

# Luteolin improves heart preservation through inhibiting hypoxia-dependent L-type calcium channels in cardiomyocytes

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**Abstract.** The current study aimed to evaluate whether luteolin could improve long-term heart preservation; this was achieved by evaluating the heart following long-term storage in University of Wisconsin solution (the control group) and in solutions containing three luteolin concentrations. The effects of different preservation methods were evaluated with respect to cardiac function while hearts were in custom-made *ex vivo* Langendorff perfusion systems. Different preservation methods were evaluated with respect to the histology, ultra-structure and apoptosis rate of the hearts, and the function of cardiomyocytes. In the presence of luteolin, the rate pressure product of the left ventricle was increased within 60 min of reperfusion following a 12-h preservation, coronary flow was higher within 30 min of reperfusion, cardiac contractile function was higher throughout reperfusion following 12- and 18-h preservations, and the left ventricle peak systolic pressure was significantly higher compared with the control group (all  $P < 0.05$ ). The expression levels of apoptosis regulator Bax and apoptosis regulator Bcl-2 in the luteolin groups were significantly decreased and increased, respectively. Lactate dehydrogenase, creatine kinase and malondialdehyde enzymatic activity was increased following long-term storage, while the activity of superoxide dismutase was significantly decreased. Furthermore, luteolin inhibited L-type calcium currents in ventricular myocytes under hypoxia conditions. Thus, luteolin demonstrated protective effects during long-term heart preservation in what appeared to be a dose-dependent manner, which may be accomplished through inhibiting hypoxia-dependent L-type calcium channels.

## Introduction

Heart transplantation is a widely accepted treatment for end stage heart disease (1). Currently, safe heart storage is restricted to 6 h (2), making it impossible to supply donor hearts in emergencies to patients who are a large distance away from donors. Thus, it is necessary to explore approaches that may extend the storage time (3).

The heart consumes a large amount of oxygen to support cardiomyocytes, which can become hypoxic within 8 min of the heart stopping (4). Thus, during preservation and transplantation, cardiomyocytes suffer pathological changes, including acidosis, autolysis and aggravated myocardial damage. These changes are caused by free radicals and calcium overload following reperfusion, leading to deteriorations in metabolism, function and the ultrastructure of the cells (5). An improved heart preservation solution with ATP precursors could be effective in preventing cellular edema, acidosis, and intercellular edema and injury. Currently, effective preservation methods, including University of Wisconsin solution (UW), histidine-tryptophan-ketoglutarate solution and Celsior solution, cannot extend effective heart preservation beyond 6 h (6-8).

Previous studies have revealed that various traditional Chinese medicines are effective in protecting organs from reperfusion-induced injury, including root of red-rooted salvia, astragalus saponin and ligustrazine (9-15). Luteolin (3',4',5,7-tetrahydroxyflavone,  $C_{15}H_{10}O_6$ ) is a widely distributed natural flavonoid and the main active ingredient in various medicinal plants (16). Luteolin has exhibited anti-oxidative, anti-bacterial, anti-inflammatory and anti-viral effects; it has been demonstrated to lower blood fat and cholesterol, and inhibit intracellular calcium elevation (17-20). However, whether luteolin may be used in heart preservation remains unknown.

In the current study, the effect of luteolin on heart preservation and the underlying mechanisms of luteolin were explored. The present study may provide a theoretical basis for preserving hearts with luteolin.

## Materials and methods

**Animal and heart dissection.** Specific pathogen free-grade Sprague Dawley rats (n=80, 40 females and 40 males; age, 12-13 weeks; weight, 250 g) were obtained from the

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Animal Center of Xi'an Jiaotong University (Xian, China). Rats were given access to a standard diet of Animalabo A 04 and water *ad libidum*, maintained under controlled conditions of light (12-h light/dark cycle), temperature ( $22\pm1^{\circ}\text{C}$ ) and humidity ( $35\pm5\%$ ) and. The animals were separated into four groups (control, low luteolin  $7.5\ \mu\text{M}$ , medium luteolin  $15\ \mu\text{M}$  and high luteolin  $30\ \mu\text{M}$ ;  $n=20/\text{group}$ ). In each group, dissected hearts were stored for 12 ( $n=10$ ) or 18 h ( $n=10$ ). Prior to heart dissection, rats were fasted for 12 h, anesthetized with pentobarbital sodium ( $30\ \text{mg/kg}$ ; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and then administered heparin ( $3\ \text{mg/kg}$ ) by intraperitoneal injection. Breathing was observed for 5 min before additional doses of pentobarbital sodium ( $10\text{--}30\ \text{mg/kg}$ ) were administered by intraperitoneal injection, and sacrifice by cervical dislocation. The chest cavity was immediately opened, the main arteries were cut and the hearts were transferred into  $37^{\circ}\text{C}$  Krebs-Henseleit (KH) solution ( $118\ \text{mM NaCl}$ ,  $4.7\ \text{mM KCl}$ ,  $0.9\ \text{mM KH}_2\text{PO}_4$ ,  $1.2\ \text{mM MgSO}_4\cdot 7\text{H}_2\text{O}$ ,  $1.5\ \text{mM CaCl}_2$ ,  $25\ \text{mM NaHCO}_3$  and  $11\ \text{mM C}_6\text{H}_{12}\text{O}_6$ ,  $\text{pH}=7.4$ ). Following the washing-out of residual blood, an aorta perfusion tube was set up and the hearts were transferred to a Langendorff system (custom-made) for perfusion with  $37^{\circ}\text{C}$  filtered KH solution balanced with  $95\%\ \text{O}_2$  and  $5\%\ \text{CO}_2$  (perfusion pressure of  $75\ \text{cm H}_2\text{O}$ ). The interval between the opening of the chest and the start of perfusion was within 50 sec. After a 1-min perfusion, left ventricular functional assessments can be made using a left ventricular balloon. One end of a latex balloon was fixed to polyethylene tubing (devoid of air bubbles) and inserted into the left ventricle, via the mitral valve through a small opening in the left atrium, whilst the other end was connected to a multi-channel recorder for data collection. The ventricular latex sphere (balloon) was filled with saline ( $\sim 0.15\ \text{ml}$ ) to maximize signal detection. The left ventricular end-diastolic pressure was maintained at  $4\ \text{mmHg}$  for 5 min and then the heart was perfused with  $15\ \text{ml}$  University of Wisconsin (UW) preservation solution ( $100\ \text{mM}$  potassium lactose,  $25\ \text{mM KH}_2\text{PO}_4$ ,  $5\ \text{mM MgSO}_4$ ,  $30\ \text{mM}$  raffinose,  $5\ \text{mM}$  adenosine,  $3\ \text{mM}$  glutathione,  $1\ \text{mM}$  allopurinol,  $50\ \text{g/l}$  hydroxyethyl starch,  $100\ \text{U}$  insulin,  $40\ \text{U}$  penicillin and  $8\ \text{mg}$  dexamethasone,  $\text{pH}=7.4$  and osmotic pressure= $320\pm5\ \text{mOsm/l}$ ) with or without luteolin (Hangzhou FST Pharmaceutical Co., Ltd., Hangzhou, China) for 1 min at  $4^{\circ}\text{C}$ . The dissected hearts were incubated in UW preservation solution for 12 or 18 h (Fig. 1). All surgical tools were purchased from Shanghai Medical Instruments Co., Ltd. (Shanghai, China). All protocols were approved by the Ethics Committee of Hainan Medical University (Haikou, China) and procedures conformed to the Directive 2010/63/EU of the European Parliament and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (21). All animal experiments were performed in an animal facility at Hainan Medical University.

**Monitoring heart function.** Heart function was measured following reperfusion in the Langendorff system. Heart rate (HR), left ventricle peak systolic pressure (LVPSP), maximal rate of rise of LVPSP ( $\text{dP}/\text{dt}_{\text{max}}$ ) and coronary flow (CF) were measured for 1 min at 3, 5, 15 and 30 min after reperfusion.

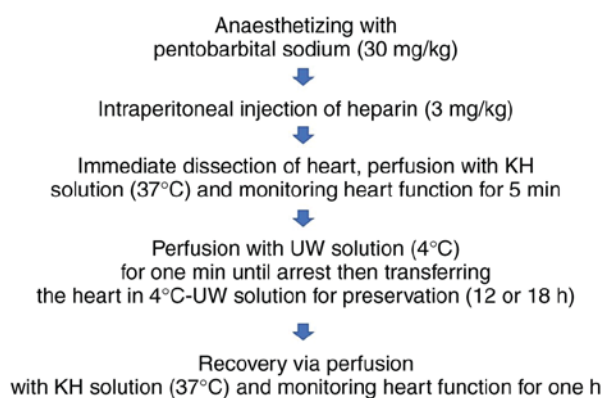


Figure 1. General workflow. UW, University of Wisconsin solution; KH, Krebs-Henseleit solution.

Rate pressure product (RPP) was calculated as  $\text{LVPSP} \times \text{HR}$ . Residual liquid was wiped away with filter papers and wet weight was measured. Following incubation at  $80^{\circ}\text{C}$  for 48 h, dry weight was measured. Water content (%) was calculated as:  $1 - (\text{dry weight}/\text{wet weight}) \times 100$ .

**Histological monitoring.** Following reperfusion,  $2\text{--}3\ \text{cm}^3$  tissue samples of the left ventricle were dissected and fixed in  $10\%$  neutral formaldehyde solution at room temperature for 24 h, embedded in paraffin and cut into  $4\text{--}5\ \mu\text{m}$ -thick sections for hematoxylin and eosin staining. Paraffin-embedded tissue samples were deparaffinised in xylene at  $25^{\circ}\text{C}$  at room temperature and rehydrated through graded concentrations of ethanol ( $100\%$  ethanol for 5 min,  $95\%$  ethanol for 1 min,  $80\%$  ethanol for 5 min,  $75\%$  ethanol for 5 min and distilled water for 2 min) at room temperature prior to staining with hematoxylin and eosin for 12 min at room temperature. Images were captured using an optical microscope (magnification,  $\times 400$ ). In addition,  $1\ \text{mm}^3$  tissue samples of the left ventricle were dissected and fixed with  $2.5\%$  glutaraldehyde for 24 h. The fixed samples were washed three times with PBS, post-fixed with  $1\%$  osmium tetroxide, dehydrated with acetone, and embedded in epoxy resin. Ultrathin sections were cut and double-stained with  $1\%$  lead citrate and  $0.5\%$  uranyl acetate. Images were captured using a transmission electron microscope (H-7700; Hitachi, Ltd., Tokyo, Japan).

**Immunohistochemistry.** Immunohistochemical staining was performed on paraffin-embedded left ventricular tissue samples using the Avidin-Biotin-Peroxidase kit (Boster Biological Technology, Ltd., Wuhan, China). Antigen retrieval was performed using a citrate buffer ( $\text{pH}\ 6.0$ ) for 20 min. Paraffin-embedded tissue sections were cut into  $3\text{--}\mu\text{m}$ -thick sections using a SM200R microtome (Leica Microsystems GmbH, Wetzlar, Germany) and mounted onto silane-coated glass slides. The dried tissue sections were subsequently deparaffinized in xylene and rehydrated in a descending alcohol series. Deparaffinised sections were incubated with  $3\%$  hydrogen peroxide for 10 min at room temperature to inhibit endogenous peroxidase activity. Tissue sections were incubated with primary antibodies directed against Bax (1:300; cat. no. BM3964) or Bcl-2 (1:100; cat. no. BA0412; both Boster Biological Technology, Ltd.) for 2 h at room temperature. Following the primary incubation, tissue sections

were incubated with a biotinylated secondary antibody (1:200; cat. no. BA1005) for 20 min at room temperature followed by incubation with peroxidase-labeled streptavidin (1:500; cat. no. BA1088; Boster Biological Technology, Ltd.) in a humidified box at 37°C for 20 min. Peroxidase activities were visualized using 3,3-diaminobenzidine peroxidase substrate. Sections were counterstained with 1% haematoxylin for 2-3 min at room temperature. Negative and positive controls were run on all sections. The intensity of Bax and Bcl-2 immunostaining was assessed using a light microscope (magnification, x400), by two independent observers. Cytoplasmic staining was considered as positive staining. Immunostaining was scored as follows: (-) negative, (+) weak, (++) moderate and (+++) strong.

**Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay.** The left ventricular tissue sections were analyzed using a TUNEL assay kit (cat. no. MK1025; Boster Biological Technology, Ltd.), according to the manufacturer's protocol. Paraffin-embedded tissue sections were subsequently deparaffinized in xylene at 58-60°C for 30 min and rehydrated in a descending ethanol series (100, 95 and 70% ethanol) for 5 min, respectively. Antigen retrieval was performed with 100  $\mu$ l of 20  $\mu$ g/ml proteinase K (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37°C for 10 min and subsequently washed twice with PBS, according to Gavrieli's method (22). Terminal deoxynucleotidyl transferase (TdT) was used to incorporate digoxigenin (DIG)-conjugated dUTP to the ends of DNA fragments. The sections were incubated with 20  $\mu$ l TdT reaction solution in a dark humidified chamber at 37°C for 120 min. Following TdT incubation, sections were incubated with anti-DIG-Biotin antibody (1:100; Boster Biological Technology, Ltd.) for 30 min at 37°C. The signal of TdT-mediated TUNEL was detected by incubation with 100  $\mu$ l streptavidin biotin-peroxidase complex (Boster Biological Technology, Ltd.) in a dark humidified chamber for 30 min at 37°C. The tissue sample slides were incubated with diaminobenzidine (DAB; Boster Biological Technology, Ltd.) solution for color development and observed using a light microscope (magnification, x400). DAB precipitates as a dark brown pigment allowing easy visualization of positively stained cells. Apoptotic cells with condensed nuclei were stained brown, whilst non-apoptotic cells were not stained. Cells with clear nuclear labeling were defined as TUNEL-positive cells. The number of TUNEL-positive cells from four randomly selected fields from each tissue section were used to determine the extent of myocardial tissue injury.

**Monitoring enzymatic activities.** Extracellular lactate dehydrogenase (LDH; cat. no. A020-2) and creatine kinase (CK; cat. no. A032) activity in the perfusion solution, as well as superoxide dismutase (SOD; cat. no. A001-3) and malondialdehyde (MDA; cat. no. A003-1) activity in ventricular homogenates were examined using kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), according to the manufacturer's protocols.

**Recording L-type calcium channels in cardiomyocytes.** Dissected hearts were placed into 4°C high potassium Tyrode's solution [143 mM NaCl, 140 mM KCl, 1.8 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 0.3 mM NaH<sub>2</sub>PO<sub>4</sub>, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) and 5 mM

glucose, pH=7.4]. The aorta was rapidly cannulated and connected to a Langendorff perfusion apparatus. The hearts were perfused retrogradely at 37°C for 5 min with normal Tyrode's solution (143 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 0.3 mM NaH<sub>2</sub>PO<sub>4</sub>, 5 mM HEPES and 5 mM glucose, pH=7.4) followed by 3-5 min perfusion with calcium-free Tyrode's solution (143 mM NaCl, 5.4 mM KCl, 0.5 mM MgCl<sub>2</sub>, 0.3 mM NaH<sub>2</sub>PO<sub>4</sub>, 5 mM HEPES and 5 mM glucose, pH=7.4) and 15-18 min perfusion with calcium-free Tyrode's solution containing Type-II collagenase (2 mg/ml). The hearts were then washed with Krebs solution (70 mM L-glutamic acid, 25 mM KCl, 3 mM MgCl<sub>2</sub>, 20 mM taurine, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.5 mM EGTA, 10 mM HEPES and 10 mM glucose, pH=7.4) until the heart expanded to increase tension, and then the tension decreased and the heart became soft. The left ventricular wall was dissected and sheared in Krebs solution; following filtration with a 200-mesh filter and incubation in Krebs solution at room temperature for 10 min, cardiomyocytes were separated following sedimentation. The supernatant was replaced with Krebs solution supplemented with bovine serum albumin (1 mg/ml, Sigma-Aldrich) and incubated at room temperature for 40 min, the myocardial cells were resuspended in Tyrode's solution supplemented with the calcium concentration gradually increased to 1.8 mmol/l. Then the cells were stored in normal Tyrode's solution at room temperature for 1 h. The myocardial cells were cultured in glucose-free Krebs solution and hypoxia was induced by bubbling the gas mixture (90% N<sub>2</sub> and 10% O<sub>2</sub>) in the cell medium for 3 h. The level of hypoxia was measured using a commercial oxygen needle electrode (Strathkelvin Instruments Ltd., North Lanarkshire, UK). Hypoxic cardiocytes were placed in an experimental perfusion chamber mounted on an inverted microscope for 5 min to allow cell adhesion. The chamber was perfused with external solution (135 mM NaCl, 1.8 mM CaCl<sub>2</sub>, 4.6 mM CsCl, 0.5 mM MgCl<sub>2</sub>, 5 mM HEPES, 10 mM glucose, pH=7.4) at a rate of 1.8 ml/min at 37°C. Cells with a rod-like appearance, good marginal refraction, clear striations and no contraction activity were selected for recording.

Action potentials were recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Data acquisition and analysis was performed by pCLAMP™ 10 Electrophysiology Data Acquisition & Analysis software (Molecular Devices). Micropipettes (resistance 2.5-3.5 M $\Omega$ ) were pulled using a two-step vertical microelectrode puller (pp-83, Narisige, Japan). Whole-cell recording micropipettes were filled with external solution (140 mM CsCl, 0.5 mM MgCl<sub>2</sub>, 4 mM Na<sub>2</sub>ATP, 1 mM EGTA, 5 mM HEPES and 5.5 mM glucose, pH=7.2). After the whole-cell configuration was achieved, action potential was recorded in current clamp mode and membrane current recorded in voltage clamp mode. Positive pressure (10 cm H<sub>2</sub>O) was applied when electrodes approached each cell, while negative pressure was applied following the attachment of each electrode. Cell membranes were broken by negative pressure (~100 cm H<sub>2</sub>O), which allowed recording in whole-cell mode. Whole-cell recording started 5 min after the cell membranes were broken and membrane potential was gradually increased from -40 mV to +50 mV in 10 mV steps (300 msec duration, 0.5 Hz) with 10 sec intervals. Cells were incubated with 7.5, 15 or 30  $\mu$ M luteolin

Table I. Heart function prior to preservation.

Group	Left ventricle peak systolic pressure (mmHg)	+dP/dt <sub>max</sub> (mmHg/sec)	-dP/dt <sub>max</sub> (mmHg/sec)	Heart rate (beats/min)	Rate pressure product (mmHg/min)
Control	85.4±9.7	1,815.2±321.9	1,429.6±320.6	344.8±45.4	30,819.5±501.0
Lu low (7.5 μmol/l)	87.3±8.3	1,785.1±283.5	1,586.5±349.3	326.1±53.1	30,973.1±410.2
Lu medium (15 μmol/l)	85.7±7.7	1,708.2±335.4	1,681.4±278.7	354.1±49.2	30,701.3±545.2
Lu high (30 μmol/l)	86.8±8.4	1,828.7±132.0	1,646.7±295.4	340.9±43.6	30,740.8±617.4

Lu, luteolin; dP/dt<sub>max</sub>, maximal rate of rise of left ventricle peak systolic pressure.

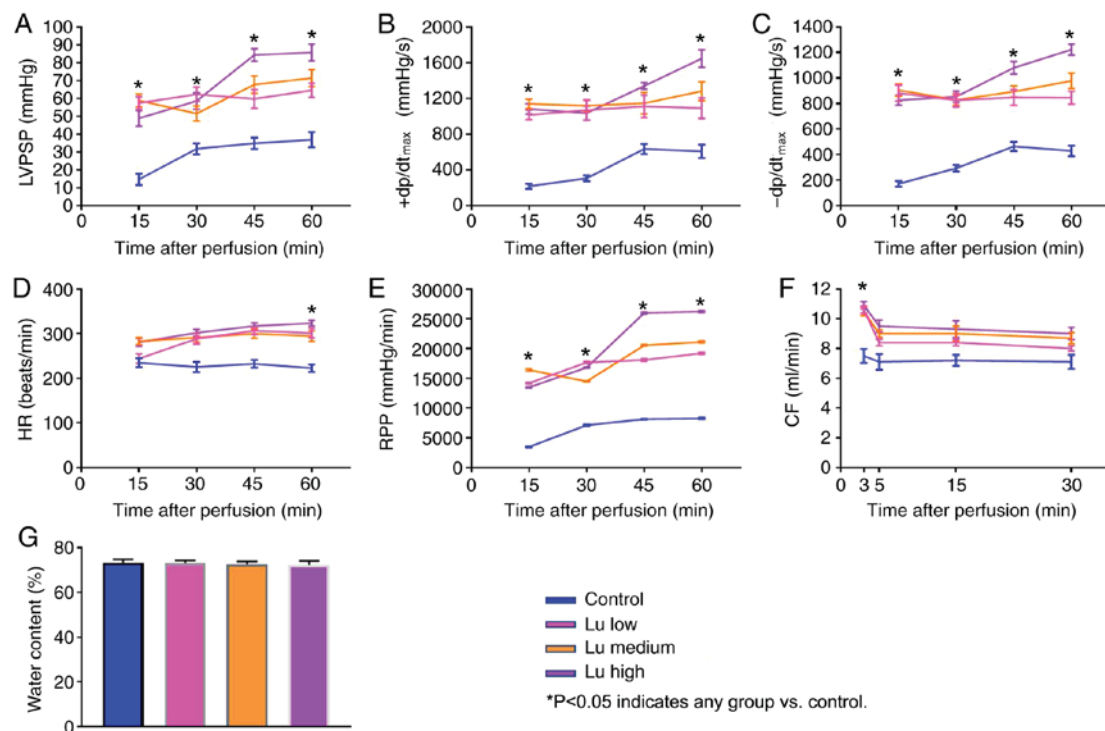


Figure 2. Lu improves heart function 12 h after preservation. (A) LVPSP, (B) +dP/dt<sub>max</sub>, (C) -dP/dt<sub>max</sub>, (D) HR, (E) RPP and (F) CF were measured 15, 30, 45 and 60 min after a 12-h preservation in a low, medium or high Lu solution. (G) Water content was measured after a 12-h preservation in a low, medium or high Lu solution. \*P<0.05 vs. Control. Lu, luteolin; low, 7.5 μmol/l; medium, 15 μmol/l; high, 30 μmol/l; LVPSP, left ventricle peak systolic pressure; dP/dt<sub>max</sub>, maximal rate of rise of LVPSP; HR, heart rate; RPP, rate pressure product; CF, coronary flow.

(Hangzhou FST Pharmaceutical Co., Ltd., Hangzhou, China) dissolved in external solution for perfusion and membrane potential was clamped at -40 mV, depolarized to 0 mV and further clamped for 300 msec.

**Statistical analysis.** Data were presented as mean ± standard deviation. SPSS 12.0 (SPSS, Inc., Chicago, IL, USA) was used for analysis. Differences between groups were evaluated using a two-way analysis of variance followed by Bonferroni's correction. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Luteolin improves heart function following preservation.** Heart function was monitored prior to preservation. No

significant differences were identified in the monitored parameters among the different groups (Table I).

To characterize heart preservation under different conditions, several parameters reflecting heart function were monitored. All parameters significantly improved in the presence of luteolin at 12 h (Fig. 2A-F) and 18 h (Fig. 3A-F) after preservation in what appeared to be a dose-dependent manner. No significant difference was identified in tissue water content among different groups (Figs. 2G and 3G). Thus, luteolin may improve heart function following long term heart storage.

**Luteolin protects hearts from structural damage.** Structural analysis using a light microscope demonstrated that, prior to preservation, cardiomyocytes were arranged in an orderly manner and branches extended to form a network (Fig. 4A). Intercalated disks were clear and no edema was evident.



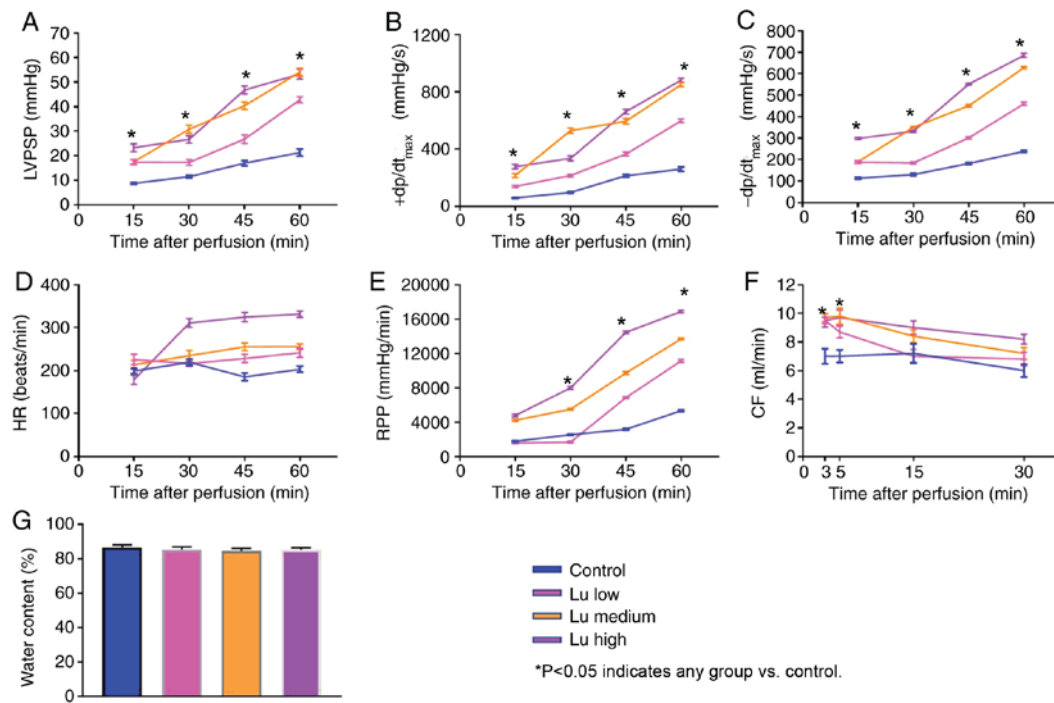


Figure 3. Lu improves heart function 18 h after preservation. (A) LVPSP, (B) +dp/dt<sub>max</sub>, (C) -dp/dt<sub>max</sub>, (D) HR, (E) RPP and (F) CF were measured 15, 30, 45 and 60 min after an 18-h preservation in a low, medium or high Lu solution. (G) Water content was measured after an 18-h preservation in a low, medium or high Lu solution. \* $P < 0.05$  vs. control. Lu, luteolin; low, 7.5  $\mu$ mol/l; medium, 15  $\mu$ mol/l; high, 30  $\mu$ mol/l; LVPSP, left ventricle peak systolic pressure; dp/dt<sub>max</sub>, maximal rate of rise of LVPSP; HR, heart rate; RPP, rate pressure product; CF, coronary flow.

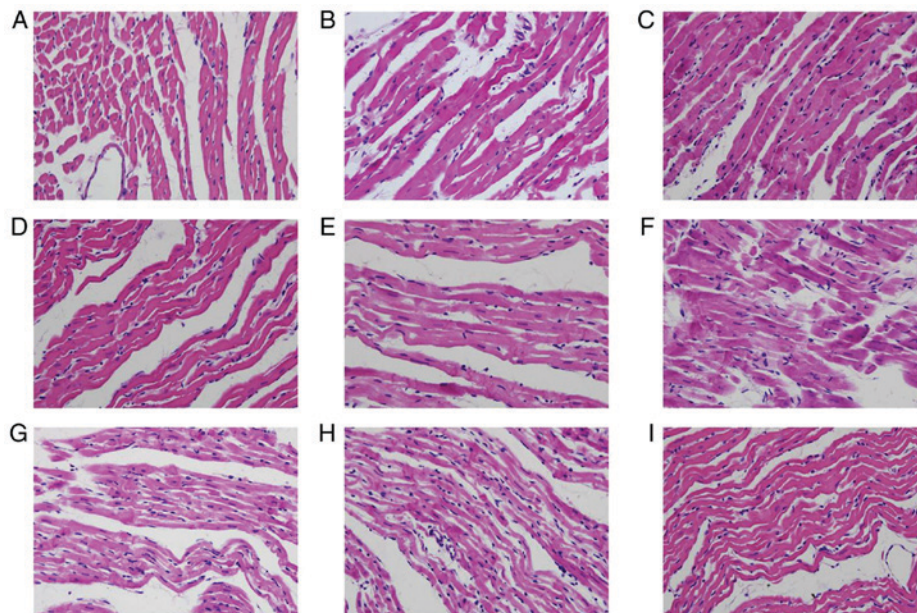


Figure 4. Lu protects rat cardiac muscle morphology from long-term storage-induced damage. The morphology of rat cardiac muscle (A) prior to preservation, in the (B) control, (C) Lu low, (D) Lu medium and (E) Lu high groups 12 h after preservation, and in the (F) control, (G) Lu low, (H) Lu medium, (I) Lu high groups 18 h after preservation. Magnification, x400. Lu, luteolin; low, 7.5  $\mu$ mol/l; medium, 15  $\mu$ mol/l; high, 30  $\mu$ mol/l.

Blood vessels were normal with intact endothelia and stratified structures. At 12 h after preservation, vacuoles and irregular structures were evident in the cytosol of cardiomyocytes, and cardiac muscle fibers were interrupted. (Fig. 4B). In the Lu low group (Fig. 4C), there was some evidence of necrosis as necrotic cardiomyocytes exhibited eosinophilic hyperchromatism. In the Lu medium group (Fig. 4D) and

Lu high group (Fig. 4E), 12 h after preservation, there were some areas of the myocardial cytoplasm that showed a small number of vacuoles. In addition, the myocardial fibers became thin and elongated, forming waves of parallel arrangement. Necrosis was evident in cardiomyocytes, the interstitial space was slightly enlarged and the endothelia were interrupted. The aforementioned pathological alterations were

more evident 18 h after preservation compared with the control group (Fig. 4F). A small number of different-sized vacuoles were observed in the cytosol of cardiomyocytes, and cardiac muscle fibers were elongated, forming waves of parallel arrangement. In some regions, myocardial fibers were broken. Some necrotic myocardiocytes exhibited eosinophilic hyperchromatism in luteolin groups 18 h after preservation (Fig. 4G-I). However, the pathological alterations observed were improved by luteolin in what appeared to be a dose dependent manner (Fig. 4G-I).

In structural analysis using TEM, cardiomyocytes exhibited a fusiform and large oval nucleus, the long axis of nucleus was parallel to muscle fibers, and chromatin was distributed normally prior to preservation (Fig. 5A). In cytosol, slight mitochondrial edema was evident, but with uniform crista. Sparse sarcoplasmic reticulum (SR), dense rough endoplasmic reticulum and Golgi complex were around each nucleus. The enlargement of SR was evident, and formed diad and triad complexes with T tubules. Intercalated disks, which form intercellular connections, were observed between neighboring cardiomyocytes. Prior to preservation it was revealed that microvasculature networks were within intercellular spaces, which exhibited flattened endothelia and intact base membranes. At 12 h after preservation, some myofibrils and sarcomeres exhibited distortions. Mitochondrial membranes were unclear, and mitochondria exhibited edema and reduced crista; Vacuoles were observed in a number of crista (Fig. 5B). In the Lu low, medium and high groups, most of the cardiac muscle fibers were arranged orderly with clear striations. There were no significant changes observed in the nucleus of the myocardiocytes, however there were some broken muscle fibers. The arrangement of myofibrils was slightly disordered, but the light and dark bands were still clear. Mitochondria appeared swollen and vacuolated 12 h after preservation, compared with the control group (Fig. 5C-E). In addition, the observed alterations were more pronounced in cells 18 h after preservation compared with the control group (Fig. 5G-I). However, those alterations could be ameliorated by luteolin in what appeared to be a dose dependent manner (Fig. 5G-I). Thus, luteolin may protect hearts from structural damage during long-term preservation.

*Luteolin protects cardiomyocytes from apoptosis.* As apoptosis of cardiomyocytes is related to functional damage during long-term storage, inhibiting the apoptotic process may serve a role in organ transplantation (23). The effect of luteolin on apoptosis in cardiomyocytes was assessed. According to the TUNEL assay, no apoptosis was observed in isolated hearts prior to preservation (Fig. 6A). The morphology of rat cardiac muscle was examined in the control, Lu low, Lu medium and Lu high groups 12 and 18 h after preservation. In addition, the number of apoptotic cells was examined at 12 and 18 h after preservation. Following long-term storage, apoptotic cells were sparsely distributed. In cardiomyocytes at 12 h after preservation there were a few apoptotic cells distributed below the epicardium (Fig. 6B). Additionally, in the presence of luteolin there was a decrease in the number of apoptotic cell observed at 12 h after preservation (Fig. 6C-E). In cardiomyocytes at 18 h after preservation there were a few apoptotic cells distributed near the endocardium (Fig. 6F). Similarly, in

Table II. Semi-quantification of apoptosis regulator Bcl-2 protein expression following long-term storage.

Group	12-h preservation	18-h preservation
Control	+	+
Lu low (7.5 $\mu$ mol/l)	++	++
Lu medium (15 $\mu$ mol/l)	++	++
Lu high (30 $\mu$ mol/l)	++	++
Lu, luteolin.		

Table III. Semi-quantification of apoptosis regulator Bax protein expression following long-term storage.

Group	12-h preservation	18-h preservation
Control	+++	+++
Lu low (7.5 $\mu$ mol/l)	++	++
Lu medium (15 $\mu$ mol/l)	++	++
Lu high (30 $\mu$ mol/l)	+	+
Lu, luteolin.		

the presence of luteolin there was a decrease in the number of apoptotic cell observed at 18 h after preservation (Fig. 6G-I). The results of TUNEL test demonstrated that luteolin administration significantly decreased the number of apoptotic cells 12 h (Fig. 6J) and 18 h (Fig. 6K) after preservation, compared with their respective control groups.

Key factors involved in anti- and pro-apoptotic signaling were examined. The endogenous expression of Bcl-2 in myocardiocytes at 12 h after preservation was very low (Fig. 7A). The expression of Bcl-2 in cardiac myocardium increased in luteolin groups compared with the control group at 12 h after preservation (Fig. 7B-D). Similarly, the endogenous expression of Bcl-2 in myocardiocytes at 18 h after preservation was very low (Fig. 7E). The expression of Bcl-2 in cardiac myocardium increased in luteolin groups compared with the control group at 18 h after preservation (Fig. 7F-H).

The positive staining of Bax protein was mainly located in the cytoplasm of myocardiocytes and smooth muscle cells. The endogenous expression of Bax in myocardiocytes at 12 h after preservation was very high (Fig. 8A). The expression of Bax in cardiac myocardium decreased in luteolin groups compared with the control group at 12 h after preservation (Fig. 8B-D). Similarly, the endogenous expression of Bax in myocardiocytes at 18 h after preservation was very high (Fig. 8E). The expression of Bax in cardiac myocardium decreased in luteolin groups compared with the control group at 18 h after preservation (Fig. 8F-H). The immunostaining results suggest that luteolin enhanced the storage-induced increase of Bcl-2 and decrease of Bax expression in what appeared to be a dose-dependent manner according to the semi-quantitative results presented in Tables II and III. The results indicated that luteolin may protect cardiomyocytes from apoptosis via increasing Bcl-2 and decreasing Bax.



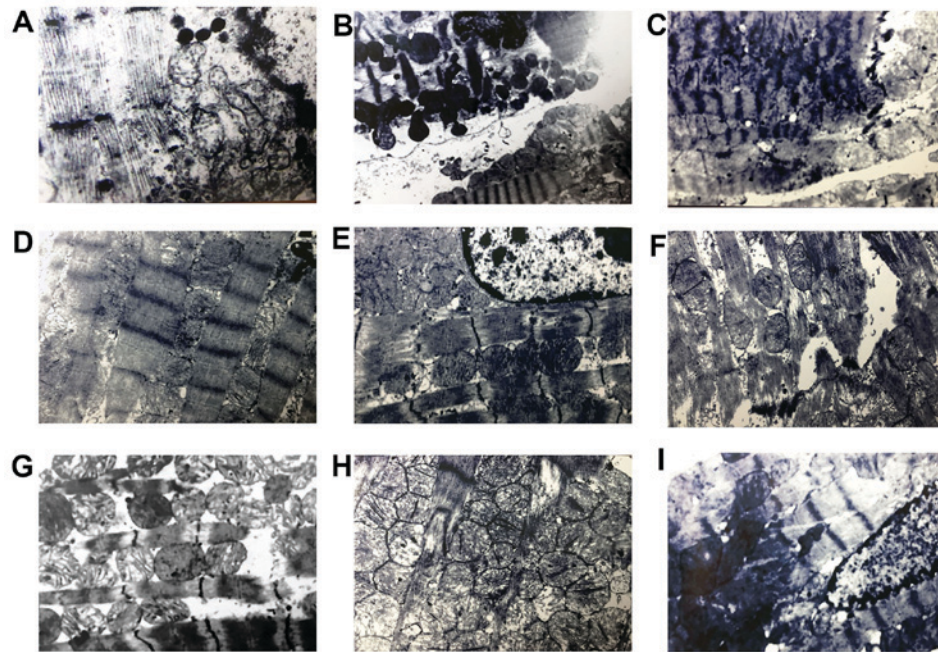


Figure 5. Lu protects rat cardiac muscle ultrastructure from long-term storage-induced damage. (A) The morphology of rat cardiac muscle prior to preservation (magnification, x10,000). The morphology of rat cardiac muscle in the (B) control (magnification, x10,000), (C) Lu low (magnification, x10,000), (D) Lu medium (magnification, x10,000) and (E) Lu high (magnification, x10,000) groups 12 h after preservation. The morphology of rat cardiac muscle in the (F) control (magnification, x10,000), (G) Lu low (magnification, x10,000), (H) Lu medium (magnification, x10,000), (I) Lu high (magnification, x10,000) groups 18 h after preservation. Lu, luteolin; low, 7.5  $\mu\text{mol/l}$ ; medium, 15  $\mu\text{mol/l}$ ; high, 30  $\mu\text{mol/l}$ .

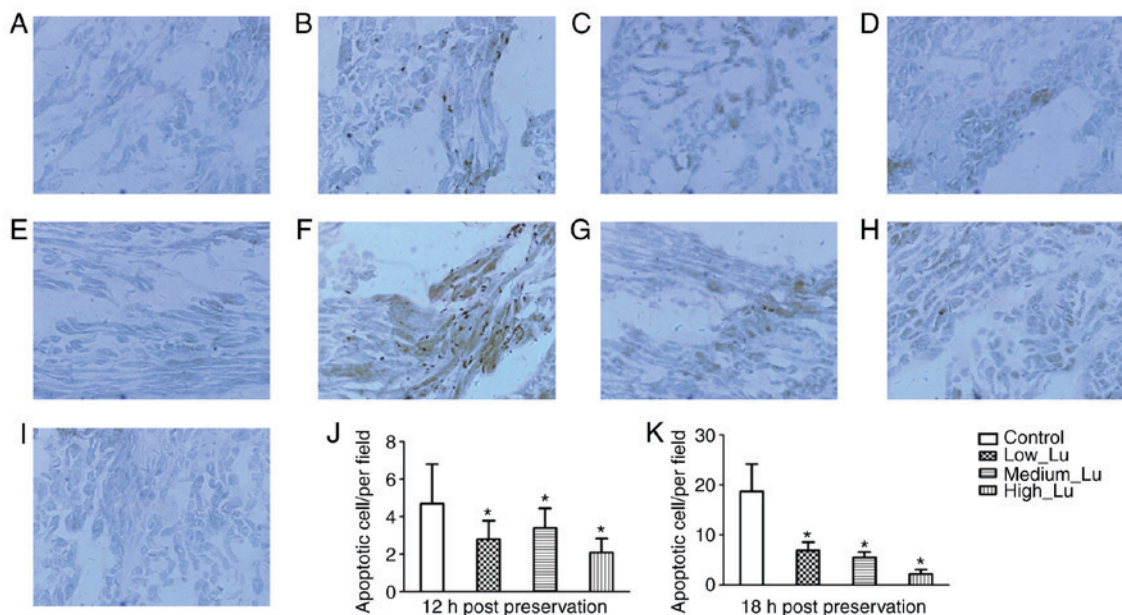


Figure 6. Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling staining reveals that Lu protects rat cardiac muscle from apoptosis. Rat cardiac muscle (A) prior to preservation, in the (B) control, (C) Lu low, (D) Lu medium and (E) Lu high groups 12 h after preservation, and in the (F) control, (G) Lu low, (H) Lu medium, (I) Lu high groups 18 h after preservation (magnification, x400). Quantification of the number of apoptotic cells (J) 12 h and (K) 18 h after preservation. \* $P < 0.05$  vs. control. Lu, luteolin; low, 7.5  $\mu\text{mol/l}$ ; medium, 15  $\mu\text{mol/l}$ ; high, 30  $\mu\text{mol/l}$ .

*Luteolin inhibits the activity of LDH, CK and MDA, and promotes the activity of SOD.* The enzymatic activity of LDH, CK, SOD and MDA may reflect damage in cardiomyocytes (24). The extracellular LDH and CK activity significantly decreased after 12 h (Fig. 9A and B) and 18 h (Fig. 9C and D) of storage in all three luteolin groups in what appeared to be a time-dependent manner. Therefore, heart damage may be positively

associated with storage duration and luteolin may be involved in reducing myocardial damage during long-term storage. By contrast, SOD activity, which is negatively associated with cell damage (25), increased after 12 and 18 h of storage in all three luteolin groups in what appeared to be a dose-dependent manner (Fig. 9E). In addition, MDA activity decreased after 12 and 18 h of storage in all three luteolin groups in what appeared

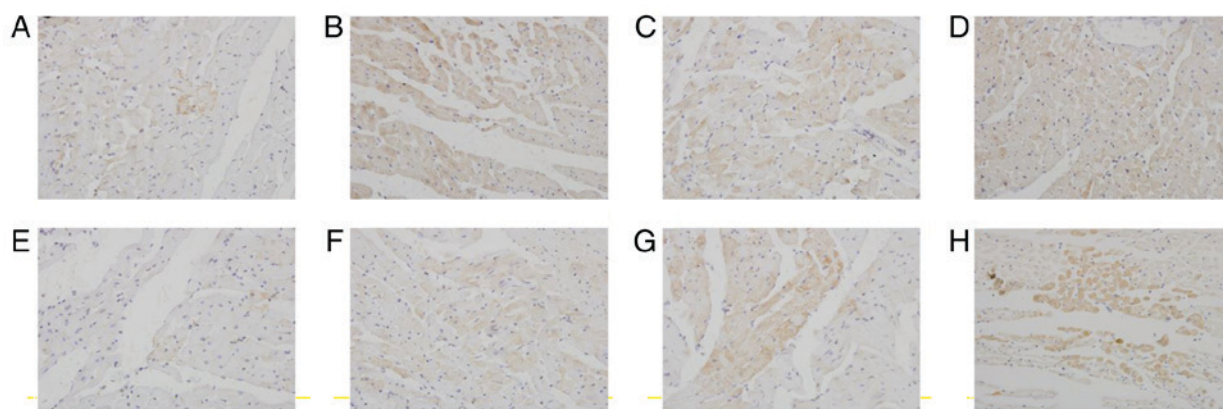


Figure 7. Lu ameliorates long-term storage-induced increases in apoptosis regulator Bcl-2 expression. Rat cardiac muscle in the (A) control, (B) Lu low, (C) Lu medium and (D) Lu high groups 12 h after preservation, and in the (E) control, (F) Lu low, (G) Lu medium, (H) Lu high groups 18 h after preservation. Magnification, x400. Lu, luteolin; low, 7.5  $\mu\text{mol/l}$ ; medium, 15  $\mu\text{mol/l}$ ; high, 30  $\mu\text{mol/l}$ .

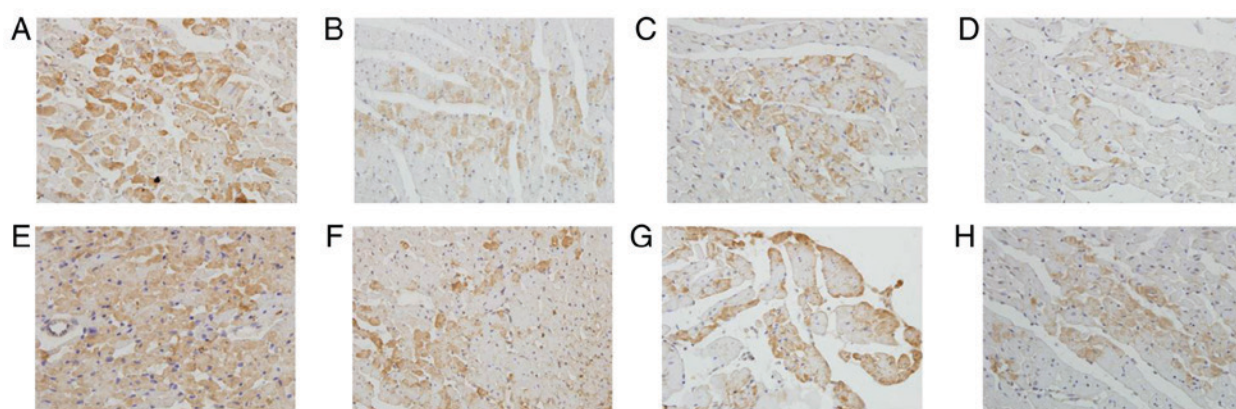


Figure 8. Lu enhances long-term storage-induced decreases in apoptosis regulator BAX expression. Rat cardiac muscle in the (A) control, (B) Lu low, (C) Lu medium (D) Lu high groups 12 h after preservation, and in the (E) control, (F) Lu low, (G) Lu medium, (H) Lu high groups 18 h after preservation. Magnification, x400. Lu, luteolin; low, 7.5  $\mu\text{mol/l}$ ; medium, 15  $\mu\text{mol/l}$ ; high, 30  $\mu\text{mol/l}$ .

to be a dose-dependent manner Fig. 9F). Thus, luteolin may protect hearts from long-term storage-induced damage.

*Luteolin inhibits L-type calcium channels during hypoxia.* As hypoxia is one of the main causes for heart damage during long-term storage and L-type calcium channels, which mediate calcium influx, are involved (26), the effect of luteolin on L-type calcium channels was assessed in cardiomyocytes experiencing hypoxia. Calcium currents mediated through L-type calcium channels exhibited typical voltage-dependency. Original current traces were elicited by depolarizing voltage to -40, 0, and 50 mV from a holding potential of -40 mV with a peak potential of 0 mV (Fig. 10A and B). A comparison of the current densities at 0 mV demonstrated that the percentage of calcium current significantly decreased in all three luteolin groups in what appeared to be a dose-dependent manner (Fig. 10C and D). These results of this study demonstrated that luteolin can inhibit L-type calcium channels during hypoxia.

## Discussion

The present study demonstrated that luteolin may protect hearts from damage induced by long-term storage (12-18 h),

including heart dysfunction, structural damage observed using a light microscope and TEM, increased apoptosis and disrupted cell membranes. The amelioration of this damage may result from the inhibitory effect of luteolin on L-type calcium channels. The current study has provided experimental evidence demonstrating that luteolin may be applied to heart preservation solutions, particularly during long-term storage.

Heart transplantation is the only effective treatment for patients with end stage cardiopathy (27). However, donor heart preservation has been a major obstacle in clinical practice (28). Currently, storage duration has been limited to 6 h (2,29-32), despite studies attempting to extend storage by supplementing preservation solutions with additional substances that are able to block calcium channels (29,32-36), making it impossible to transport donor hearts over long distances. Luteolin may be a plausible supplement to improve long-term heart preservation.

It is well known that ischemia and reperfusion-induced injuries are the main cause for heart injury; free radical oxygen species and calcium overload are two widely recognized factors leading to heart injury (37). Free radical oxygen species can result in the over-oxidation of membrane lipids and cause heart damage (38); thus, scavenging free radical oxygen species and



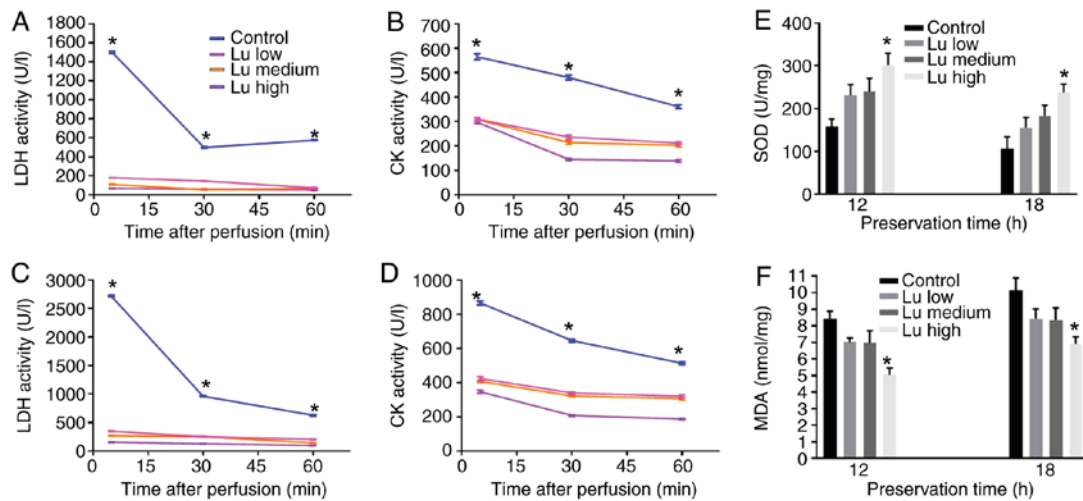


Figure 9. Luteolin inhibits the enzymatic activity of LDH, CK and MDA, and promotes the enzymatic activity of SOD. The enzymatic activities of (A) LDH and (B) CK were measured 15, 30, 45 and 60 min after a 12-h preservation in a low, medium or high Lu solution. The enzymatic activities of (C) LDH and (D) CK were measured 15, 30, 45 and 60 min after an 18-h preservation in a low, medium or high Lu solution. The enzymatic activities of (E) SOD and (F) MDA were measured 15, 30, 45 and 60 min after a 12 and 18-h preservation in a low, medium or high Lu solution. \* $P < 0.05$  vs. control. Lu, luteolin; low, 7.5  $\mu\text{mol/l}$ ; medium, 15  $\mu\text{mol/l}$ ; high, 30  $\mu\text{mol/l}$ ; LDH, lactate dehydrogenase; CK, creatine kinase; MDA, malondialdehyde; SOD, superoxide dismutase.

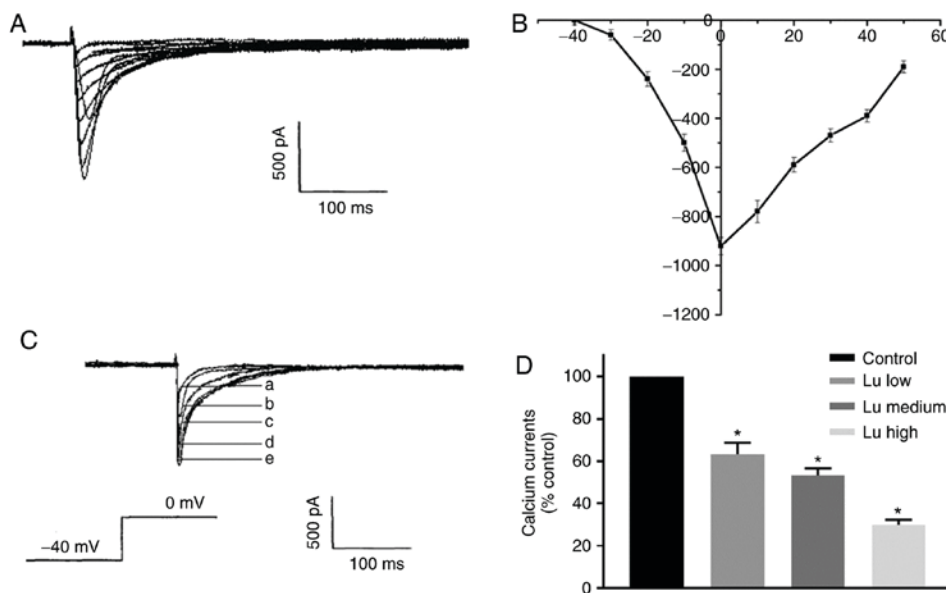


Figure 10. Luteolin inhibits L-type calcium channels during hypoxia. (A) Representative traces of the ramp currents recorded. (B) A summary of the amplitude-voltage association in rat cardiac cells. (C) Traces of calcium currents following the incubation of cells in (a) Lu high, (b) Lu medium and (c) Lu low solutions, (d) following washout and (e) in the control group. (D) Percentage of calcium current through L-type calcium channels. \* $P < 0.05$  vs. control. Lu, luteolin; low, 7.5  $\mu\text{mol/l}$ ; medium, 15  $\mu\text{mol/l}$ ; high, 30  $\mu\text{mol/l}$ .

blocking oxidation are effective strategies to prevent heart injury. Previous studies have revealed that luteolin is capable of clearing superoxide anion, inhibiting lipid peroxidation and blocking oxidation of low-density lipoprotein (39-42), making luteolin a promising candidate for heart protection. On the other hand, there are a large number of calcium channels on cardiomyocytes. During excitation under physiological conditions, calcium influx through L-type calcium channels is important and responsible for further calcium influx from internal stores (43-45). However in the presence of ischemia and hypoxia, calcium influx through L-type calcium channels increases and leads to further calcium overload from internal stores during reperfusion, leading to irreversible pathological

changes, including cell necrosis and apoptosis (46,47). Thus, the inhibition of L-type calcium channels may also contribute to the protection of the heart. Additionally, luteolin can lead to vasculature dilation (39), which may be involved in the inhibition of voltage-gated calcium channels, ligand-gated calcium channels, internal calcium release and potassium channel activation; this is consistent with the results of the current study, which demonstrate an increase in CF in the presence of luteolin. Luteolin-induced dilation may minimize endothelial cell injury caused by UW-induced vessel contraction and improve the penetration of protective components in UW, thereby improving heart protection (31,48). Thus, during long term-storage, luteolin may protect against heart injury

through its effect on anti-oxidation, and by preventing calcium overload and increasing vessel dilation.

In conclusion, the current study demonstrated that luteolin may improve long-term ( $\leq 18$  h) heart preservation. However, whether the stored hearts are suitable enough for transplantation requires verification. For longer storage, it is hypothesized that ultra-low or low temperature storage may be better alternative approaches (29). A recent study demonstrated that luteolin mitigated the myocardial inflammatory response induced by a high-carbohydrate/high-fat diet, thus supporting the conclusion of the current study (49). Although there are several technical obstacles, such as irreversible injury during freezing, developments in cellular and molecular techniques may provide breakthroughs in long-term heart preservation in the near future.

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### Availability of data and materials

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

### Authors' contributions

QY and JY prepared the manuscript. JY designed the study. YLi and YLiu performed the experiments and collected the data. YZ analyzed the data. SL made substantial contributions to the study conception.

### Ethics approval and consent to participate

All protocols were approved by the Ethics Committee of Hainan Medical University and procedures conformed to the Directive 2010/63/EU of the European Parliament and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996).

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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