and protein expression levels in acrolein-induced H9c2 cells by down regulating caspases cascade reaction.

DISCUSSION

As a highly reactive hazardous pollutant, acrolein poses serious threats to human health. It was reported that acrolein increase the risk of cardiovascular disease (Moghe *et al.*, 2015). Because of increasing exposure to acrolein, there is an urgent need to understand how acrolein influences cardiovascular system. Hence there is interest in developing potential therapies that can repair and restore the cardiovascular system against toxic environmental pollutant. In this work, we explored the effect of PEF on acrolein-induced H9c2 cardiomyocytes injury and investigated the potential mechanisms. These results indicated that PEF has the protective effect on acrolein-induced cardiomyocytes injury by inhibiting cardiomyocyte apoptosis involving a mechanism of suppressing ROS generation and down regulating caspases cascade reaction.

ROS, a series of cellular molecules generated during metabolism, are generally considered to be important

mediators of oxidative stress injury (Zhang *et al.*, 2013). Acrolein was possibly the most toxic aldehyde due to its high reactivity and regarded as a perpetrator of oxidative stress. Moreover, both *in vivo* and *in vitro* researches have revealed that acrolein can trigger tissue damage, cytotoxicity and apoptosis, which have been suggested to be linked to oxidative stress (Moghe *et al.*, 2015). Our experiment showed that acrolein treatment dramatically resulted in excessive ROS accumulation in H9c2 cardiomyocytes, clearly supporting the involvement of oxidative stress in the process of acrolein damaging. Whereas PEF pretreatment resulted in decreasing ROS generation, indicating a role of PEF in oxidative stress.

Previous studies indicated that apoptosis was an important event during the whole process of exposure to acrolein in cardiomyocyte (Xu *et al.*, 2018). Apoptosis was featured by the activation of apoptotic signaling pathways and expression of related genes (McStay and Green, 2014). Caspases are cysteine aspartate proteases that play a key role in apoptotic signaling pathway. Caspase-9 is one of the upstream regulators of caspase-3, which is the main effector and the most important executioner of apoptosis. Once the initiator caspase-9 activated, the effector caspase-3 was directly activated and subsequently executed apoptosis (Brentnall *et al.*, 2013; Ozacmak *et al.*, 2018). Our study proved that PEF could reduce the expressions of cleavage caspase-9 and caspase-3 in H9c2 cells exposure to acrolein. Therefore, we conjectured that PEF could decrease cell apoptosis, which may be the mechanisms for acrolein-induced cardiotoxicity.

As the major phytochemicals in the genera of paeonia

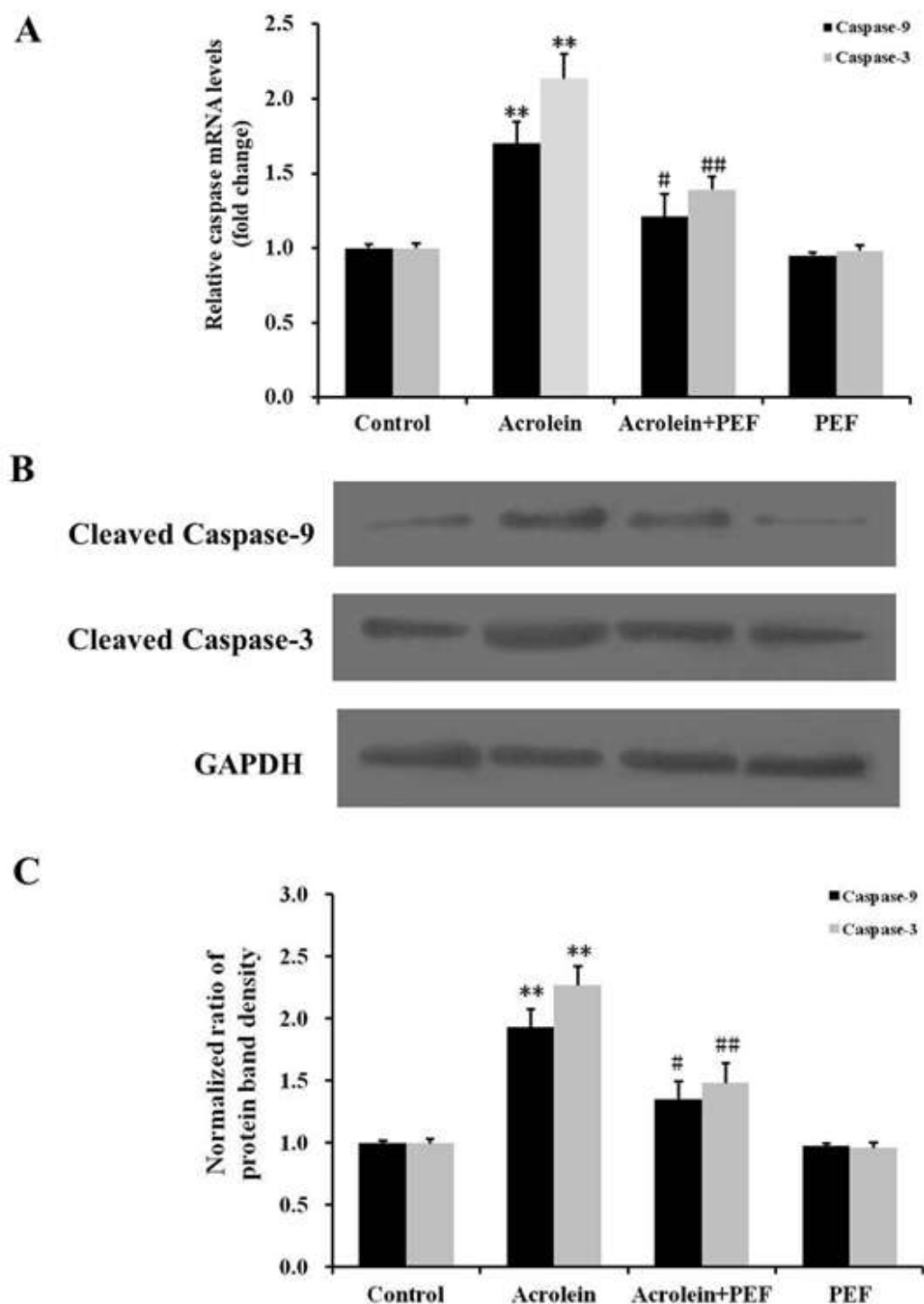


Fig. 4: Effect of PEF on cell apoptosis-regulating genes and protein expression in H9c2 cardiomyocytes induced by acrolein. (A) The mRNA expression levels of caspase-9 and caspase-3 were analyzed by real-time PCR. (B) representative image of cleaved caspase-9 and caspase-3 protein expression by western blot; (C) optical density of protein bands. Each result shown is representative of three independent experiments. All data are expressed as mean $\pm$ SD. ** P <0.01 vs. the control group; ## P <0.01 vs. the acrolein group; # P <0.05 vs. the acrolein group. PEF, peoniflorin.

lactiflora pall, PEF posses various beneficial bioactivities (Chu *et al.*, 2011; Hu *et al.*, 2017; Lu *et al.*, 2014; Wang

et al., 2011). Our previous investigation has revealed that PEF may protect against doxorubicin-induced myocardial

cell apoptosis through preventing oxidative stress (Li *et al.*, 2012). In the present study, the investigations performed further elucidated the protective effects of PEF on myocardial injury *in vitro*.

CONCLUSION

In summary, our current study revealed that PEF could significantly reverse acrolein-induced cardiomyocyte apoptosis by alleviating oxidative stress and down regulating caspases cascade reaction. These findings provided evidence that PEF is a potential therapeutic agent for cardiac toxic environmental pollutant injury. Whereas the mechanisms underlying the antioxidant and anti-apoptotic properties of PEF on toxic environmental pollutant are not fully elucidated, and curative effects based on animal model and possible mechanisms are still worth continuing in further study.

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