Reduction of Cold Ischemic Injury with the Addition of Compound Glycyrrhizin in HTK Solution in a Mouse Heart Transplantation Model

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Summary
Cold ischemic injury in heart storage is an important issue pertaining to heart transplantation. This study aims to evaluate the addition of compound glycyrrhizin (CG) in histidine-tryptophan-ketoglutarate (HTK) solution on chronic isograft injury in comparison to traditional HTK solution.

Hearts of mouse were stored for 8 h in 4°C cold preservation solution and then transplanted heterotopically into mouse. Five groups were evaluated: HTK, low dose of CG solution (LCG), medium dose of CG solution (MCG), high dose of CG solution (HCG), and hearts without cold ischemia (sham). Survival was assessed. Time to restoration of heartbeat and strength of the heartbeat was measured. Lactate dehydrogenase (LDH) and creatine kinase (CK) levels in the preservation solution were determined. The myocardial damage and interstitial fibrosis of transplanted hearts were evaluated. TGF-β1 expression in the transplanted hearts was assessed.

Addition of CG to HTK solution significantly attenuated cold ischemic injury during cold storage, as evidenced by the lower time to restoration of heartbeat, higher strength of the heartbeat, lower LDH, and CK leakage. After transplantation, hearts stored in HTK solution containing CG had decreased the myocardial damage and interstitial fibrosis, compared with those stored without CG. The percentage of TGF-β1-positive cells and TGF-β1 level in the transplanted hearts were also decreased when stored in CG-containing HTK solution.

The addition of CG to HTK solution attenuates cold ischemic injury during cold storage.

Key words: Heart storage, Chronic isograft injury, Myocardial protection

Heart transplantation has become the most favorable therapeutic approach for patients with terminal cardiac failure. However, nonspecific right heart failure, arrhythmia, coronary artery disease, chronic rejection, and other complications are challenges surgeons must overcome to perform heart transplantation.1,2) Previous study demonstrated that these complications were closely related to cold ischemic injury of donated organs during cold preservation.3) To date, due to convenience for transporting and low cost, static cold storage with preservation solution is the preferred organ preservation method in most centers.4) Hypothermia during organ preservation has beneficial effects by delaying hypoxia-induced ATP decline and slowing down the subsequent organ damage.5) However, it has also been identified to induce cell injury in a variety of cell types.6,7) Meanwhile, the heart has a poor tolerance of prolonged cold ischemia with only a safe preservation period of 4-6 h with the currently available preservation solutions.8) Therefore, new strategies should be considered to further improve the outcome of cold storage.

Compound glycyrrhizin (CG) is a compound preparation composed of glycyrrhiza extract, cysteine hydrochloride, and glycine. This compound has anti-inflammatory properties and has been used commonly in Asia to treat patients with chronic liver disease. In addition, several studies reported that CG can significantly reduce ischemia/reperfusion injury in the liver and kidney.9-11) However, to date, no study has described its use in the heart cold ischemic injury. Therefore, this study aims to investigate the effect of CG on heart cold ischemic injury.

Methods

Animals: Male C57BL/6J mice (25-30 g) were obtained from the central animal laboratory of Southwest Medical University. All animals were housed under standard conditions at constant temperature, humidity, and a 12 h light/dark cycle. All animals received care in compliance with the Principle of Laboratory Animal Care, and the protocol...
was approved by the local Animal Care and Research Committee.

**Surgery:** The surgery was performed as described previously. Briefly, the thorax was opened under general anesthesia with ketamine and xylazine i.p., and a cannula was inserted retrogradely from the descending aorta toward the aortic arch. The heart was then flushed with cardioplegic solution (histidine-tryptophan-ketoglutarate (HTK) solution or HTK with addition of CG solution at 4°C) via the descending aorta until complete cardioplegia. After a single-band ligation of the superior vena cava, the pulmonary artery, and the pulmonary veins, the donor heart was procured with the dissection of the aorta and inferior vena cava and stored in the respective preservation solution for 8 hours at 4°C. Then, the graft was implanted to the recipient intraperitoneally. In a word, the abdomen of recipient was opened and the donor’s ascending aorta was anastomosed end-to-side to the recipient’s abdominal aorta using a 10-0 nylon running suture. Likewise, the donor’s inferior vena cava was anastomosed end-to-side to the recipient’s inferior caval vein. After transplant, the time between the beginning of heart reperfusion and heartbeat restoration was recorded. All the surgeries were performed by a single, experienced microsurgeon.

**Preservation solutions:** The preservation solution was HTK solution without CG (HTK group; n = 6) and with CG, designated as low-dose (LCG; 50 mg/L; n = 6), medium-dose (MCG; 100 mg/L; n = 6), or high-dose (HCG; 150 mg/L; n = 6) groups, respectively. Sham group (n = 6) composed of hearts that upon harvest were subjected immediately to transplant, without prior storage in HTK solution.

**Survival of the transplanted hearts:** Function of the transplanted heart was assessed as previously described. An investigator assessed the heartbeat’s strength on a 4-point scale (0 = no heartbeat to 3 = very strong heartbeat) by palpating the transplanted heart in the abdomen of the recipient mice.

**Serological parameters:** After cold storage (except for the sham group), 2 mL preservation solution was collected for the determination of lactate dehydrogenase (LDH) and creatine kinase (CK) levels. Both measurements were performed in the central laboratory of the Southwest Medical University using the commercial kits (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions.

**Histopathological analysis and damage score:** The grafted hearts were harvested at day 60 post-transplantation. A part of tissue was formalin fixed, embedded in paraffin, and cut into 3-μm sections. Tissue sections were then stained with hematoxylin and eosin and Sirius red. Myocardial injury was scored: score 0, no lesion; score 1, focal vacuolization (myocyte becomes vacuolated); score 2, diffuse vacuolization and mild mononuclear infiltration; score 3, focal necrosis of myocyte (irregular border; fragmented sarcoplasm, debris, myocyte dropout); and score 4, extensive myocyte necrosis (interstitial hemorrhage and eosinophil infiltration). Cardiac fibrosis was measured as percentage of fibrotic area in each field. Six random fields were evaluated in each slide. Two observers, blinded to the preservation solution, evaluated the slides separately. The average scores were calculated and the data was expressed as mean ± standard deviation.

**Immunohistochemistry:** The expression of TGF-β1 was analyzed using immunohistochemical staining. Briefly, paraffin slides were deparaffinized with xylene, hydrated in ethanol, and incubated in 1.0% hydrogen peroxide for 10 minutes. The slides were then incubated with a rabbit anti-TGF-β1 polyclonal antibody (1:150, Santa Cruz Biotechnology, Santa Cruz, CA) for 30 minutes at room temperature. After washing with PBS, sections were incubated for 30 minutes with goat anti-rabbit biotinylated secondary antibody. Sections were incubated for 30 minutes with AB enzyme reagent followed by peroxide substrate and counterstained with hematoxylin for 10 seconds. An observer blinded to the experimental groups evaluated the slides. The slides were observed under a microscope and images were captured and analyzed using a digital imaging system. The percentage of positive cells in each field was counted. Five random fields were evaluated in each slide and the mean percentage of each slide was calculated.

**Enzyme-linked immunosorbent assay:** The heart tissues were collected at day 60 post-transplantation. TGF-β1 involved in myocardium was directly measured by ELISA kit according to the manufacturer’s instructions (Bio-source, Camarillo, CA). The PBS solution used for grinding tissue was analyzed as a control. The absorbance was measured at 450 nm. The concentration of TGF-β1 was calibrated with the TGF-β1 standard curve.

**Statistical analysis:** The results are presented as mean ± SD. Data were tested for Gaussian distribution using the Kolmogorov-Smirnov test. Normally distributed data were analyzed using one-way analysis of variance with a Bonferroni post hoc test. All tests were performed using SPSS 19.0 (SPSS Inc., Chicago, IL) and P < 0.05 was considered significant.

**Results**

**Survival:** All recipients survived the entire 60 days after transplantation and all the grafts were determined alive by palpation until harvested.

**Re-beating time and palpation score:** Time for restoration of heartbeat after transplantation in HTK preservation solution was 14.1 ± 1.6 minutes. The addition of CG to HTK solution significantly shortened the re-beating time (LCG, 10.1 ± 0.95 minutes; MCG, 8.43 ± 0.65 minutes; HCG, 6.43 ± 0.76 minutes; all P < 0.05 versus HTK group). Hearts of the sham group without cold ischemia scored lowest (1.03 ± 0.25 min; P < 0.05 versus all other groups; Figure 1A). The palpation score was significantly higher in hearts stored in the HTK solution with CG (LCG, 2.13 ± 0.25; MCG, 2.27 ± 0.25; HCG, 2.9 ± 0.3) than in hearts stored in HTK solution only (1.2 ± 0.3; all P < 0.05). Sham hearts were scored highest (3.73 ± 0.42; all P < 0.01 versus all other groups; Figure 1B).

**LDH and CK release:** The leakage of LDH and CK from myocardium is an important indicator of heart damage. As shown in Figure 2A, after cold storage in HTK solution for 8 hours, the addition of CG to HTK solution
significantly inhibited LDH release (LCG, 15.93 ± 1.6 U/L; MCG, 13.13 ± 2.4 U/L; HCG, 8.8 ± 1.0 U/L) compared with HTK solution only (23.47 ± 2.1 U/L, all P < 0.05). Meanwhile, the CK levels in the preservation solutions with CG (LCG, 41.33 ± 5.45 U/L; MCG, 38.63 ± 2.89 U/L; HCG, 24.97 ± 4.29 U/L) were significantly decreased when compared with HTK solution only (63.13 ± 4.8 U/L; all P < 0.05; Figure 2B).

**Histology:** Histologic assessment was carried out in grafted hearts 60 days after transplantation. Representative images of myocardial injury are shown in Figure 3A-E. The histologic score of hearts in the sham group was the lowest (0.2 ± 0.45), suggesting minimal heart damage. The hearts stored in cold HTK solution showed a significantly higher score (3.0 ± 0.71), whereas the addition of CG reduced the histopathologic scores (LCG, 1.6 ± 0.55; MCG, 1.2 ± 0.45; HCG, 0.8 ± 0.45; all P < 0.05 versus HTK solution only; Figure 4A). As shown in Figure 4B, the interstitial fibrosis of hearts in the sham group was the lowest (1.0 ± 0.71%). The addition of CG to HTK solution significantly reduced interstitial fibrosis (LCG, 5.2 ± 0.84%; MCG, 4.2 ± 0.84%; HCG, 3.4 ± 0.55%) compared with HTK solution only (7.8 ± 1.48%, all P < 0.05). Representative images of interstitial fibrosis are shown in Figure 3F-J.

**TGF-β1 expression:** As shown in Figure 5B, after cold storage in HTK solution, the percentage of TGF-β1-positive cells significantly increased when compared with sham hearts (13.8 ± 2.39% versus 3.6 ± 1.14%, P < 0.05). Meanwhile, the addition of CG to HTK solution significantly reduced the percentage of TGF-β1-positive cells (LCG, 9.6 ± 2.07%; MCG, 8.2 ± 1.3%; HCG, 5.4 ± 1.14%) compared with HTK solution without CG (all P < 0.05). Representative images of TGF-β1-positive cells are shown in Figure 5A. The TGF-β1 level in the sham group was the lowest (12 ± 4 pg/mL). The hearts stored in HTK solution showed a significantly higher TGF-β1 level (52 ± 12 pg/mL), whereas the addition of CG reduced the TGF-β1 level (LCG, 37 ± 8 pg/mL; MCG, 32 ± 5 pg/mL; HCG, 21 ± 4 pg/mL; all P < 0.05 versus HTK solution only; Figure 6).

**Discussion**

Graft storage plays a decisive role in the recovery of cardiac function after heart transplantation. Hypothermia is widely used for its protective effect on organs and tissues during their storage. Although hypothermia has
strong beneficial effects, it also triggers cellular injury to cardiomyocytes, which limits the long-term preservation of grafted organs. In our study, the effect was evaluated whether the addition of CG to HTK solution reduces chronic isograft injury in comparison to HTK solution only in a heterotopic mouse heart transplant model.

CG is a compound preparation which is mainly composed of glycyrrhizin. This compound has been associated with numerous pharmacologic effects, including anti-inflammatory, anti-allergic, protective membrane structure, and immunomodulatory properties. Ogiku, et al. reported that CG can significantly reduce ischemia/reperfusion injury in the liver. Meanwhile, our previous study also found that glycyrrhizin is protected against cold ischemic injury of the liver by inhibiting high mobility group box 1-Toll-like receptor-4 signaling. In addition, Ye, et al. also found that CG can protect mice against renal ischemia-reperfusion injury through inhibition of apoptosis and inflammation.

In this study, CG has already shown superior early postoperative results in heart transplant models: CG improved myocardial contractility and relaxation, reduced rebeating time, and decreased LDH and CK levels. Meanwhile, the histologic score of hearts was significantly re-

![Figure 3](image-url)

Figure 3. Representative histologic images as examined by hematoxylin and eosin staining (A-E) and Sirius red (F-J). Heart stored in solutions with/without the addition of the different dosage of compound glycyrrhizin, sham (A, F), histidine-tryptophan-ketoglutarate (HTK) (B, G). Low-dose compound glycyrrhizin (LCG) (C, H). Medium-dose compound glycyrrhizin (MCG) (D, I). High-dose compound glycyrrhizin (HCG) (E, J). Original magnification, × 400

![Figure 4](image-url)

Figure 4. A: Myocardial injury score after transplantation. *P < 0.05 versus sham; †P < 0.05 versus histidine-tryptophan-ketoglutarate (HTK); ‡P < 0.05 versus high-dose compound glycyrrhizin (HCG). B: Interstitial fibrosis of hearts after transplantation. *P < 0.05 versus sham; †P < 0.05 versus histidine-tryptophan-ketoglutarate (HTK); ‡P < 0.05 versus high-dose compound glycyrrhizin (HCG)
Reduced TGF-β1 expression of hearts stored in the new HTK solution with addition of CG indicates that CG may reduce chronic graft injury. The above results indicated that the addition of CG can significantly reduce the cold ischemic injury of heart graft. Furthermore, the protective activity of CG was dose dependent. The anti-ischemic effect of CG is mainly related to two aspects: First, CG can inhibit the activation of nuclear factor NF-κB, thus inhibiting the synthesis and release of multiple inflammatory factors, and second, CG can selectively inhibit the activities of lipoxygenase and phospholipase and increase the production of leukotrienes and prostaglandins, thus achieving the effect of anti-inflammation and protection of cell membranes.

**Conclusion**

In conclusion, based on the data of our study, the addition of CG to HTK solution markedly attenuates cold-induced heart injury and improves graft survival compared...
with the HTK solution only. The potential limitation of this study was that mainly the chronic graft injury was analyzed. These results have to be confirmed in clinical trials.

Disclosure
Conflicts of interest: No conflicts of interest or sources of funding exist.

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