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Protective mechanisms of hypaconitine and glycyrrhetinic acid compatibility in oxygen and glucose deprivation injury*

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Abstract: This study investigated the protective effect of the compatibility of hypaconitine (HA) and glycyrrhetinic acid (GA) on H9c2 cells under oxygen and glucose deprivation (OGD)-induced injury, and the possible mechanisms. We found that HA+GA significantly improved pathology and morphology of the nucleus and ultrastructure of H9c2 cells under OGD as determined by Hoechst 33342 staining and transmission electron microscopy (TEM) tests. It also reduced the releases of lactate dehydrogenase (LDH), creatine kinase-myocardial band isoenzyme (CK-MB), and aspartate transaminase (AST) from the cultured supernatant of H9c2 cells, which were tested by enzyme-linked immune sorbent assay (ELISA) kits. In addition, it lessened the apoptotic rate as determined by a fluorescein isothiocyanate-annexin V/propidium iodide (FITC-AV/PI) double staining assay. It was also found that HA+GA might regulate the protein expression associated with the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway. Overall, the study demonstrated that HA+GA protected H9c2 cells against OGD-induced injury, and the signaling mechanism might be related to the PI3K/Akt signaling pathway.

Key words: Hypaconitine (HA); Glycyrrhetinic acid (GA); H9c2 cells; Apoptosis; PI3K/Akt
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1 Introduction

Cardiovascular diseases (CVDs) are disorders of heart and blood vessels, including ischemic heart disease, with high morbidity and mortality around the world (Menezes *et al.*, 2011; Nabel and Braunwald, 2012). It is generally accepted that ischemic heart injury is a pathological process, which will cause extensive death of cells (Yucel *et al.*, 2011). Furthermore, ischemic heart diseases can produce apoptosis, and the apoptosis might be related to both extrinsic and intrinsic apoptotic pathways. However, preconditioning may reduce apoptosis via regulation of the expression of Bcl-2 and Bax (Lai *et al.*, 2015). There are also many risk factors. For example, inflammatory reactions have been proved to participate in CVD progression (Munk and Larsen, 2009). Thus it is important to investigate possible therapeutic and prevention strategies for improving CVD patients' quality of life. Drug treatment is still an effective method for enhancing both the quality and length of life (van der Hoeven *et al.*, 2012). It has been reported that the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway is an amenable pharmacological target for CVD protection, and it achieves its effect mainly through regulating caspase activation, Bcl-2 family activity, and so on (Wang J. *et al.*, 2015). In

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the past few years, many traditional Chinese medicines have been claimed to be useful for controlling CVD, particularly for the injury induced by ischemia (Quan *et al.*, 2014; Zheng *et al.*, 2014). Therefore, searching for anti-necrosis and anti-apoptotic compounds with minimal side effects from traditional Chinese medicines probably is an ideal way for CVD patients, and to help create a much safer and more effective treatment.

Aconitum carmichaelii Debeaux is a well-known traditional Chinese herb, including diester diterpenoid alkaloids, which are responsible for not only biological activity but also toxicity. It is widely applied in treatments for the cardiovascular system, such as heart failure, hypertension, and arrhythmia (Zhao *et al.*, 2012; Yu B. *et al.*, 2015), and for anti-inflammation and analgesic action, such as in rheumatism and painful joints (Cai *et al.*, 2013). Then there are other applications, including for anti-aging, protecting the kidney, as an anti-diabetic and for lipid-lowering, helping the immune system, and so on (Zhou *et al.*, 2015). Hypaconitine (HA) (Fig. 1) is one of the major active alkaloids of *Aconitum carmichaelii* Debeaux, which has both therapeutic and high toxic activity, with cardiotoxity and evident side-effect (Xie *et al.*, 2015). It is frequently prescribed with other ordinary herbal medicines, such as the *Glycyrrhiza uralensis* Fisch. It is largely used in clinics as an adjuvant, and in some cases for reducing toxicity. Glycyrrhizic acid (GL) and glycyrrhetinic acid (GA) (Fig. 2) have aglycone saponins. It has been proved that GA can ameliorate ventricular arrhythmia (Wu *et al.*, 2015) and GL transforms to GA in the absorption process (Fan *et al.*, 2016). HA is considered to be effective on CVD, and its heat stability in water is better than that of other alkaloids (Yue *et al.*, 2008; Zhang *et al.*, 2015). Studies have also found that HA and GA are the major compounds after oral administration of Fuzi-Gancao decoction (Gao *et al.*, 2004; Zhang *et al.*, 2013; Yang *et al.*, 2014). It is known that oxygen and glucose deprivation (OGD) is a generally applicable issue in CVD studies, since OGD treatment can cause necrosis and apoptosis of cells. Though the effects of HA and GA have been elucidated separately in H9c2 cells, their combination effects and mechanisms are not so well understood. Hence we chose to use the compatibility of HA and GA (HA+GA) to interpret the effects and mechanisms on H9c2 cells.

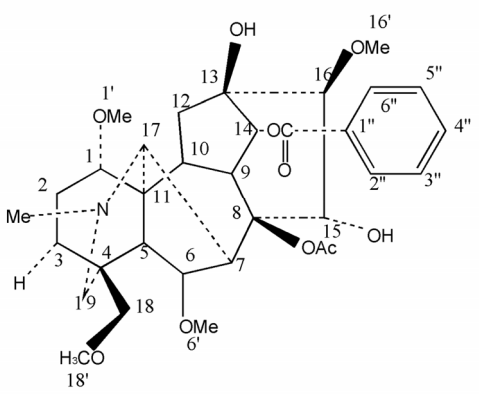

Fig. 1 Structure of hypaconitine (HA)

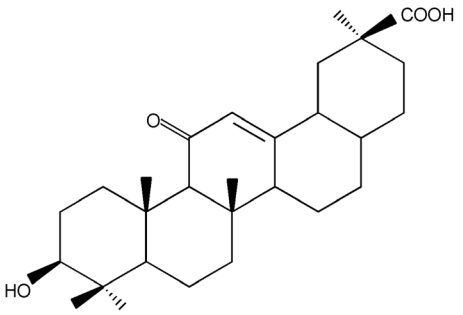

Fig. 2 Structure of glycyrrhetinic acid (GA)

2 Materials and methods

2.1 Materials

HA (molecular formula: $C_{33}H_{45}NO_{10}$; molecular weight: 615.71; purity: ≥98%) and GA (molecular formula: $C_{30}H_{46}O_4$; molecular weight: 470.64; purity: ≥98%) were purchased from Baoji Chenguang Biotechnology Co., Ltd. (Xi'an, China). LY-294002 (molecular formula: $C_{19}H_{17}NO_3$; molecular weight: 307.34; purity: ≥98%) was obtained from Calbiochem Novabiochem Co. (San Diego, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from GIBCO Co. (Grand Island, NY, USA). Fetal bovine serum (FBS) was provided by the Shanghai Shengnabao Biological Technology Development Co., Ltd. (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Beijing dingguochangsheng Biotechnology Co., Ltd. (Beijing, China). Phosphate buffered saline (PBS) was obtained from Wuhan Boster Bio-Engineering Co., Ltd. (Wuhan, China). The kits for determination of lactate dehydrogenase

(LDH), creatine kinase-myocardial band isoenzyme (CK-MB), and aspartate transaminase (AST) were obtained from Jiancheng Bioengineering Institute (Nanjing, China). Anti-phospho-Akt (anti-p-Akt), anti-Akt, anti-Bax, anti-Bcl-2, anti-caspase-9, and anti-β-actin antibodies were obtained from Santa Cruz Biotechnology Co. (CA, USA). The fluorescent kit for Hoechst 33342 was obtained from Beyotime Institute of Biotechnology Co., Ltd. (Hangzhou, China). The fluorescein isothiocyanate-annexin V/propidium iodide (FITC-AV/PI) apoptosis detection kit was provided by BD Pharmingen (San Diego, USA).

2.2 Cell culture

The H9c2 cells were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (IBCB, CAS). The H9c2 cells were maintained in DMEM supplemented with 10% FBS and penicillin plus streptomycin (both 100 μg/ml), incubated at 37.0 °C in a 5% CO_2 atmosphere. The medium was replaced every 2–3 d, and cells were subcultured or subjected to experimental procedure when developed into 80%–90% density.

2.3 OGD model building and experimental grouping

OGD has been used as a cellular technique of ischemic heart damage in cultured myocardial cells (Marambio *et al.*, 2010). According to a described protocol (Rizvi *et al.*, 2010), the OGD injury was produced by incubating with a glucose-free balanced salt solution (116 mmol/L NaCl, 5.4 mmol/L KCl, 0.8 mmol/L $MgSO_4$, 1.0 mmol/L NaH_2PO_4, 1.8 mmol/L $CaCl_2$, 26.2 mmol/L $NaHCO_3$, 0.025 mmol/L phenol red, and 20 mmol/L sucrose) and exposing to a hypoxic environment of 5% CO_2 and 95% N_2 in air tight gas chambers at 37 °C for 4 h. These basic experimental conditions had been confirmed in our previous work. After 4 h culture, H9c2 cells were used in the experiment. H9c2 cells were randomly divided into seven groups: (1) control group, without any treatment; (2) OGD group, being developed under OGD for 4 h with no treatment; (3) OGD+HA group, being pretreated with HA (120 μmol/L) before being cultured under OGD for 4 h; (4) OGD+GA group, being preconditioned with GA (120 μmol/L) before being incubated under OGD for 4 h; (5) OGD+HA+GA group, being pretreated with HA+GA (120 μmol/L, separately) before being cultivated under OGD for 4 h; (6) OGD+LY group, being incubated under OGD for 4 h with LY-294002 (10 μmol/L) pretreatment 1 h before OGD; (7) OGD+HA+GA+LY-294002 group, being preprocessed with LY-294002 (10 μmol/L) 1 h before being developed with HA+GA (120 μmol/L, separately) under OGD for 4 h.

2.4 Cell viability assay

H9c2 cell viability was measured by MTT assay as described by Chen *et al.* (2013). The optical density (OD) values were detected at 490 nm by a Molecular Devices SpectraMAX plus-384 microplate reader (MD, USA), and the survival ratio of H9c2 cells was obtained as a percentage of the control. On the basis of results of previous experiments (Liu *et al.*, 2013), both the concentrations of HA and GA were selected at 120 μmol/L for the further investigation.

2.5 Hoechst 33342 staining assay

H9c2 cells were washed in ice-cold PBS, fixed in 10% neutral buffered formalin for 10 min at room temperature, and washed again with ice-cold PBS. Then the H9c2 cells were exposed to Hoechst 33342 (2 μg/ml in PBS) and incubated for 20 min at room temperature. The H9c2 cells were then washed three times with pre-cooled PBS, and lastly the stained cells were observed in an appropriate filter of the fluorescence microscope.

2.6 Transmission electron microscopy

The ultrastructure presences of H9c2 cells were surveyed by transmission electron microscopy (TEM), and cells were gathered from 6-well plates, and washed with PBS prior to fixing in a fixative buffer. Then cells were centrifuged at 3000 r/min for 10 min, suspended and incubated for 2 h in 2.5% glutaraldehyde. Cell samples were processed with 2% osmium tetroxide in a 0.1 mol/L sodium cacodylate buffer, dehydrated through a graded series of acetone, and embedded in resin. Then the cell samples were sliced into 65-nm sections, and ultrastructures were observed via TEM.

2.7 LDH, CK-MB, and AST activity assays

To be confident of the level of cell necrosis in the cultured supernatant of H9c2 cells, the activity of LDH, CK-MB, and AST was measured using enzyme-linked immune sorbent assay (ELISA) kits.

The procedures were conducted according to the manufacturers' instructions.

2.8 FITC-AV/PI assay

FITC-AV/PI doubles staining assay was used to examine the apoptosis of H9c2 cells. H9c2 cells were collected, washed with calcium-free PBS, resuspended with binding buffer, and incubated with annexin V at room temperature in the dark for 15 min. Then the H9c2 cells were centrifuged and resuspended with binding buffer. PI was added to the resuspended cells before they were analyzed by flow cytometry.

2.9 Western blot analysis

The relevant protein 50 μg was resolved on a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel. The fractionated proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membranes and probed with rabbit anti-rat polyclone anti-p-Akt, anti-Akt, anti-Bcl, anti-Bax, and anti-caspase-9 at 4 °C overnight, and then incubated with the peroxidase-conjugated secondary antibodies at room temperature for 2 h. The blots were visualized with ECL (enhanced chemiluminescent) Plus reagent (GE Healthcare, Piscataway, NJ, USA). In all these experiments, the blots were reprobed with an anti-β-actin antibody to control the protein loading.

2.10 Statistical analysis

Routine statistical analysis was completed using IBM SPSS 19.0. Data were presented as the mean± standard deviation (SD). One-way analysis of variance (ANOVA) was applied to evaluate between group differences, followed by Tukey's test. In this study, values of $P<0.05$ were considered statistically significant.

3 Results

3.1 Morphological characteristics of apoptosis by Hoechst 33342 staining

Nucleus morphologies of H9c2 cells in the first five groups were performed by Hoechst 33342, which is a permeable membrane dye. As shown in Fig. 3, the nuclei of cells in the control group appeared with normal contours and were elliptical or round in shape with few bright spots, which represented the apoptotic cells. However, in the OGD group, cells were revealed with many more bright spots, together with condensed chromatin and smaller cell nuclei. The

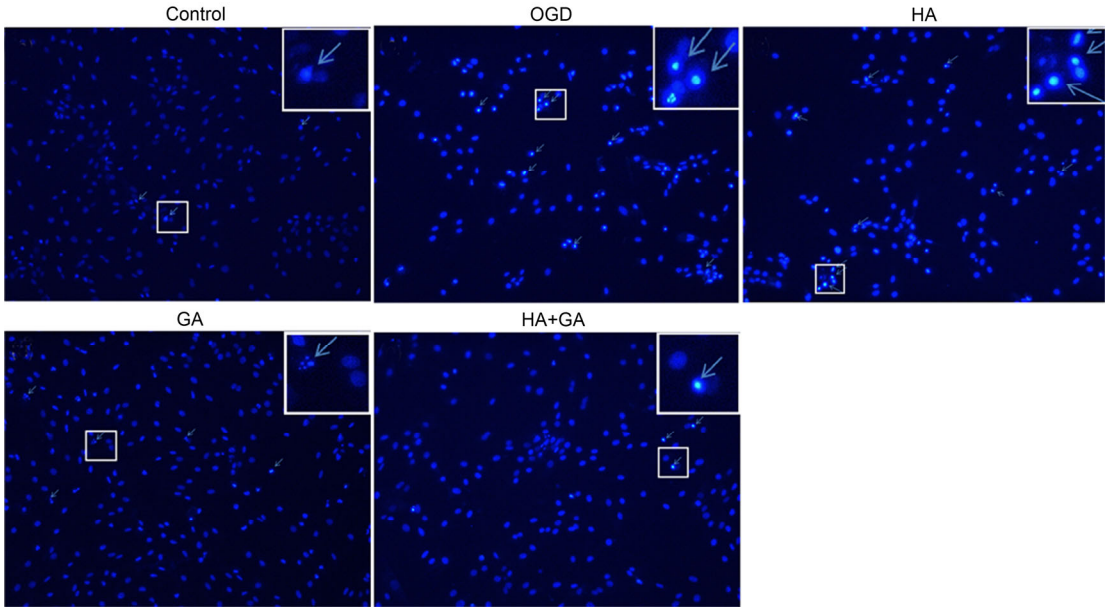

Fig. 3 Effects of HA+GA on histochemical characterizations of H9c2 cells in OGD injury
Arrowheads in the pictures indicate the nuclei of apoptotic cells (magnification ×100, enlarged part ×200)

other three pretreatment groups had ameliorated morphological characteristics and reduced number of apoptotic cells, but the HA group had slight differences from the OGD group. The HA+GA and GA groups had apparent changes, and the HA+GA group emerged with a better appearance.

3.2 Ultrastructure changes of H9c2 cells by TEM observation

To observe the ultrastructure changes of OGD-induced injury on H9c2 cells by the pretreatments of HA+GA, TEM was used. As presented in Fig. 4, the control group had a generally normal cell ultrastructure and shape, the cell nuclei membrane was intact and clear, and most of the mitochondria were in a nearly clear and integrated structure with compact cristae. Compared with the control group, the OGD group had some different and damaged forms of cell apoptosis; for example, the mitochondria were swollen with disrupted and lysed cristae, even many of hypervacuolization, with cell nuclei chromatin condensed and migrated to the cell edge. Meantime, the HA group revealed a similar morphological change with the OGD group, but the pretreatment groups with GA and HA+GA displayed apparent morphological improvements, with a small amount of swollen mitochondria, which meant GA and HA+GA could help protect the H9c2 cells from OGD-induced injury.

3.3 Protective activity of HA+GA against OGD-induced LDH release

To address the protective activity of HA+GA on cultured H9c2 cells from OGD injury, LDH assay was performed to assess whether HA+GA can secure H9c2 cells against OGD-induced membrane damage. As demonstrated in Fig. 5a, a larger increase of LDH was observed in the OGD group than in other groups, and the OGD group had a significant difference with the control group (P<0.05). In contrast, GA and HA+GA pretreatment groups revealed markedly inhibited LDH releases, and the value of the LDH release in the HA+GA group was lower than that of the GA group. Compared with the OGD group, the HA group had no significant difference (P>0.05), yet the HA+GA group had a significant difference (P<0.05).

3.4 Protective activity of HA+GA against OGD-induced CK-MB and AST releases

CK-MB and AST are enzymes that can assess the range and degree of myocardial injury, and a release of them was performed to reveal the OGD-induced damage or necrosis of H9c2 cells. As shown

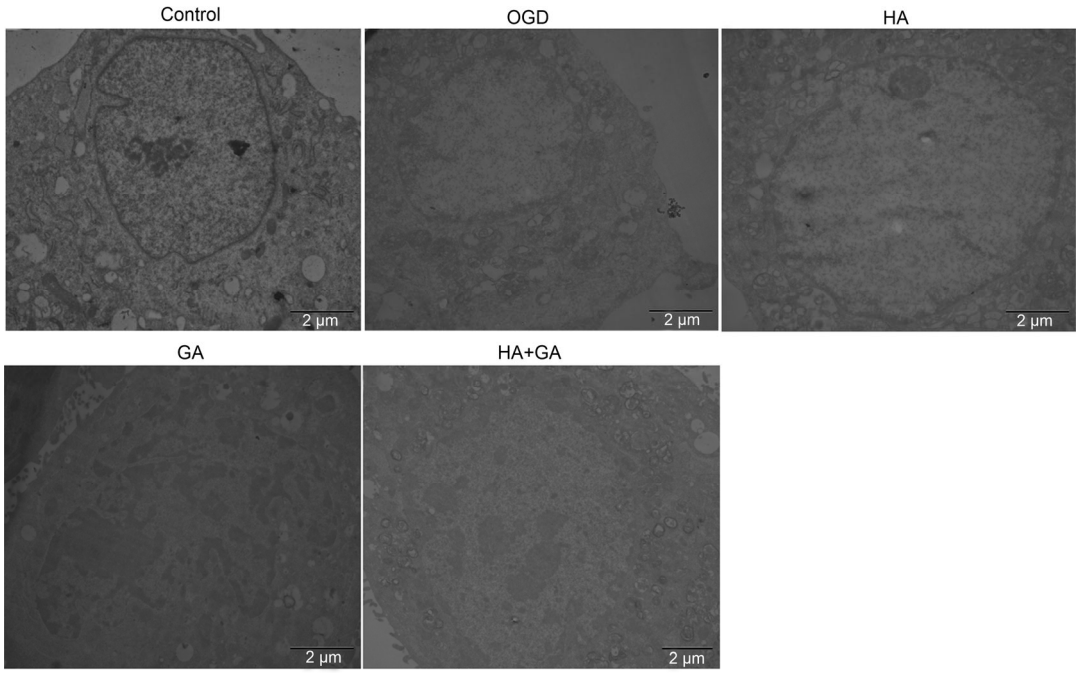

Fig. 4 Effects of HA+GA on ultrastructure changes of H9c2 cells in OGD injury

in Figs. 5b and 5c, marked improvements of CK-MB and AST were discovered in the OGD group compared with the other groups, and most of all had a highly significant difference compared with the control group (P<0.05). GA and HA+GA pretreatment groups showed tremendous inhibition of CK-MB and AST releases, and the HA+GA group revealed slightly better results than the GA group. Compared with the OGD group, the HA group had no significant difference (P>0.05), but the GA and HA+GA groups had a significant difference (P<0.05).

3.5 Anti-apoptotic effects of HA+GA on H9c2 cells with OGD injury

To analyze the possible anti-apoptotic capability of HA+GA under OGD conditions, the apoptotic rate was detected by flow cytometry using FITC-AV/PI double staining methods. As shown in Fig. 6, a small number of apoptotic cells were found in the control group. Compared to that, the apoptotic rate of H9c2 cells was significantly increased in the OGD group (P<0.05). However, the number of apoptotic cells was

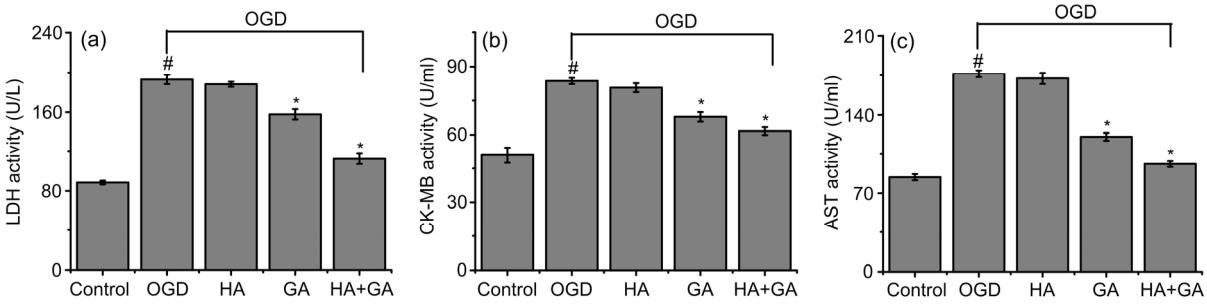

Fig. 5 Effects of HA+GA on releases of LDH (a), CK-MB (b), and AST (c) of H9c2 cells in OGD injury

Values were expressed as mean±SD (n=6). # P<0.05 compared with the control group; * P<0.05 compared with the OGD group

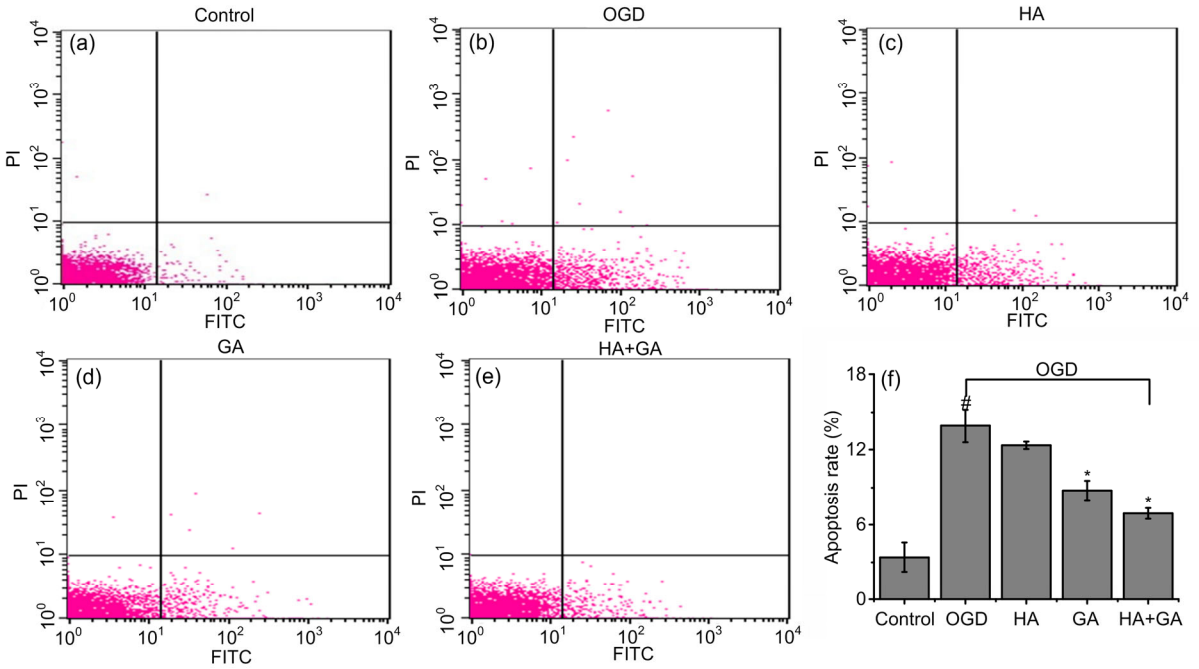

Fig. 6 Effects of HA+GA on apoptosis rates of H9c2 cells in OGD injury

(a–e) FITC-AV/PI double staining of the control, OGD, HA, GA, and HA+GA groups. (f) Apoptotic rates of different groups. Values were expressed as mean±SD (n=6). # P<0.05 compared with the control group; * P<0.05 compared with the OGD group

decreased by the pretreatments of HA+GA, GA, and HA; particularly, the HA+GA and GA groups had a significant difference (P<0.05), the HA group had no significant difference (P>0.05), and the HA+GA group was better than the GA group.

3.6 Intracellular mechanisms of the anti-apoptosis by HA+GA

To further explore mechanisms underlying the protection effects of HA+GA, we examined the expression of the apoptosis-related proteins, such as Akt, p-Akt, caspase-9, Bax, and Bcl-2. As shown in Fig. 7, the ratio of p-Akt/Akt of the OGD group was markedly higher than that of the control group (P<0.05). However, the group pretreated with HA+GA before OGD significantly increased p-Akt level compared with the OGD group (P<0.05). When pretreated with LY-294002 for 1 h before HA+GA and OGD, the level of p-Akt was greatly decreased compared with the HA+GA group (P<0.05), but the OGD and OGD+LY-294002 groups had no significant difference (P>0.05). Furthermore, as shown in Fig. 8, compared with the control group, the protein level of caspase-9 was enhanced remarkably in the OGD group

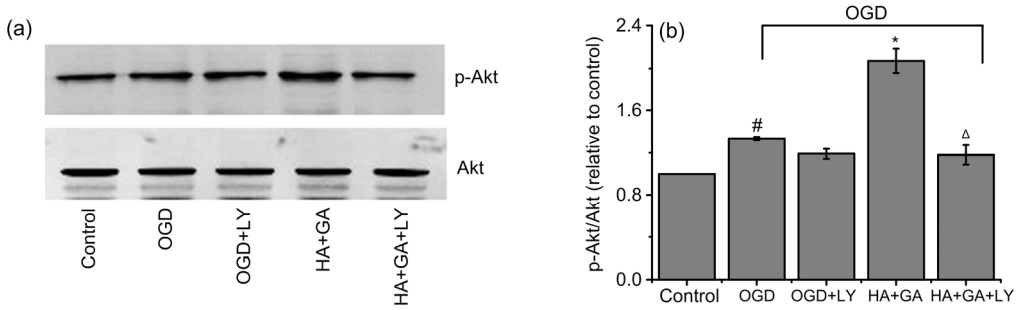

Fig. 7 Changes of p-Akt after the use of LY-294002

(a) Band diagrams; (b) p-Akt/Akt. HA+GA activates PI3K and phosphorylation of Akt. Values were expressed as mean±SD (n=6). $^{\#}P$<0.05 compared with the control group; $^{*}P$<0.05 compared with the OGD group; $^{\Delta}P$<0.05 compared with the HA+GA group

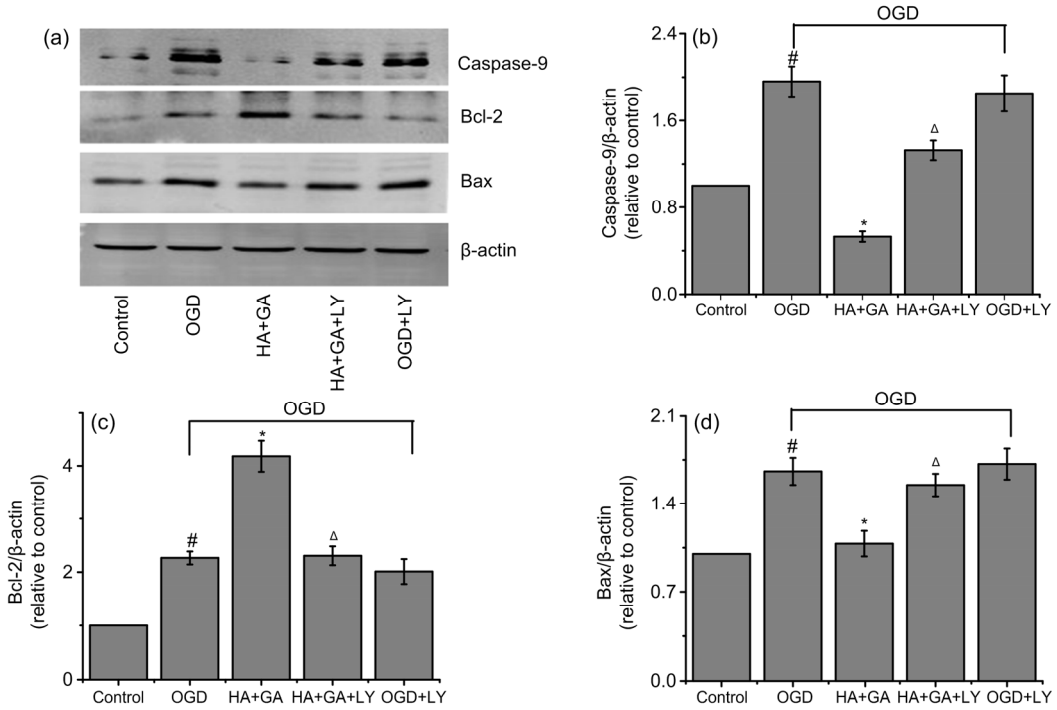

Fig. 8 Regulation of proteins caspase-9, Bcl-2, and Bax associated with apoptosis by HA+GA

(a) Band diagrams; (b) Caspase-9/β-actin; (c) Bcl-2/β-actin; (d) Bax/β-actin. Values were expressed as mean±SD (n=6). $^{\#}P$<0.05 compared with the control group; $^{*}P$<0.05 compared with the OGD group; $^{\Delta}P$<0.05 compared with the HA+GA group

(P<0.05), whereas, in contrast with the OGD group, the groups pretreated with HA+GA declined significantly (P<0.05). The HA+GA+LY-294002 group increased the protein levels of caspase-9 substantially when compared with the HA+GA group at a remarkable level of difference (P<0.05). The OGD group increased Bcl-2 protein expression and promoted Bax expression in cells compared with the control group. However, the HA+GA group dramatically changed the effects of OGD on Bcl-2 and Bax expression, but the HA+GA+LY-294002 group notably reversed the expression of Bcl-2 and Bax of the HA+GA group. At the same time, the OGD and OGD+LY-294002 groups had no significant difference (P>0.05).

4 Discussion

There has been some research illustrating the benefits of HA+GA treatment in CVD and pointing out the possibility of multiple targets where HA+GA may have protective effects. However, the direct effects of HA+GA on the H9c2 cells under OGD conditions were not clearly stated, and the relevant pathway and potential mechanisms were also not well expounded. In this study, the observations were displayed by fluorescence microscope and TEM, and the H9c2 cells and nucleus demonstrated prominent morphological injuries under OGD. Yet, these morphological changes and damages were mitigated in the group pretreated with HA+GA. Therefore, the results from morphological analysis suggested that preconditioning with HA+GA exerted a protective effect on H9c2 cells against OGD injury.

In previous research, the releases of LDH, CK-MB, and AST were assessed to investigate whether myocardial injury could be inhibited (Shi *et al.*, 2007; Wakayama *et al.*, 2012). Accumulating evidence indicates that necrosis is one of the main forms of cardiomyocyte death in CVD (Wang J.X. *et al.*, 2015). As we know, under a normal physiological state, LDH, CK-MB, and AST cannot cross cytoplasmic membranes. Nevertheless, when a cell is damaged or dead, they are released out of the cell (Qiao *et al.*, 2016). In this present study, LDH, CK-MB, and AST releases were downregulated, which proved that HA+GA protected H9c2 cells from OGD-induced cell damage.

It is said that a wide variety of regulatory stimuli can influence apoptosis (a programmed cell death) in a cell, displaying a crucial role in normal development of cells, tissue remodeling, immune response, and the stability of an organism. When the cell apoptosis process is disordered, it will become the pathogenesis of many human diseases, including cancer, viral infections, autoimmune diseases, and so on (Thompson, 1995). Meanwhile, apoptosis has an important effect on many CVD (Freude *et al.*, 2000). The apoptosis rates collected by flow cytometry in this paper confirmed that HA+GA might exert a protective effect against OGD-induced apoptosis. Estimates of apoptosis rates of H9c2 cells widely make use of FITC-AV/PI double staining analyzed by flow cytometry (Shang *et al.*, 2013). The annexin V assay is sensitive and supplies the possibility of detecting early phases of apoptosis before the loss of cell membrane integrity, and annexin V in polyethylenimine film can hold its high affinity following with phosphatidylserine altered from the inner to the outer cell membrane of the apoptotic cells in in vitro experiments (Vermes *et al.*, 1995; Liu *et al.*, 2009). In this study, the cardiomyocyte protection of HA+GA may be associated with the suppression of the cell apoptosis through the decreased apoptotic rates.

On the basis of results on the protective activity of HA+GA against OGD, further studies focused on the mechanisms of the anti-apoptotic effects of HA+GA. The PI3K/Akt signaling pathway regulates many processes in mammals, such as modulating cell survival, hypertrophy, contractility, and metabolism, inhibiting cell apoptosis, and controlling cell proliferation (Oudit and Penninger, 2009). It has been proved that the PI3K/Akt signaling pathway was critical in ischemic defense and pharmacologic preconditioning of cardioprotection (Qiao *et al*., 2016). In order to be cardioprotective and prevent the development of heart failure, the damage of myocardial cells could be lowered via inhibiting the apoptosis process (Lee and Gustafsson, 2009). Studies also revealed that part of the mechanisms of protecting cardiomyocytes relied on the activity of Akt, which can activate the downstream spot targets, improve energy generation of mitochondria, and then decrease the pro-apoptotic factors (Tong *et al.*, 2000). Researchers have found PI3K/Akt to be a powerful survival pathway in numerous systems; cells can accelerate apoptosis by

inhibiting PI3K and decelerate apoptosis by activating Akt (Hemmings and Restuccia, 2012). It was found that a decrease of caspase-9 activation could indicate the cardioprotective effects against diabetic cardiomyopathy by inhibiting cardiomyocyte apoptosis (Sun *et al.*, 2014; Yu H.T. *et al*., 2015). Meanwhile, studies have demonstrated that an increased expression of pro-apoptotic Bax family proteins and a decreased expression of anti-apoptotic Bcl-2 family proteins were connected with the process of cell apoptosis (He *et al.*, 2010; Yu *et al.*, 2010). In the whole pathway system, Bax and Bcl-2 are the relevant downstream indexes of PI3K/Akt signaling pathway (Gao *et al.*, 2010) and their regulation can induce the activity of caspase-9, so we evaluated the protein expression of Akt, p-Akt, caspase-9, Bax, and Bcl-2 to elucidate the possible mechanism pathway. Our data showed that HA+GA inhibited the cell apoptosis through suppressing Bax and caspase-9 expression and increasing Bcl-2 synthesis. In addition, to dissect the role of HA+GA in protecting cells against OGD-induced injury, the LY-294002, which is the specific inhibitor of PI3K/Akt pathway, was used. The data demonstrated that LY-294002 prevented HA+GA to generate cardioprotection by inhibiting the p-Akt expression and decreasing the amount of caspase-9, together with increasing the ratio of p-Akt/Akt. So, these results suggested that HA+GA might protect H9c2 cells from OGD-induced injury through the PI3K/Akt signaling pathway.

5 Conclusions

The results indicated that GA could reduce the toxicity of HA through the anti-necrotic and anti-apoptotic ways, and that the mechanism of this herb-pair's effect might be correlated with the PI3K/Akt signaling pathway. These results also provided strong evidence for clinical use of *Aconitum carmichaelii* and *Radix Glycyrrhizae*. Furthermore, this study method might be a guide for researching mechanisms of other compatibility medicines.

Contributors

Jie-hong YANG and Hai-tong WAN guided the experiment progress, Li-qin WANG implemented the experiment and wrote the paper, Yu HE participated in the pharmacodynamics experiment and the modification of this paper, Hao-fang WAN performed the cell culture work, and Hui-fen ZHOU performed the fluorescence microscope and flow cytometry work.

Compliance with ethics guidelines

Li-qin WANG, Yu HE, Hao-fang WAN, Hui-fen ZHOU, Jie-hong YANG, and Hai-tong WAN declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题　目：次乌头碱配伍甘草次酸对缺氧缺糖损伤 H9c2 心肌细胞的保护作用及其分子机制

目　的：探讨次乌头碱（HA）配伍甘草次酸（GA）对缺氧缺糖（OGD）损伤 H9c2 心肌细胞的保护作用及其作用机制。

创新点：首次在 OGD 模型中证明 HA+GA 对 H9c2 心肌细胞有明显的保护作用。此作用与减少细胞坏死和凋亡有关系，且其作用机制与磷脂酰肌醇-3-激酶/蛋白质丝氨酸-苏氨酸激酶（PI3K/Akt）信号通路有关。

方　法：采用 H9c2 心肌细胞为研究对象，将其分为七组：正常组、OGD 模型组、OGD+HA 组、OGD+GA 组、OGD+HA+GA 组、OGD+LY294002 组、OGD+HA+GA+LY294002 组。采用 Hoechst 33342 染色荧光显微镜及透射电镜观察前五组的 H9c2 心肌细胞的形态学改变；采用酶联免疫吸附测定法（ELISA）检测前五组细胞上清液中乳酸脱氢酶（LDH）、肌酸激酶同工酶（CK-MB）以及天门冬氨酸氨基转移酶（AST）的释放量的改变；采用异硫氰酸荧光素-磷脂结合蛋白 V/碘化丙啶（FITC-AV/PI）双染色法检测前五组细胞凋亡率的情况；采用蛋白质免疫印迹法（Western blot）检测加入抑制剂 LY294002 前后丝苏氨酸蛋白激酶（Akt）、磷酸化丝苏氨酸蛋白激酶（p-Akt）、B 细胞淋巴瘤/白血病-2 相关 x 蛋白（Bax）、B 细胞淋巴瘤/白血病-2（Bcl-2）及半胱氨酸天冬氨酸蛋白酶-9（caspase-9）等细胞作用信号通路 PI3K/Akt 相关蛋白的情况。

结　论：（1）Hoechst 33342 染色荧光显微镜显示 OGD+HA+GA 组抗凋亡作用最明显；（2）透射电镜观察 OGD+HA+GA 组凋亡现象改善最多；（3）LDH、CK-MB 及 AST 的含量变化显示 OGD+HA+GA 组心肌细胞损伤指标降低最多（$P<0.05$）；（4）Western blot 法检测结果显示 HA+GA 可以减少 OGD 对 H9c2 心肌细胞的损伤，其作用机制与 PI3K/Akt 信号通路有关。

关键词：次乌头碱；甘草次酸；H9c2 心肌细胞；凋亡；PI3K/Akt