

Astragalin and dihydromyricetin as adjuncts to histidine-tryptophan-ketoglutarate cardioplegia enhances protection during cardioplegic arrest

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Abstract. The present study used an *in vitro* model of cold cardioplegia in isolated working rat hearts to evaluate the possible effects of two flavonoids, astragalin and dihydromyricetin, as adjuncts to histidine-tryptophan-ketoglutarate (HTK) cardioplegia. The following three groups of male Sprague Dawley rats were evaluated: The HTK group, treated with HTK alone; the HTK-A group, treated with 10 μ mol/l astragalin; and the HTK-D group, treated with 10 μ mol/l dihydromyricetin. Isolated rat hearts were perfused with Krebs-Henseleit buffer for 30 min and incubated with the respective cardioplegic solution for 6 h at 4°C. Subsequently, astragalin or dihydromyricetin was added to the cardioplegic solutions. Following 30 min of reperfusion, the left ventricular developed pressure (LVDP), maximum up/down rate of left ventricular pressure ($\pm dp/dt_{max}$) and heart rate were documented as indices of myocardial function using a physiological recorder. Myocardial infarct size (IS) was estimated using 2,3,5-triphenyltetrazolium chloride staining. Lactate dehydrogenase (LDH) and creatine kinase (CK) levels were also determined to assess the degree of cardiac injury. Cardiomyocyte apoptosis analysis was performed using an *in situ* cell death detection kit. In addition, malondialdehyde (MDA), superoxide dismutase (SOD), interleukin-6 (IL-6), tumor necrosis factor- α (TNF- α), C-reactive protein (CRP) levels, as well as the glutathione/glutathione disulfide (GSH/GSSG) ratio were determined and analyzed using ELISA kits. The protein levels of caspase-9 and B-cell lymphoma-2 (Bcl-2) were determined using western blot analysis. The results demonstrated that exposure to astragalin

or dihydromyricetin significantly improved the recovery of LVDP ($P<0.05$ and $P<0.01$, respectively), the $+dp/dt_{max}$ ($P<0.05$ for dihydromyricetin only) and the $-dp/dt_{max}$ ($P<0.05$ and $P<0.01$, respectively), increased SOD levels ($P<0.05$ and $P<0.01$, respectively) and GSH/GSSG ratios ($P<0.05$), reduced myocardial IS ($P<0.05$ and $P<0.01$, respectively), decreased CK, LDH, IL-6 (all $P<0.05$ and $P<0.01$, respectively), MDA ($P<0.05$), CRP ($P<0.05$) and TNF- α levels ($P<0.05$ and $P<0.01$, respectively), increased Bcl-2 levels ($P<0.01$) and decreased caspase-9 levels ($P<0.01$). The results indicated that the addition of either flavonoid (particularly dihydromyricetin) to HTK enhances protection during ischemia, decreases myocardial dysfunction by enhancing anti-inflammatory activities, attenuates myocardial oxidative injury and prevents apoptosis during ischemia/reperfusion.

Introduction

At present, heart transplantation is considered the critical standard for end-stage heart failure treatment (1). Following retrieval, the donor heart is maintained in cold (4°C) cardioplegic solution, and then introduced to the recipient (2). Therefore, a period of cold ischemia and ischemia/reperfusion (I/R) are inevitable due to tissue matching and transportation. However, inadequate myocardial protection associated with prolonged cold ischemia and I/R injury will result in postoperative myocardial dysfunction (2,3). Therefore, minimization of ischemia and I/R injury during transport is essential to reduce the risk of primary graft failure and improve short- and long-term outcomes (4,5).

Histidine-tryptophan-ketoglutarate (HTK) solution is clinically used for effective organ preservation (6). A previous study revealed that the HTK solution is a valid alternative solution for organ preservation due its superior role in compensating for cellular acidosis and prolonging anaerobic glycolysis (7). However, studies have demonstrated that reperfusion following resuscitation from cardiac arrest increases reactive oxygen species (ROS) production, leading to oxidative stress (8,9). Oxidative stress is a factor involved in epithelial apoptosis (10). Previous research has also demonstrated that

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the inflammatory response may be induced during myocardial I/R, and that the cold ischemic donor organ is a major source of pro-inflammatory mediators (11,12). However, it is known that the HTK solution is unable to effectively reduce hypothermia-induced oxidative stress (13,14).

Astragalín (kaempferol-3-O-glucoside, a flavonoid that is extracted from leaves of persimmon, *rosa agrestis*, or green tea seeds) and dihydromyricetin (a flavonoid that is extracted from *ampelopsis grossedentata*) flavonoids, present a wide range of pharmacological activities, including anti-oxidative (15,16) and anti-inflammatory effects (10,17), and they have been demonstrated to ameliorate apoptosis (18,19). In addition, astragalín and dihydromyricetin may protect cells from hydrogen peroxide-induced oxidative stress damage (16,20). Furthermore, supplementation with dihydromyricetin improves glucose and lipid metabolism, and decreases the serum level of tumor necrosis factor- α (TNF- α) in nonalcoholic fatty liver disease patients (17). Previous studies have revealed that astragalín or dihydromyricetin may protect endothelial cells from oxidative stress damage via anti-oxidative, anti-inflammatory and anti-apoptotic signaling pathways *in vitro* (10,16). However, whether administration of astragalín or dihydromyricetin as an additive to the HTK solution during the hypothermic storage stage, exerts a significant protective effect on isolated cardiac grafts subject to cold ischemia, remains unclear.

Based on the pharmacological potential of astragalín and dihydromyricetin, the authors of the present study hypothesized that the addition of astragalín or dihydromyricetin to HTK may reduce cold ischemia and I/R induced injuries, thereby improving cardioprotective potential. To investigate this hypothesis, an isolated perfused heart model was generated in the present study, whereby rat hearts were perfused in the working state following preservation in the respective cardioplegic solution for 6 h at 4°C. To the best of our knowledge, the present study is the first to investigate the cardioprotective effects of cardioplegic solution containing astragalín or dihydromyricetin. In addition, the current study compared the effects of astragalín and dihydromyricetin on myocardial preservation.

Materials and methods

Chemicals. Astragalín and dihydromyricetin (purity, $\geq 98\%$) were purchased from Chengdu Must Bio-Technology Co., Ltd. (Sichuan, China). Astragalín and dihydromyricetin were dissolved in dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) prior to use. The dimethyl sulfoxide concentration in the working solutions was $<0.1\%$, which was found to exert no effects on the rats/cardiomyocytes in preliminary experiments. HTK was obtained from Beijing Witton Economic and Trade Co., Ltd. (Beijing, China).

Animals. A total of 24 adult male Sprague Dawley rats (weight, 260 ± 22 g; age, 2-3 months) were obtained from Jinan Pengyue Experimental Animal Breeding Co., Ltd. [license number, SCXK (Lu) 2014-0007; Jinan, China] and housed in a specific environment (temperature, 22-25°C; relative humidity, 50-60%; 12-h light/dark cycle; and free access to standard rodent food and water). All experimental protocols were

performed in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals and approved by the Ethics Committee of Animal Laboratory and Experimental Management Center of Shandong University [Shandong, China; license number SYXK (Lu) 20130001, revised 2013].

Study groups. Astragalín and dihydromyricetin doses used in the present study were determined by preliminary experiments; 5, 10 and 20 $\mu\text{mol/l}$ of these agents were selected for preliminary analysis. Measurement of heart hemodynamic parameters revealed that 10 $\mu\text{mol/l}$ of each agent significantly improved functional recovery during early reperfusion (data not shown). This is consistent with the results of some studies indicating that this moderate dose may demonstrate protective effects (21,22). Therefore, 10 $\mu\text{mol/l}$ was selected for the experiments performed in the current study.

The hearts from rats subjected to 6 h ischemia followed by 30 min perfusion were divided into the following three groups according to the cardioplegic solution used: The HTK group, treated with HTK alone; the HTK-A group, treated with HTK containing 10 $\mu\text{mol/l}$ astragalín; and the HTK-D group, treated with HTK containing 10 $\mu\text{mol/l}$ dihydromyricetin.

Model of the isolated and perfused working rat heart. Ischemia was performed according to procedures described previously (5). Rats were anesthetized with 10% chloral hydrate (300 mg/kg body weight) through intraperitoneal injection, and then administered with 250 U/kg heparin (Solebo Biotechnology Co., Ltd., Beijing, China) through sublingual venous injection to prevent coagulation. The chest was then opened using a bilateral sternocostal triangle. The hearts were immediately excised and placed into 40 ml of respective cardioplegic solution. The hearts were carefully washed for 10 sec to remove blood from the coronaries and to avoid the formation of blood clots. Subsequently, the hearts were placed in a conical tube with 10 ml ice-cold cardioplegic solution at 4°C for 6 h. A Langendorff perfusion system (Powerlab, Australia; www.adinstruments.com/) was then used to perform heart perfusion. Hearts were perfused via their aortas at a constant pressure of 75 mmHg using Krebs-Henseleit buffer (120 mM NaCl, 1.2 mM KH_2PO_4 , 1.2 mM CaCl_2 , 1.2 mM MgSO_4 , 25 mM sodium acetate and 11 mM glucose, pH 7.4). All chemicals and reagents were purchased from Solebo Biotechnology Co., Ltd.) at 37°C for 30 min. A water-filled latex balloon connected to a pressure transducer was inserted into the left ventricle through an incision in the left atrium and through the mitral valve, and the volume was adjusted to achieve a stable end-diastolic pressure (8-12 mmHg). A number of cardiac parameters, including heart rate (HR), left ventricular developed pressure (LVDP) and maximum up/down rate of left ventricular pressure ($\pm dp/dt_{\text{max}}$), were continuously monitored and recorded using the 4S AD Instruments biology polygraph data acquisition system and Chart5 software (both Powerlab). Following 30 min reperfusion, the hearts were stored at -80°C until downstream analysis.

Measurement of cellular injury. The myocardium contains an abundance of diagnostic marker enzymes that can be used to detect myocardial infarction. Following metabolic damage to the myocardium, intracellular contents are released into

the extracellular fluid (23). Therefore, the levels of these marker enzymes reflect alterations in membrane integrity and/or permeability. In the present study lactate dehydrogenase (LDH) and creatine kinase (CK) levels were measured to evaluate the degree of cardiac injury. LDH ELISA kits (Nanjing Jiancheng Biological Products, Co., Ltd., Nanjing, China. Cat. no. A02020150421) and CK ELISA kits (Nanjing Jiancheng Biological Products, Co., Ltd., Nanjing, China. Cat. no. A03220150501) were used to measure LDH and CK levels according to the manufacturer's protocol. Samples were collected from the respective cardioplegic solutions prior to reperfusion and from the coronary effluent following 30 min of reperfusion.

Evaluation of myocardial infarct size (IS). Myocardial IS was evaluated using triphenyltetrazolium chloride (TTC) staining, as previously described (24). At the end of the experiments, the hearts were removed from the cardioplegic solutions, washed in phosphate-buffered saline and stored at -20°C for 30 min. Subsequently, the hearts were sliced perpendicularly into 1 mm-thick sections along the long axis from apex to base and incubated in 1% TTC at 37°C for 15 min. Slices were imaged using a digital camera following fixation in 10% formaldehyde solution at 25°C for 24 h. Image-Pro Plus 7.0 software (Media Cybernetics, Inc., Rockville, MD, USA) was used to measure the IS area. Red regions indicated non-ischemic areas, whereas white regions indicated ischemic areas. The IS (%) was calculated using the following equation: Infarct volume (%)=(infarct volume/total volume of slice) x100.

Analysis of oxidative stress in heart tissue homogenates. Following 30 min perfusion, the hearts were harvested and maintained at -80°C for subsequent analysis. The frozen ventricles were crushed to a powder using liquid nitrogen-chilled tissue pulverizer. For tissue analysis, weighed quantities of frozen tissues (100 mg) were homogenized in a stroke-physiological saline solution using a microcentrifuge tube homogenizer. Superoxide dismutase (SOD) activity and the glutathione/glutathione disulfide (GSH/GSSG) ratio are typically used to represent the antioxidant molecules of antioxidant systems (24). In addition, malondialdehyde (MDA) is the end-product of lipid peroxidation metabolism, and its content directly reflects the rate and extent of lipid peroxidation (25,26). Therefore, the MDA level, SOD activity and GSH/GSSG ratio in heart homogenates were measured using MDA kit (cat. no. A00320150624), SOD kit (cat. no. A00120150221), GSH kit (cat. no. A00620150214) and GSSG kit (cat. no. A06120150207), according to the manufacturer's protocol.

Analysis of inflammation in heart tissue homogenates. TNF- α , C-reactive protein (CRP) and interleukin-6 (IL-6) were analyzed using ELISA TNF- α kit (cat. no. MI002126), CRP kit (cat. no. MI105432) and IL-6 kit (cat. no. MI012815) following the manufacturer's protocol (Enzyme-linked Biotechnology Co., Ltd., Shanghai, China). The concentrations of the cytokines were quantified by referencing standard curves.

Evaluation of apoptosis. Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labelling (TUNEL) assay was performed according to the manufacturer's instructions

using the In Situ Cell Death Detection kit (Roche Diagnostics GmbH, Mannheim, Germany) according to a previously described method (27). Following deparaffinization and rehydration, the sections were treated with 10 mM protease K at 37°C for 15 min. Slides were immersed in TUNEL reaction mixture for 60 min at 37°C in a humidified atmosphere in the dark and converter-peroxide was used to incubate the slides at 37°C for 30 min to reveal blue nuclear staining. The slides were subsequently analyzed using light microscopy. The TUNEL index (%) was obtained as the ratio of the number of TUNEL-positive cells divided by the total number of cells and used to evaluate the apoptotic index of TUNEL-stained heart tissues. For each sample, eight randomly selected areas of TUNEL-stained slices were counted and the mean value was calculated.

Western blot analysis. Protein expression levels of caspase-9 and B-cell lymphoma-2 (Bcl-2) were determined using western blot analysis. Following 30 min of perfusion with the Langendorff apparatus, the ventricular apical of the rats was cut, homogenized in appropriate buffer (50 mM Tris-HCl, pH 7.6, 0.5% Triton X-100, 20% glycerol. All chemicals and reagents were purchased from Solebo Biotechnology Co., Ltd.) and centrifuged at 15,000 g for 15 min at 4°C. Supernatant was extracted and boiled for 15 min to promote protein denaturation. Protein extracts (the protein concentration was determined by bicinchoninic method) were separated using 12% SDS-PAGE. Proteins were transferred to nylon membranes using an electrophoretic transfer system. The membranes were blocked with 5% skimmed milk blocking buffer at 25°C for 1 h and then incubated with primary antibodies overnight (18 h) at 4°C. The membranes were subsequently washed with Tris-buffered saline with Tween-20 (3 times, 5 min each) at 25°C and the corresponding secondary antibodies were used to identify primary antibody binding at 25°C for 60 min. Finally, the blots were visualized with enhanced chemiluminescence-plus reagent (Beijing Solarbio Biological Products, Co., Ltd.) to visualize protein bands, and imaged using the Bio-Rad Gel Doc 2000 imaging system.

Statistical analysis. Data are presented as mean \pm or + standard deviation as indicated (n=8 for each condition). A Student's t-test and two-way analysis of variance, followed by Tukey's test were used to analyze the results. Statistical analysis was performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Addition of dihydromyricetin improves the recovery of cardiac function. Cardiac mechanical performance was evaluated by measuring LVDP, $\pm dp/dt_{max}$ and HR Table I indicates the LVDP, $\pm dp/dt_{max}$ and HR obtained during reperfusion of donor hearts. LVDP ($P<0.01$), $\pm dp/dt_{max}$ ($P<0.05$), $-dp/dt_{max}$ ($P<0.01$) and HR ($P<0.05$) were significantly increased in the HTK-D group when compared with the HTK group (Table I). Hearts in the HTK-A group also demonstrated a significant increase in LVDP, $-dp/dt_{max}$ and HR compared with those in the HTK group ($P<0.05$; Table I).

Table I. Hemodynamic variables among different groups following 6 h of storage and 30 min of reperfusion.

Physical index	HTK group	HTK-A group	HTK-D group
LVDP (mmHg)	64.8±0.38	77.90±3.21 ^a	91.51±5.21 ^b
+dp/dt _{max} (mmHg/sec)	1,234.89±100.13	1,409.38±90.13	1,613.84±126.13 ^a
-dp/dt _{max} (mmHg/sec)	-806.21±70.13	-868.01±60.13 ^a	-1,040.09±101.13 ^b
HR (beats/min)	238.43±43.34	266.80±40.13 ^a	274.70±60.13 ^a

^aP<0.05 and ^bP<0.01 vs. HTK group. Values are presented as the mean ± standard deviation (n=8). LVDP, left ventricular developed pressure; ±dp/dt_{max}, maximum up/down rate of left ventricular pressure; HR, heart rate; HTK, histidine-tryptophan-ketoglutarate; HTK-A, histidine-tryptophan-ketoglutarate + astragaline; HTK-D, histidine-tryptophan-ketoglutarate + dihydromyricetin.

Addition of astragaline or dihydromyricetin attenuates I/R-induced enzyme release. Necrosis was assessed by the release of LDH and CK into the coronary effluent among the three groups. As indicated in Table II, LDH and CK levels released in the three cardioplegic solutions were not significantly different among the groups during cold storage. However, significantly increased LDH and CK levels were observed in the HTK-A (P<0.05) and HTK-D (P<0.01) groups compared with the respective HTK groups following 30 min reperfusion. These results reflect the cell death of myocytes during reperfusion. The present findings suggested that the addition of astragaline to HTK significantly reduced the release of LDH and CK following reperfusion. In particular, administration of dihydromyricetin significantly prevented the release of LDH and CK to a greater extent when compared with astragaline.

Addition of astragaline or dihydromyricetin to HTK-induced IS. Myocardial IS may be used as an indicator of myocardial injury (24). Representative images of heart sections from rats stained with TTC are indicated in Fig. 1. The addition of astragaline significantly reduced I/R-induced myocardial IS by 26.67±1.98% (P<0.05), whereas the addition of dihydromyricetin significantly reduced I/R-induced myocardial IS by 18.36±1.67% (P<0.01) compared with the HTK group (Fig. 1).

Addition of astragaline or dihydromyricetin to HTK alleviates oxidative stress. As indicated in Fig. 2, the addition of astragaline (P<0.05) or dihydromyricetin (P<0.01) to HTK significantly decreased MDA levels (P<0.05; Fig. 2A), and significantly increased SOD activities (Fig. 2B) and GSH/GGSS ratios (P<0.05; Fig. 2C) compared with the HTK group. These results indicated that the addition of astragaline or dihydromyricetin may increase the antioxidant ability of HTK.

Addition of astragaline or dihydromyricetin to HTK weakens cardiomyocyte apoptosis. To test whether the addition of astragaline or dihydromyricetin enhanced the protection against apoptosis during storage and reperfusion, TUNEL assays were performed. Under an optical microscope, TUNEL staining revealed a large number of apoptosis (42.58±4.29%) in the HTK group (Fig. 3A). The number of apoptotic cells was significantly decreased in the HTK-A (36.51±3.63%) and HTK-D (28.13±3.36%) groups (Fig. 3B). The HTK-D group exhibited the greatest reduction in the number of apoptotic cells (Fig. 3B).

Table II. Effect of astragaline or dihydromyricetin on levels of LDH and CK in cardioplegic solution prior to reperfusion and in the coronary effluent following reperfusion.

Physical index	In cardioplegic solution	At 30 min following reperfusion
CK (U/l)		
HTK group	3.90±0.13	57.24±6.13
HTK-A group	4.50±0.24	48.97±4.13 ^a
HTK-D group	4.23±0.19	38.67±5.13 ^b
LDH (U/l)		
HTK group	3.16±0.23	65.31±5.13
HTK-A group	2.98±0.16	50.12±4.32 ^a
HTK-D group	3.36±0.21	41.12±3.62 ^b

^aP<0.05 and ^bP<0.01 vs. HTK group. Values are presented as the mean ± standard deviation (n=8). LDH, lactate dehydrogenase; CK, creatine kinase; HTK, histidine-tryptophan-ketoglutarate; HTK-A, histidine-tryptophan-ketoglutarate + astragaline; HTK-D, histidine-tryptophan-ketoglutarate + dihydromyricetin.

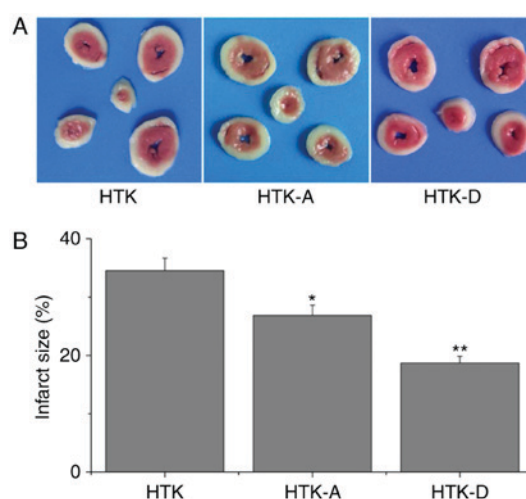


Figure 1. Effects of astragaline and dihydromyricetin on IS. (A) Representative images of heart sections stained with triphenyltetrazolium chloride from the HTK, HTK-A and HTK-D groups. Red regions indicate non-ischemic areas, whereas white regions indicate ischemic areas. (B) Quantitative analysis of IS percentage. Values were presented as the mean ± standard deviation (n=8). *P<0.05 and **P<0.01 vs. HTK group. HTK, histidine tryptophan keto-glutarate; HTK-A, histidine tryptophan keto-glutarate + astragaline; HTK-D, histidine tryptophan keto-glutarate + dihydromyricetin; IS, infarct size.

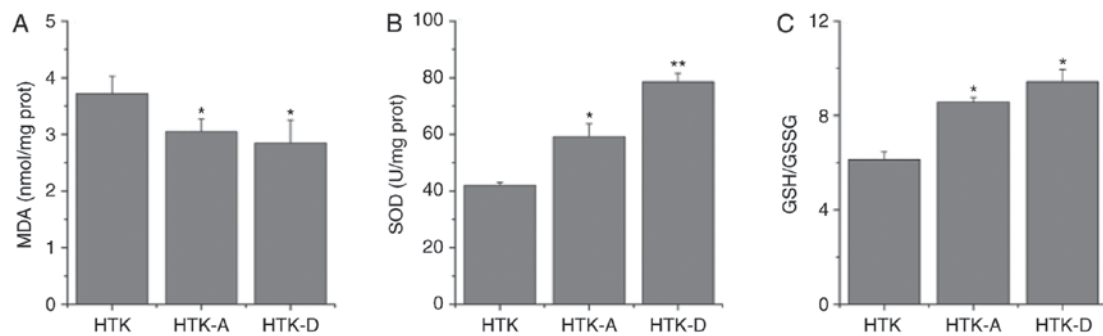


Figure 2. Effects of astragaline and dihydromyricetin combined with HTK on oxidative injury of cardiac grafts subjected to 6 h of cold ischemia followed by 30 min of reperfusion. (A) MDA levels. Addition of astragaline or dihydromyricetin significantly reduced MDA levels compared with HTK treatment alone. (B) Tissue SOD activity. Addition of astragaline or dihydromyricetin to HTK significantly increased SOD activity. (C) GSH/GSSG ratio. HTK with astragaline or dihydromyricetin significantly increased the GSH/GSSG ratio. Values were presented as the mean + standard deviation (n=8). *P<0.05 and **P<0.01 vs. HTK group. HTK, histidine tryptophan ketoglutarate; HTK-A, histidine tryptophan ketoglutarate + astragaline; HTK-D, histidine tryptophan ketoglutarate + dihydromyricetin; SOD, superoxide dismutase; MDA, malondialdehyde; GSH/GSSG, glutathione/glutathione disulfide; prot, protein.

Addition of astragaline or dihydromyricetin to HTK reduces the inflammatory response. Inflammation is an important mechanism underlying myocardial injury. The presence of inflammatory cytokines, including CRP, IL-6 and TNF- α , is associated with myocardial injury (24). Therefore, the present study determined the levels of these inflammatory cytokines in myocardial tissues to identify the possible mechanisms underlying the cardioprotective activity of astragaline or dihydromyricetin. As demonstrated in Fig. 4A, the content of TNF- α in the HTK-A group (708.59 ± 51.15 ng/l) and HTK-D group (623.93 ± 51.15 ng/l) were significantly decreased (P<0.05 and P<0.01, respectively) compared with that in the HTK group (772.10 ± 47.53 pg/ml; Fig. 4A). Secretion of CRP (Fig. 4B) was also significantly decreased following astragaline (P<0.05) or dihydromyricetin (P<0.05) treatment compared with the HTK group. In addition, secretion of IL-6 (Fig. 4C) was significantly decreased following astragaline (P<0.05) or dihydromyricetin (P<0.01) treatment compared with the HTK group.

Effect of astragaline and dihydromyricetin preconditioning on the protein expression levels of caspase-9 and Bcl-2. The expression levels of caspase-9 and Bcl-2 proteins determined by western blot analysis from the left ventricular cavity of the rats was determined (Fig. 5A). Quantification of the western blot results indicated that astragaline and dihydromyricetin preconditioning inhibited the increase of proapoptotic protein caspase-9 (P<0.01; Fig. 5B). Furthermore, astragaline and dihydromyricetin preconditioning significantly increased the protein expression levels of antiapoptotic Bcl-2 compared with the HTK group (P<0.01; Fig. 5C). The results indicated that the effect of dihydromyricetin on the expression levels of the two proteins is superior compared with astragaline.

Discussion

The present study demonstrated that donor heart preservation solution containing astragaline or dihydromyricetin improves cardio protection compared with HTK alone during the first 6 h of cold static storage and 30 min of reperfusion. Improved cardio protection was indicated by normalized cardiac function, reduced intracellular oxidation status and inflammatory response, and inhibited myocardial apoptosis. Furthermore, the

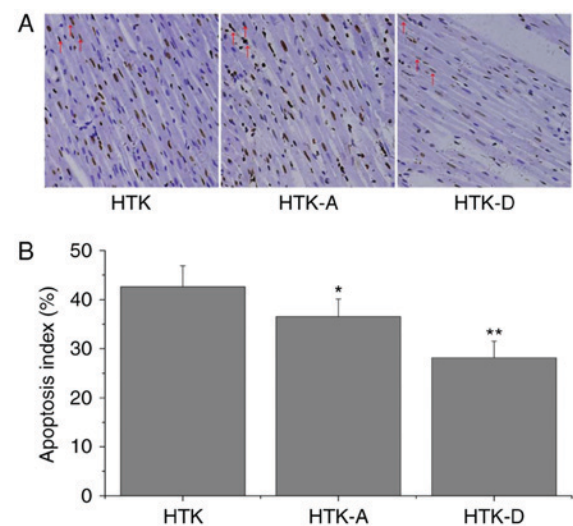


Figure 3. Effects of astragaline or dihydromyricetin on cardiomyocyte apoptosis (magnification, x400). (A) Representative immunohistochemical staining results of apoptotic cells in rat myocardium among the different groups, as demonstrated by the appearance of dark brown cell nuclei (indicated by red arrows). (B) Quantitative measurement of the terminal deoxynucleotidyl-transferase-mediated dUTP nick end labelling index (apoptosis percentage) among different groups. Values were presented as the mean + standard deviation (n=8). *P<0.05 and **P<0.01 vs. HTK group. HTK, histidine tryptophan ketoglutarate; HTK-A, histidine tryptophan ketoglutarate + astragaline; HTK-D, histidine tryptophan ketoglutarate + dihydromyricetin.

present results suggested that the effect of dihydromyricetin may exceed that of an equivalent dosage of astragaline as an adjunct to the HTK solution. However, further research is required to investigate this phenomenon in larger and longer-term studies.

Developments in science and technology have significantly improved the methods of graft preservation, and the availability of donor hearts has also increased (28). However, the composition of the cardioplegic solution is one of the key factors influencing the success of cold static preservation, and the methods of minimizing graft dysfunction caused by IR injury remain one of the primary objectives for myocardium protection (2,29).

The coupled effects of ischemia and hypothermia during organ preservation result in inevitable deterioration

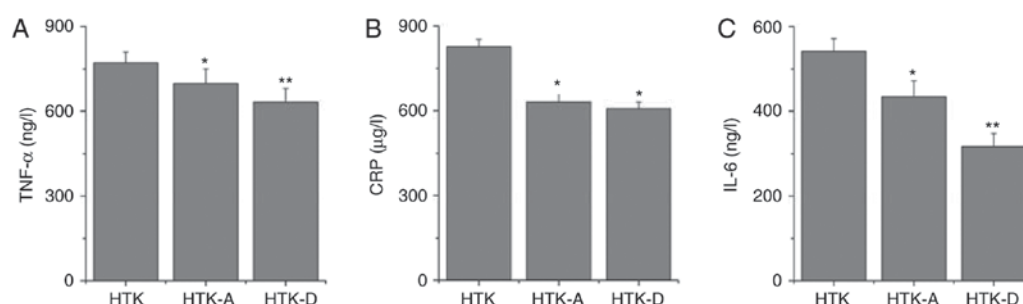


Figure 4. Effects of astragaloside or dihydromyricetin on myocardial concentrations of pro-inflammatory mediators in grafts subjected to 6 h of cold ischemia followed by 30 min of reperfusion. (A) TNF- α , (B) CRP and (C) IL-6 levels were determined in rat heart tissue using ELISA. Values were presented as the mean + standard deviation (n=8). *P<0.05 and **P<0.01 vs. HTK group. HTK, histidine tryptophan ketoglutarate; HTK-A, histidine tryptophan ketoglutarate + astragaloside; HTK-D, histidine tryptophan ketoglutarate + dihydromyricetin; IL-6, interleukin-6; TNF- α , tumor necrosis factor- α ; CRP, C-reactive protein.

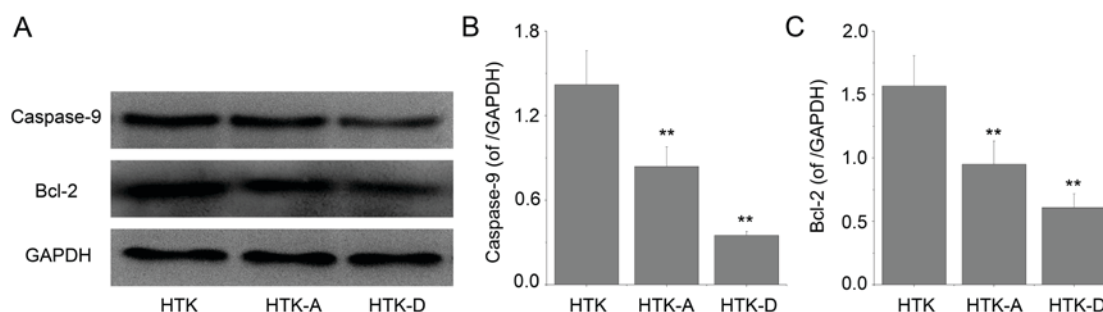


Figure 5. Effects of astragaloside or dihydromyricetin on protein expression levels of anti-apoptotic mediators caspase-9 and Bcl-2 in grafts subjected to 6 h of cold ischemia followed by 30 min of reperfusion. GAPDH was used as a loading control. (A) Representative image of the western blot. Quantitative results of (B) caspase-9 and (C) Bcl-2 protein expression levels. Values were presented as the mean + standard deviation (n=8). *P<0.05 and **P<0.01 vs. HTK group. HTK, histidine tryptophan ketoglutarate; HTK-A, histidine tryptophan ketoglutarate + astragaloside; HTK-D, histidine tryptophan ketoglutarate + dihydromyricetin.

prior to organ transplantation (2,3). Previous studies have demonstrated that cold ischemia alone may induce the marked generation of ROS, which are decisive mediators of cold storage-induced injury (2,30). When the amount of ROS available exceeds the capacity of the enzymes (such as GSH and SOD) and cannot be diminished during reperfusion, oxidative stress occurs (26). Oxidative stress-associated alterations in several intracellular signaling pathways have been implicated in the pathophysiology of severe donor heart damage during reperfusion (31). In line with these findings, preventing or at least controlling the increase in the oxidative stress levels of donor hearts during cold storage seems to be a crucial part of heart transplantation in cardiac surgery. Thus, the capability of astragaloside or dihydromyricetin to alleviate oxidative injury was assessed by investigating the myocardial levels of MDA, GSH/GSSG ratio and SOD activity in the present study.

The present results demonstrated the improved antioxidant status of astragaloside or dihydromyricetin-rich HTK preserved grafts, which was consistent with the results of the number of released myocardial enzymes following reperfusion. Although the quantity of enzymes released was not significantly different during cold storage, levels were significantly increased following reperfusion. This finding may demonstrate that the period immediately following reperfusion is the most crucial stage for generating ROS (32). Studies have also indicated that the histidine component in HTK exerts antioxidant effects (33). Thus, enhancement of antioxidant activity and inhibition of free radical peroxidation in the

myocardium may be partially involved in the cardioprotective mechanisms of HTK combined with astragaloside or dihydromyricetin. Previous studies have demonstrated that the biological markers of hemodynamic parameters, including LVDP, $\pm dp/dt_{max}$ and HR and IS, are major determinants of myocardial function and viability (5,34). In the present study, HTK combined with dihydromyricetin or astragaloside significantly improved the recovery of hemodynamic parameters and attenuated IS.

Inflammation and apoptosis, particularly the former, are typical consequences of myocardial damage during I/R, and inflammation may result in activation of the innate immune response (5,26,35). An under-explored area that may hold great potential for improving transplantation outcomes is the design of novel strategies to apply to organs specifically to reduce intra-graft inflammation (36). To investigate the association between the anti-inflammatory effects and the cardioprotective effects of the astragaloside or dihydromyricetin flavonoids, an experiment was performed to explore whether they can affect changes in CRP, IL-6, and TNF- α induced by I/R.

In the present study, that addition of astragaloside or dihydromyricetin significantly reduced the concentrations of CRP, IL-8, IL-6 and TNF- α compared with HTK alone. A significant decrease in TUNEL-positive nuclear staining in the sectioned left ventricular myocardium was observed in the HTK-D group and HTK-A group when compared with the group treated with HTK alone. ROS have been reported to be potent inducers of various cytokines, including TNF- α

and IL-6, and promote cell apoptosis (2,10). Notably, the present study indicated that astragaloside and dihydromyricetin demonstrate antiapoptotic effects. However, further investigation is required to investigate whether the observed anti-inflammatory and anti-apoptotic effects of astragaloside and dihydromyricetin are direct or only secondary to their anti-oxidative effects.

In conclusion, the results of the present study indicated that the addition of astragaloside or dihydromyricetin to HTK significantly reduces myocardial injury, decreases oxidative stress, prevents the apoptotic process, reduces inflammatory response and enhances cardiac performance. In particular, the present study provided preliminary evidence that HTK combined with dihydromyricetin may have potential clinical applications in cardiac transplantation. However, as heart transplantation using donor hearts stored in HTK combined with adjuncts has not yet been performed, other cardiac parameters, including echocardiograms, should also be tested. Studies investigating the biological activities of astragaloside and dihydromyricetin are in the initial stages, and further studies are necessary to fully understand the molecular mechanisms involved in the protective effects of astragaloside or dihydromyricetin combined with cardioplegic solutions.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DW and XZ designed the experiments. DW, XZ, DQ, JH, FM, MX performed the research. DW and QZ analyzed the data and wrote the paper, which was revised by DW and XZ.

Ethics approval and consent to participate

All experimental protocols were performed in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals and approved by the Ethics Committee of Animal Laboratory and Experimental Management Center of Shandong University [Shandong, China; license no. SYXK (Lu) 20130001, revised 2013].

Competing interests

The authors report they have no competing interests.

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