

Full Length Research Paper

## Danshen prevents hypoxia-induced cardiomyocyte death: A hypoxia inducible factor-1 $\alpha$ (HIF-1 $\alpha$ )-dependent glucose metabolism pathway

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Hypoxia-induced cardiomyocyte death plays a critical role in the deterioration of many heart diseases. Danshen (*Salvia miltiorrhiza*), a famous traditional Chinese medicine, has shown a cardio-protective effect in both clinical and animal studies. Nevertheless, the mechanism underlying its cardioprotective effects, particularly the improvement of energy metabolism, is rarely known. Hence, the purposes of this study were to determine the active glucose metabolism involved in cardio-protection of Danshen and its possible mechanisms. Cardiomyocytes from neonatal rat were pretreated with Danshen or vehicle for 24 h, and then exposed to 1% O<sub>2</sub> for 12 h. Cellular viability, lactate dehydrogenase release, glucose uptaking, lactate leakage, pH, cell death, and the mRNA levels of hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) and glucose transporter 1 (Glut 1) were investigated. After pretreating with Danshen, the hypoxia-induced cellular damages in cardiomyocytes were significantly attenuated. Besides, the mRNA levels of HIF-1 $\alpha$  and Glut 1 were up-regulated in parallel with the enhancement of glucose uptake and metabolism in Danshen-treated cardiomyocytes. 2-Methoxyestradiol (2-ME), a HIF inhibitor, obviously reduced these protections. Our results indicate that Danshen prevents hypoxia-induced cardiomyocyte death by enhanced glucose uptaking in a HIF-1 $\alpha$  dependent manner.

**Key words:** Danshen, hypoxia, hypoxia inducible factor (HIF).

### INTRODUCTION

Hypoxia is known to be the major pathogenesis of many heart diseases, which induces cardiomyocyte necrosis and apoptosis (Danial and Korsmeyer, 2004; Mehrhof et al., 2001). As cellular loss usually leads to functional degeneration, it becomes extremely important to protect cardiomyocytes from cell death besides regeneration of injured heart.

Traditional Chinese medicine (TCM) is an essential component of complementary and alternative medicines, receiving a broad popularity in both Asia and U.S. *Salvia*

*miltiorrhiza* Bunge, named Danshen in Chinese, is widely used in clinical practice to relieve ischemic cardiovascular and cerebrovascular diseases (Fu et al., 2007; Ling et al., 2005; Zhou et al., 2005). Accumulating evidences suggest that Danshen can protect cardiomyocyte in hypoxia/ischemic condition, however, its underlying mechanism remains largely unclear, especially in the molecular and cellular level. Previous study (Xu et al., 2009) has proven that Danshen prevents myocardial ischemia injury by the elevated mRNA level of hypoxia

inducible factor-1 (HIF-1). HIF-1, the transcriptional factor involved in the hypoxic response of mammalian cells, is believed to play a key role in the regulation of hypoxic response. The activation of HIF-1 regulates downstream genes expression, including many metabolic genes, which are the key genes of glucose transporter and glycolysis. (Chi et al., 2006) Furthermore, several studies have suggested that the enhancement of glucose uptake and glycolysis can prevent cardiomyocytes from ischemic or hypoxic injury *in vivo* and *in vitro* (Brosius, 1997; Malhotra and Brosius, 1999). Therefore, considering the facts from the aforementioned, the present study aims to investigate whether HIF-1 has a role in the cardio-protection of Danshen and the profile of glucose metabolism. This study extrapolates that Danshen attenuates hypoxia-induced cardiomyocytes injuries by elevated glucose uptaking via up-regulating HIF-1 $\alpha$  in parallel with Glut 1.

## MATERIALS AND METHODS

Danshen injection (State Drug Approval Number: Z51021303) was purchased from a GMP producer Sanchine-Sunnyhope Pharmaceutical Co., Ltd (Sichuan, China). As a prescription drug, its preparation method and quality control have been standardized.

Dulbecco's Modified Eagle medium (DMEM), fetal bovine serum (FBS) and trypsin were purchased from GIBCO (USA). The 2-methoxyestradiol (2-ME), 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and collagenase II were obtained from Sigma (St. Louis, USA). All the reagents and kits for real-time polymerase chain reaction (PCR) were purchased from TaKaRa (Dalian, China). Annexin V-FITC FLOUS staining kit and Roche modular DDP System were purchased from Roche Diagnostics (Indianapolis, USA).

### Neonatal rat cardiomyocyte culture

Three days Sprague-Dawley rats were purchased from Experimental Animal Center of Sichuan University. Neonatal rat cardiomyocytes were isolated as described previously with a minor revision (Müller-Ehmsen et al., 2002). In detail, the hearts were isolated and minced into 1 to 2 mm<sup>3</sup> fragments, and were then digested with 0.1% collagenase and 0.1% trypsin (in phosphate-buffered saline (PBS) without Ca<sup>2+</sup>) for 3 to 4 times, 5 min each time. The cells from each digestion were collected in DMEM supplemented with 10% FBS in a cell culture flask for 30 min cultivation (37°C in a 5% CO<sub>2</sub> incubator) for purification. After that, the culture medium with unbonded cardiomyocytes were plated onto 6- and 96-well plates and were maintained in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. 100 mM 5-Bromo-29-deoxyuridine (BrdU) was added during the first 2 days to inhibit proliferation of non-myocytes. The cells were used after cultured for 3 days.

### Dose-response effect assays

To determine the most suitable dosage, Danshen was administrated at different concentrations (0.18, 0.9, 1.8 and 18 mg/ml), base on its clinical dose. After culture in 1% O<sub>2</sub> for 12 h, cellular viability was measured to determine the dose-response effect.

### *In vitro* hypoxia model and experimental grouping

The hypoxic condition was produced by oxygen-control incubator (Thermo Fisher Scientific Inc, USA) with humidified gas mixtures of composition 1% O<sub>2</sub>+5% CO<sub>2</sub>+94% N<sub>2</sub>. Based on our preliminary result, hypoxic time was set for 12 h. For HIF-1 signaling study, 10  $\mu$ M 2-methoxyestradiol (2-ME), a well documented HIF-1 $\alpha$  down-regulator, is used to assay the role of HIF-1 $\alpha$ . Cells were divided into 4 groups: C group: normoxia culture; H group: hypoxic culture (1% O<sub>2</sub> for 12 h); DH group: cells were pretreated with 1.8 mg/ml Danshen for 24 h prior to hypoxic culture; DH2 group: cells were pretreated with 10  $\mu$ M 2-ME and 1.8 mg/ml Danshen for 24 h prior to hypoxic culture

### Cell viability assays

MTT method was performed to evaluate the cell viability after seeded on 96-well culture plates and incubated with different treatments for indicated time period. Following exposure to hypoxia, MTT solution was added into each well with a final concentration at 0.5 mg/ml, and then the plates were incubated for an additional 4 h. After the medium was removed, 150  $\mu$ l dimethyl sulfoxide (DMSO) was added into each well, and oscillatory mixing 5 min before colorimetric reading at 490 nm by microplate reader (Molecular Devices, USA). The release of lactate dehydrogenase (LDH) was also determined by enzymatic rate method (Roche modular DDP System).

### Glucose uptake and lactate production assay

Glucose and lactate were determined using Roche modular DDP System according to the manufacturer's instructions. The values of glucose and lactate concentrations in the cultured supernatant were used to determine glucose uptake and lactate production in order to indicate the status of glucose uptake and glycolysis of cardiomyocytes in the four groups.

### Nuclear staining assay

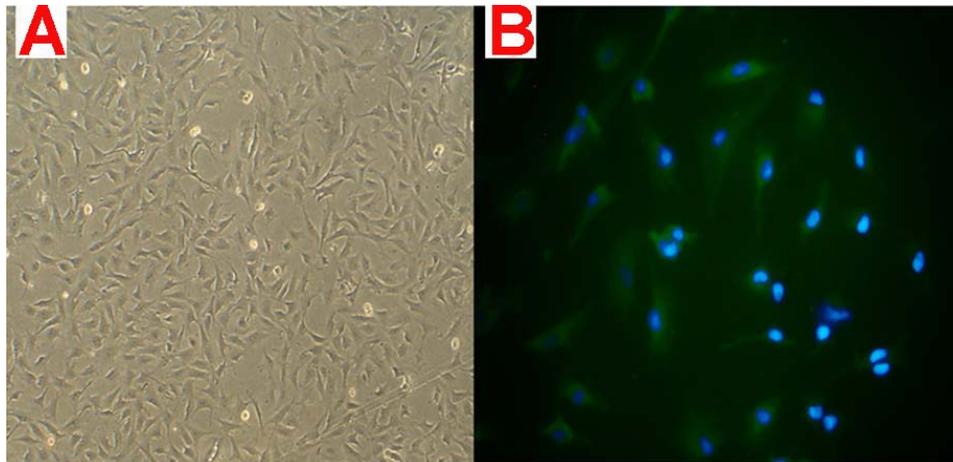
Hoechst 33258 staining was used for the assessment of apoptotic cardiomyocytes. Briefly, 4% paraformaldehyde was used to fix cells for 10 min at room temperature. After several washes with PBS, cells were exposed to Hoechst 33258 solution (5  $\mu$ g/ml in PBS) and incubated for 10 min at room temperature. Following by a final rinse with PBS, cells were examined under a fluorescence microscope with an appropriate filter. The apoptotic cells are manifested by displaying chromatin condensation and nuclear fragmentation.

### Flow cytometry analysis

Cell death was analyzed by flow cytometry, following manufacturer's guidelines. In detail, after the end of hypoxia, cells grown adherence were detached by trypsin. After being washed and resuspended three times in binding buffer (HEPES), cells were incubated for 10 min in the dark with Annexin V-FITC (1  $\mu$ g/ml) and PI solution (5  $\mu$ g/ml) at 4°C, and analyzed with a FACScan flow cytometer (BD FACSCalibur, New Jersey, USA).

### Immunofluorescence

To examine expression of Sarcomeric actin, cells cultured on glass coverslips were fixed in 4% paraformaldehyde for 30 min. After several wash with PBS, the cells were blocked in goat serum for 1



**Figure 1.** Primary culture and Immunofluorescence of cardiomyocyte. (a) Primary culture of cardiomyocyte ( $\times 100$ ); (b) Sarcomeric actin protein was revealed by immunofluorescence using a mouse polyclonal antibody to Sarcomeric actin (green) ( $\times 200$ ).

h, and then incubated overnight at  $4^{\circ}\text{C}$  with mouse monoclonal anti-Sarcomeric actin antibody (Santa Cruz, USA). After several washes by PBS, the FITC-conjugated antibody (goat anti-mouse IgM-FITC) was applied for 25 min in dark place. Fluorescent images were captured using a fluorescence microscope.

#### Real-time PCR analysis

The mRNA levels of genes were measured by fluorescent quantitative real-time polymerase chain reaction (RT-PCR). Briefly, total RNA was isolated using Trizol reagent followed by DNase treatment according to the manufacturer's instructions. One microgram of total RNA was used for cDNA synthesis, and cDNA samples were diluted in a ratio of 1:10, RT-PCR was performed using SYBR@ Premix Ex Taq™ II and the iCycler real-time PCR detection system. The following primers were used: HIF-1 $\alpha$  (Forward primer, 5'-CCA GAT TCA AGA TCA GCC AGC A-3'; reverse primer, 5'-GCT GTC CAC ATC AAA GCA GTA CTC A-3'; amplicon size, 100 bp), Glut 1 (Forward primer, 5'-GAC CCT GCA TCT CAT TGG TCT G-3'; reverse primer, 5'-AAG CCA AAG ATG GCC ACG ATA C-3'; amplicon size, 123 bp) and  $\beta$ -actin (Forward primer, 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3'; reverse primer, 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'; amplicon size, 349 bp). The mRNA levels of the gene were relative to  $\beta$ -actin and were expressed as fold of control.

#### Statistical analysis

The results were presented as Means  $\pm$  standard deviation (SD). Statistical significance was determined using one-way analysis of variance (ANOVA) followed by a least significant difference (LSD)-test and  $P < 0.05$  was considered significant.

## RESULTS

### Cell culture and identification

As shown in Figure 1A, most of the cells presented typical cardiomyocyte-like morphosis. In addition, in immunofluo-

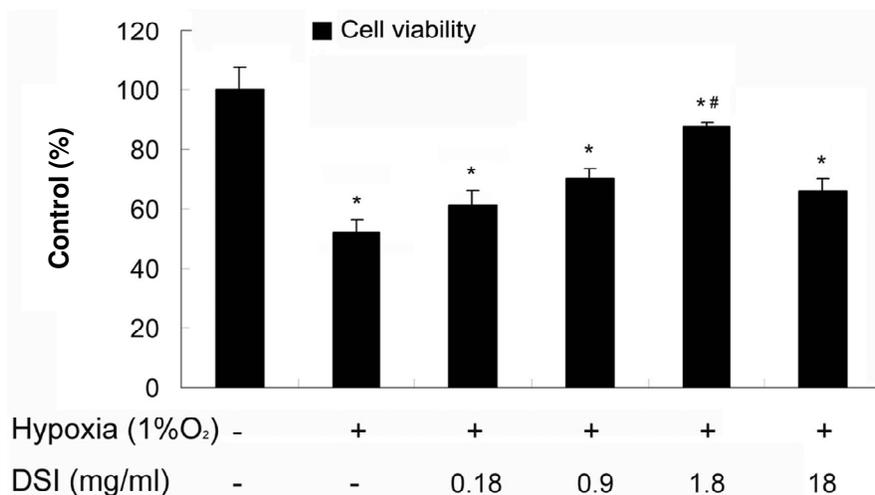
rescence detection (Figure 1B), it was found out that the  $\alpha$ -Sarcomeric actin, a myocardium-specific protein, had a wide distribution in almost all the cells (Eight high-power fields were randomly counted, and the fluorescence-positive cell accounted for 98%), which means the high-purity of the primary cultures of cardiomyocytes.

### Dose-response effect assays

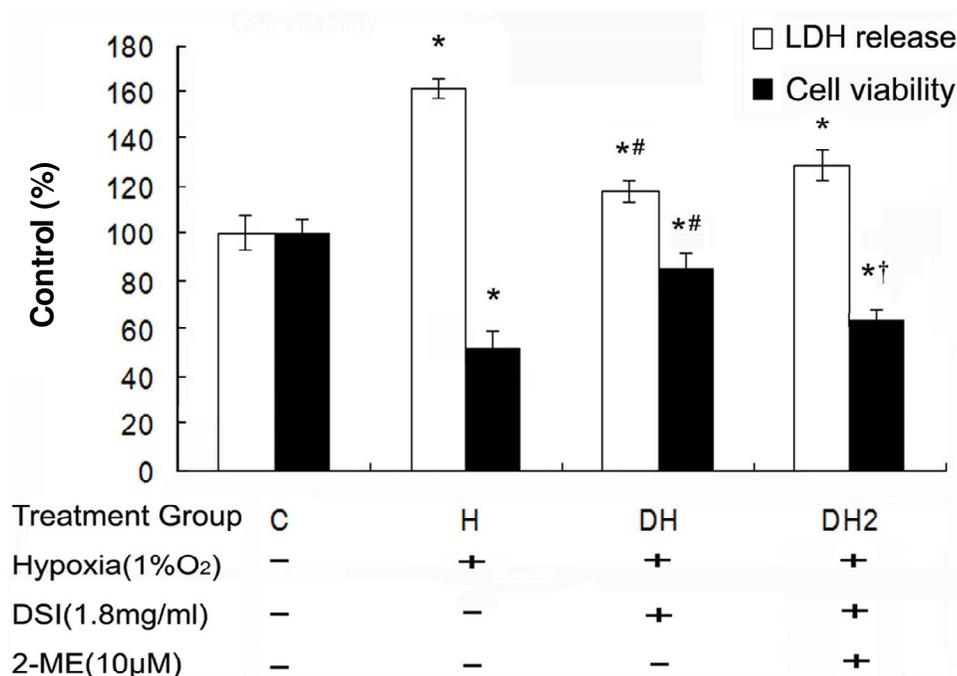
As shown in Figure 2, Danshen (0.18, 0.9, and 1.8 mg/ml) prevented cells from hypoxia-induced damage in a dose-dependent manner, restoring cell survival rate to  $66.75 \pm 1.69$ ,  $70.53 \pm 2.82$  and  $87.44 \pm 1.93\%$  of the control group, respectively. However, 18 mg/ml of Danshen did not appear better protection than 1.8 mg/ml (only  $68.44 \pm 3.13\%$ ). We also performed a dose-response curve evaluating the toxic effect of 2-ME on cardiomyocyte, and no significance was found in cell viability, after 10  $\mu\text{M}$  of 2-ME treatment ( $P > 0.05$ , data not shown).

### Danshen attenuated hypoxia-induced cell viability losing

To examine the effect of Danshen on cell viability, optical density (OD) point of MTT assay was used, and the release of lactate dehydrogenase, a necrosis indicator, as indicators of cell viability (Figure 3). The results indicate that Danshen markedly improved the cell viability and LDH release in hypoxia. In detail, the cell viability and LDH release of cells in hypoxia reach at  $58.26 \pm 6.82$  and  $161.94 \pm 5.07\%$  of normoxic control group. Cells in DH group, the Danshen treatment group, their parameter is  $87.44 \pm 6.83$  and  $117.91 \pm 7.31\%$ , respectively. Significant difference in cell viability could be found in the



**Figure 2.** Dose-response effect of variation final concentration of Danshen. Cell viability was seen as indication for the Dose-response effect. The data of Cell viability are expressed as a percentage of the control Group value. \*P<0.05 compared with C group; #P<0.05 compared with H group (n=12 for Cell viability).

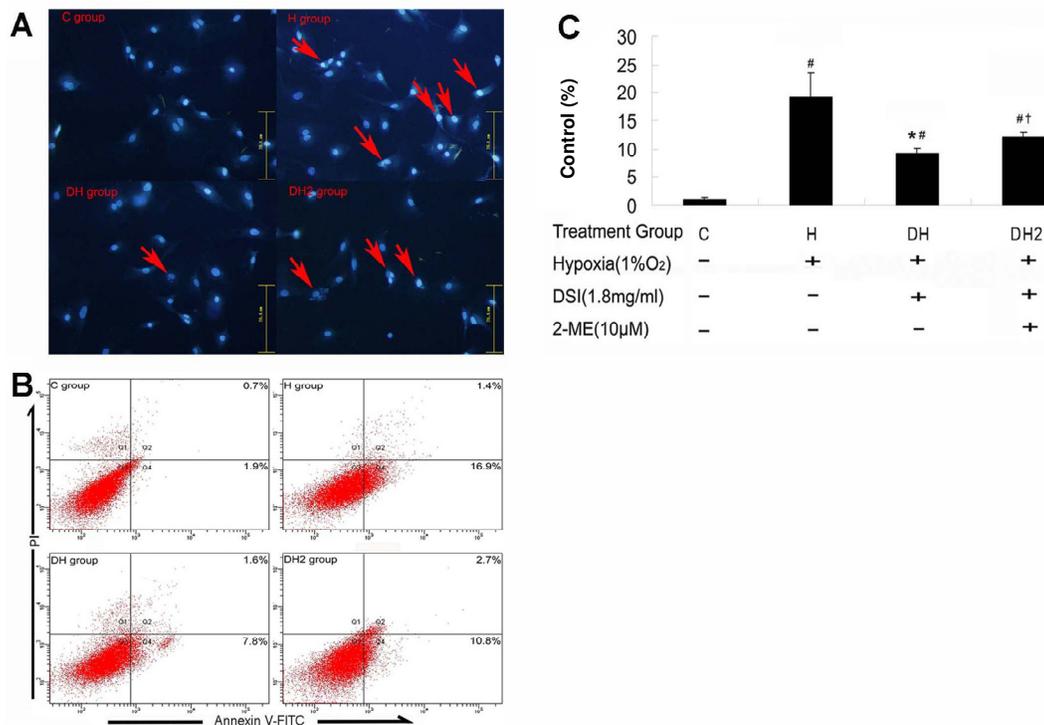


**Figure 3.** Cell viability and LDH release of different treatment group. After pretreatment, primary culture cells were incubated for 12 h in hypoxia or in normoxia, as described under Materials and Methods. The data of Cell viability and LDH release are expressed as a percentage of the control Group value. \*P<0.05 compared with control group; #P<0.05 compared with hypoxia control group; †P<0.05 compared with DH group (n=9 for Cell viability; n=9 for LDH release).

comparison of C versus H, H versus DH and DH versus DH2 (P < 0.05). However, the significant difference in the release of LDH in DH versus DH2 was not seen (P > 0.05).

### Danshen attenuated hypoxia-induced cell death

The effect of Danshen on hypoxia-induced was analyzed on cell death by Hoechst 33258 staining and flow



**Figure 4.** Glucose uptake, lactate production and pH of different treatment group. The results of glucose uptake were achieved by the total contents of glucose minus the concentrations of glucose in culture medium. All data are expressed as a percentage of the control Group value, n=5. \*P<0.05 compared with C group; <sup>#</sup>P<0.05 compared with H group; <sup>†</sup>P<0.05 compared with DH group.

cytometry. In Figure 4A, the Hoechst 33258 staining indicated that cells exposed in hypoxia showed a great nuclear fragmentation and condensation, and Danshen improve this state.

These results from Hoechst 33258 staining were further confirmed by flowcytometry analysis. As shown in Figure 4 (B and C), hypoxia increased both the apoptosis and necrosis rate of cardiomyocytes (about 9 fold elevation compare to C group, P < 0.01), Danshen significantly attenuated the hypoxia-induced cell death (about 2 fold descend compare to H group, P < 0.05), but 10  $\mu$ M 2-ME markedly attenuated this protection, as measured by flow cytometry (P < 0.01 compare to DH group).

#### Danshen enhanced glucose uptake and glycolysis

To investigate the effect of Danshen on glucose metabolism, the levels of glucose and lactate and the pH in cell cultured supernatant were tested.

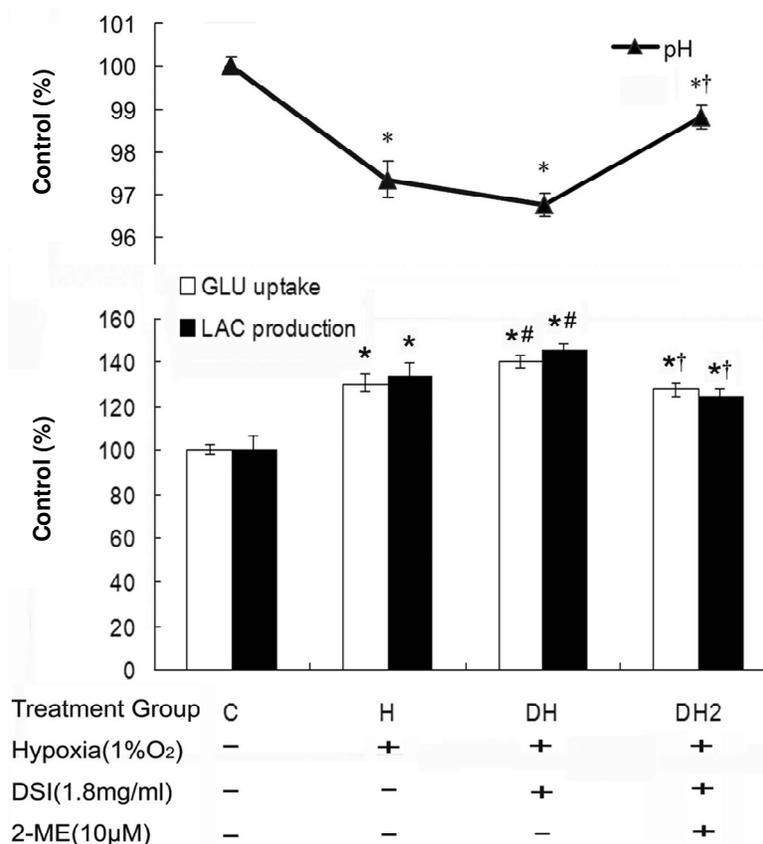
As a result, hypoxia enhanced glucose metabolism, and Danshen treatment significantly amplifies these effects from  $117.23 \pm 7.20$  to  $141.80 \pm 4.5\%$  (the uptake of glucose) and from  $118.94 \pm 7.85$  to  $148.62 \pm 4.12\%$  (the production of lactate) (P < 0.05). However, 2-ME, a HIF inhibitor, markedly inhibited these results (P < 0.05). In Figure 5, the pH point was decreasing in hypoxia, even

the pretreatment of danshen injection did not stop the decrease; however, after the addition of 2-ME, the pH increased markedly (from  $96.2 \pm 0.7$  to  $98.7 \pm 0.8\%$ , P<0.05).

These results about glucose metabolism from biochemistry assays were further confirmed by Real-Time PCR analysis in molecular level.

#### Danshen up-regulated the expression of HIF-1 $\alpha$ and its downstream gene Glut 1

To confirm the effects of Danshen on glucose uptake and metabolism, quantitative real-time PCR analysis was adopted to determine the changes in the mRNA level of HIF-1 $\alpha$  and Glut 1. As shown in Figure 6, pretreatment cardiomyocytes with Danshen significantly increased the mRNA level of HIF-1 $\alpha$  and Glut 1, compared with the hypoxia control. However, the up-regulation of HIF-1 $\alpha$  and Glut 1 mRNA level were completely abolished by pretreated cells with 10  $\mu$ M of 2-ME (P < 0.01). In detail, fluorescent quantitation of the real-time PCR revealed a 2.70 and 4.54 -fold increase in the mRNA levels of HIF-1 $\alpha$  and Glut1 of the 1.8 mg/ml of Danshen pretreated cells relative to cells in H group. After pretreatment by 10  $\mu$ M of 2-ME, the mRNA level of HIF-1 $\alpha$  and Glut 1 were dramatically reduced and the fluorescent quantitation



**Figure 5.** Cell death in different treatment group. In Hoechst 33258 staining results, apoptotic cells show nuclear fragmentation and condensation (arrow). Similar results were obtained in at least three other independent experiments.

attenuated to 0.46 and 1.17-fold compared with the cells in H group.

## DISCUSSION

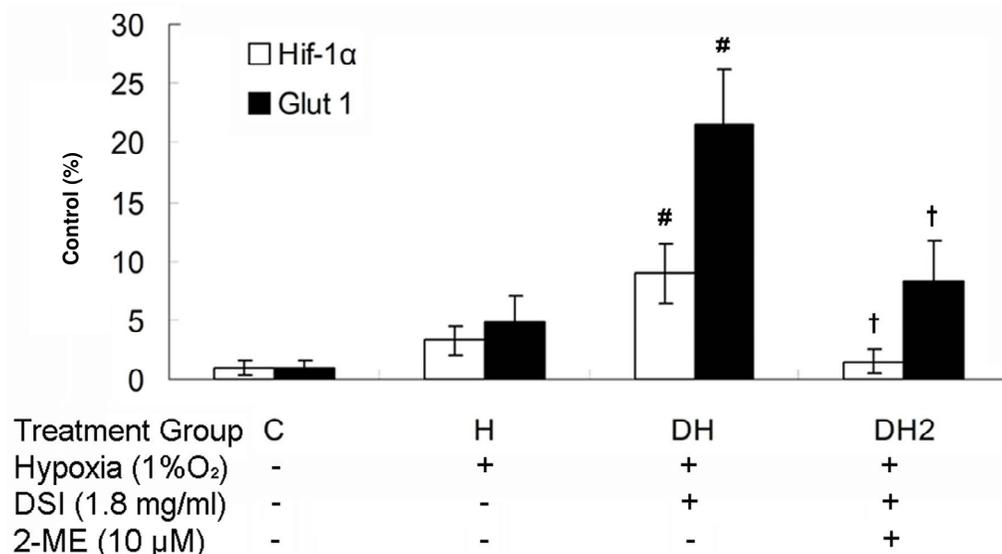
This study provides, to our knowledge for the first time, evidence that Danshen promoted the glucose uptake and glycolysis capacity of cardiomyocytes in hypoxia through enhancing the expression of Glut 1, which was regulated by the HIF-1 $\alpha$  signaling pathway.

It is well known that the fatty acid metabolism is inhibited in hypoxia. In order to maintain the energy homeostasis, glucose uptake and anaerobic glycolysis have been improved to achieve a greater amount of ATP production per mole of oxygen consumed, thus the enhancing of glucose uptake and metabolism has long been shown to be protective for ischemic myocardium (Owen et al., 1990). The data of this study found that exposure of cardiomyocytes to hypoxia for 12 h significantly increased their glucose uptake and glycolysis, and the glucose metabolism in cells pretreated with Danshen were further elevated (Figure 4). At the

same time, Danshen treatment-cells showed higher cell viability and lower cell death in hypoxia (Figures 3 and 5). These results indicated that Danshen protected hypoxia-induced cardiomyocyte injury via enhanced glucose metabolism. Furthermore, it is of interest to mention that the significant enhancement of glycolysis did not lead to the rapid decreasing of pH. In most cases, the enhancing lactate production due to the increase of anaerobic glycolysis leads to the decrease of pH, and then lower pH greatly limiting this source of ATP despite sufficient glucose supply. However, our studies interestingly found that although there was a higher lactate production in DH group, the pH of DH group did not markedly lower than H group, this phenomenon may be caused by transmembrane carbonic anhydrates (Wykoff et al., 2000), a HIF-1 target gene, regulating the pH and allowed this energy metabolism pathway to continue.

Having identified that a pharmacological action such as Danshen is capable of upregulating the glucose uptake and glycolysis in mesenchymal stem cells (MSCs), the molecular signaling pathways that might be involved in this phenomenon was further explored.

It is well known that the HIF-1 pathway is activated



**Figure 6.** mRNA levels of target gene in different treatment group. The relative mRNA levels for HIF-1 $\alpha$  (white bar) and Glut 1 (dark bar) in cells were calculated as described in the text and expressed as fold increase over control. #P<0.05 compared with H group; †P<0.05 compared with DH group (n=5).

when cells are exposed to lower oxygen tension, which render the cells adaptive response to hypoxia. In agreement with the previous studies (Jung et al., 2000), the real-time PCR data showed that the mRNA level of HIF-1 $\alpha$  in the present study was significantly increased in hypoxia, and Danshen pretreatment (DH group) markedly up-regulated the expression of HIF-1 $\alpha$  and Glut 1, as shown in Figure 6. Much of the HIF-dependent responses were considered to be associated with protecting cells against hypoxic/ischemic injury, including the induction of erythropoietin, vascular endothelial growth factor (VEGF) and glucose transporters 1 (Huang et al., 2004). Glut 1 is responsible for basal glucose transport. The elevated level of Glut 1 is considered to be an important adaptive response to minimize subsequent hypoxia-injury due to its expression improved glucose utilization and energy supply for cells (Brosius, 1997).

Since the data of this study showed that the preculture of cardiomyocytes in Danshen prior to hypoxic treatment activated HIF-1 $\alpha$  and Glut 1 while maintaining their viability and glucose metabolism. Further investigate on whether HIF-1 $\alpha$  has a central role in Danshen-induced Glut 1 expression and subsequent upregulation of glucose uptake and glycolysis in cardiomyocytes was necessary. To this end, special HIF-1 $\alpha$  inhibitor was used. As expected, the increased cell viability and glucose metabolism in Danshen treatment-cells were strongly suppressed by 2-ME (Figures 3, 4, 5 and 6; compare DH group with DH2 group), and real-time PCR results imply its mechanism may involve the down-regulating of HIF-1 $\alpha$  expression.

In summary, this study reveals new insights into the cardioprotective mechanism of Danshen in hypoxic injury

which may involves the enhancement of HIF-dependent glucose uptake, glycolysis and pH buffering pathway. These findings may be of particular interest, owing to the high prevalence of using Danshen as a conventional adjuvant to relieve ischemic diseases in clinic.

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## ABBREVIATIONS

**HIF-1**, Hypoxia inducible factor-1; **TCM**, traditional Chinese medicine; **Glut 1**, glucose transporter 1; **2-ME**, 2-methoxyestradiol.

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