Superior myocardial preservation with HTK solution over Celsior in rat hearts with prolonged cold ischemia

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Background. Increasing allograft ischemic time is a significant risk factor for mortality following heart transplantation (HTx). The purpose of this study was to evaluate the protective effects of histidine-tryptophan-ketoglutarate (HTK) and Celsior (CEL) using a rat HTx model with prolonged cold storage.

Methods. The hearts were excised from donor rats, stored in cold preservation solution for either 6 or 18 hours, and heterotopically transplanted into syngeneic recipients. Serum creatine phosphokinase (CPK), serum troponin I, graft-infiltrating cells, graft mRNA levels for inflammatory mediators, and tissue adenosine triphosphate (ATP) levels were analyzed, as markers of graft injury.

Results. The recipients of grafts stored in HTK for 18 hours of prolonged cold ischemia had lower levels of serum CPK and tissue malondialdehyde, less upregulation of the mRNAs for IL-6 and inducible nitric oxide synthase, less apoptosis, and higher ATP levels than those receiving grafts stored in CEL and Saline. Cardiac contraction 3 hours after reperfusion was observed in 43% of the cardiac grafts stored in HTK for 18 hours, while no cardiac wall movement was seen in grafts stored in either saline or CEL.

Conclusion. Cold storage in HTK exhibited superior protective effects against prolonged cold ischemia in a syngeneic rat transplantation model. (Surgery 2010;148:463-73.)

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INCREASING ALLOGRAFT ISCHEMIC TIME continues to be a significant risk factor for mortality following heart transplantation.1 Organ preservation solutions have been developed to mitigate the organ injury during procurement, preservation, transportation, and implantation.2 However, preservation of donor hearts after cardioplegic arrest is still limited to 4–6 hr and an ischemic period exceeding 4–5 hr adversely affects graft survival in the current clinical program.3 Therefore, developing methods to protect the heart from ischemia/reperfusion (I/R) injury following prolonged ischemia is critically important to improve outcomes after heart transplantation and to expand the donor pool by increasing the time available for transportation and may help overcome the disparity between the demand for and the supply of cadaver donors.

The formulations of the preservation solutions currently used in a clinical setting target the 4 major goals of effective preservation: prevention of cell swelling, limitation of calcium overload, counteraction of free radical injury, and prevention of contracture due to energy loss.4 Celsior (CEL) solution contains impermeant inert osmotic carriers (lactobionate and mannitol) and a strong buffer (histidine buffer).5 Furthermore, CEL contains reduced glutathione, one of the most effective antioxidants currently available for clinical use, for prevention of oxygen-derived free radical injury.6 Histidine-tryptophan-ketoglutarate (HTK) is an extracellular-type solution with a low sodium concentration (15 nmol/liter) and slightly higher potassium concentration (10 nmol/liter). The high concentration of histidine (198 nmol/liter) in HTK solution acts as a high-capacity buffer, maintaining extracellular and intracellular pH during ischemia (Table).7

Clinical studies in the U.S. and Europe have demonstrated that CEL provides satisfactory
results for heart transplantation (HTx). HTK has also been used for clinical HTx by some transplant programs in the U.S. However, preclinical and clinical comparisons of HTK and CEL are still limited and controversial. In this study, we examined the protective efficacies of these 2 low potassium solutions (HTK and CEL) during ischemia and reperfusion using a rat heterotopic HTx model with prolonged cold ischemia and paid special attention to the myocardial content of energy-rich phosphates during and after cold ischemia.

METHODS

Animals. Inbred male Lewis rats (LEW, RT-1, 200–250 g) were purchased from Harlan Sprague Dawley, Indianapolis, IN. Animals were maintained in laminar flow cages in a specific pathogen-free animal facility at the University of Pittsburgh and fed a standard diet and water ad libitum. All procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee at the University of Pittsburgh and the National Research Council’s Guide for the Humane Care and Use of Laboratory Animals.

Heterotopic heart transplantation. Heterotopic HTx was performed as described previously. Shortly after anticoagulation with 300 units of heparin, 3–5 mL of normal saline (Hospira Inc., Lake Forest, IL), Celsior (CEL: Sangstat Medical, Menlo Park, CA), or HTK (Custodiol, Alsbach-Hähnlein, Germany) was infused into the donor heart through the inferior vena cava. The excised grafts were stored in either saline, CEL, or HTK at 4°C for 6 or 18 hr. End-to-side anastomoses between the graft ascending aorta and the recipient infrarenal aorta, and between the graft pulmonary artery and the recipient vena cava, were performed with 10-0 suture. The number of experiments per group (n) refers to the number of individual animals used for each endpoint.

Measurement of myocardial injury enzymes. Serum creatine phosphokinase (CPK) and troponin I levels were measured using a Beckman autoanalyzer (Beckman Instruments, Fullerton, CA).

Functional and macroscopic observations on cardiac grafts. We evaluated the gross morphology of the grafts, with their identities masked, and assigned a transplant score based on contractility (0: none; 1: moderate; 2: best), color (0: dark; 1: partially dark; 2: healthy) and turgor (0: hard; 1: soft) at 10 min and 3 hr post-reperfusion. The failure of graft function was diagnosed by the cessation of the beat and confirmed by direct visualization and histopathology.

Detection of apoptosis. Formalin-fixed, paraffin-embedded cardiac graft tissue and recipient heart tissue, taken after 18 hr of cold ischemia and 3 hr of reperfusion, were cut into 6-μm sections. Apoptosis was investigated using the ApopTag Peroxidase Kit (Intergen Co., Purchase, NY) for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay. TUNEL-positive cells in 5 random high-power fields per section were counted with the sample identities masked.

Transmission electron microscopy. Heart grafts were harvested and immersion-fixed in 2.5% glutaraldehyde overnight at 4°C. Following fixation, the tissue was dehydrated through a graded series of 30–100% ethanol, 100% propylene oxide, then infiltrated with a 1:1 mixture of propylene oxide: Polybed 812 epoxy resin. After several changes of 100% resin, the tissue was embedded and cured. Ultrathin (70 nm) sections were collected on 200 mesh copper grids and stained with 2% uranyl acetate and 1% lead citrate. Sections were visualized using a JEOL JEM 1210 transmission electron microscope at 80 kV.

Realtime reverse transcription-polymerase chain reaction. The levels of mRNA for interleukin (IL)-6, intercellular adhesion molecule-1 (ICAM-1), inducible nitric oxide synthase (iNOS) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were quantified using SYBR Green 2-step, real-time reverse transcription-polymerase chain reaction (RT-PCR) in duplicate, as previously described. Gene expression was normalized to GAPDH mRNA content.

<table>
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<th>Compounds</th>
<th>Saline</th>
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<th>HTK</th>
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Enzyme-linked immunosorbent assay (ELISA) for serum IL-6. Serum IL-6 concentrations were measured in duplicates using commercially available ELISA assays (Rat Interleukin-6 ELISA Kit; BioSource, Camarillo, CA) according to the manufacturers' instructions.

Serum nitrite (NO$_2^-$) and nitrate (NO$_3^-$). Nitrite and nitrate levels in plasma were measured using a tri-iodide/ozone-based chemiluminescent nitric oxide analyzer (Model 280 NO analyzer; Sievers, Boulder, CO).

Infiltrating neutrophil stain. Grafted heart tissue, taken after 6 hr of cold ischemia and 12 hr of reperfusion, was fixed in 10% formalin, embedded in paraffin, cut into 6 μm sections, and stained with a naphthol AS-D chloroacetate esterase staining kit (Sigma Diagnostics, St. Louis, MO) for the presence of granulocytes according to the manufacturers' instructions. Positively stained cells were counted with the samples' identities masked. Data are presented as the number of positive cells per 400 × HPF.

Western blot analysis for caspase-3 and adenosine monophosphate (AMP)-activated protein kinase. Thirty micrograms of cytosolic proteins were electrophoresed on 6-15% acrylamide sodium dodecyl sulfate gels and transferred to nitrocellulose membranes (Scleicher & Schuell, Keene, NH). To block nonspecific binding, 5% nonfat dry milk in phosphate buffered saline (PBS)-Tween (0.1%) was added to the membrane for 1 hr at room temperature. Membranes were washed in PBS-Tween and then incubated overnight with anti-cleaved caspase-3 rabbit polyclonal antibodies, anti-β-actin mouse...
polyclonal antibodies, anti-AMP-activated protein kinase (AMPK)-alpha rabbit monoclonal antibodies and anti-phosphorylated (p-Thr 172) AMPK-alpha (dilution 1:1,000; Cell Signaling, Boston, MA) followed by incubation with an appropriate secondary antibody for 1 hr. After repeated washings with PBS-Tween, membranes were developed with the SuperSignal detection system (Pierce Chemical, Rockford, IL) and exposed to film.

**Assessment of graft oxidative injury.** Tissue malondialdehyde (MDA) level, an indicator of oxidative stress in cells and tissues, was assessed using the Bioxytech MDA-586 Kit (Oxis Research, Portland, OR), according to the manufacturer’s protocol.

**Measurement of tissue adenosine triphosphate levels.** Cellular adenosine triphosphate (ATP) level was quantified using the ENLITEN ATP luciferin/luciferase bioluminescence assay system (Promega, Madison, WI). Myocardial tissue specimens were excised after 0, 6, 12 and 18 hr of cold ischemia, immediately frozen in liquid nitrogen, and individually pulverized into a fine powder by hand grinding with a dry ice-chilled steel mortar and pestle. Ten milligrams of myocardium were homogenized with 1 mL of precooled extractant (1.0% trichloroacetic acid) and centrifuged at 4,500 rpm for 10 min. Supernatant (100 μL) was diluted 10-fold with 50 mmol/L Tris-acetate buffer containing 2 mmol/L ethylenediaminetetraacetic acid (pH 7.75). Then, 20 μL of sample extract or reference standard solution was placed in a 96-well microplate, followed by the injection of 100 μL of ATP luciferin/luciferase assay mix for ATP quantification. Luminescence was measured at a set lag time of 1 s (1420 VICTOR™ multilabel counter; PerkinElmer Life Sciences, Waltham, MA).

**Statistical analysis.** The results are expressed as mean with standard deviation. All data were analyzed using the SPSS Version 12 statistical software package (SPSS Inc., Chicago, IL). When ANOVA indicated a significant overall effect, differences among individual means were assessed using the Bonferroni post hoc test for multiple comparisons. A probability level of $P < .05$ was considered statistically significant.

**RESULTS**

Preservation in HTK attenuates myocardial injury of the grafts with prolonged cold ischemia. Serum concentrations of CPK and troponin I were determined 3 hr after reperfusion following 6 or 18 hr of cold ischemia in either preservation solution. Although graft preservation in saline for 6 hr resulted in increases in serum CPK and troponin I, HTK and CEL significantly reduced the serum levels of these myocardial injury markers ($n = 5–7$; Fig 1, A and B). As expected, extended cold ischemia for 18 hr led to more myocardial injury than 6 hr of storage and was associated with deterioration of the gross appearance of the cardiac grafts. Cardiac contraction 3 hr after reperfusion and improved gross structural appearance were observed in 43% of the cardiac grafts stored in HTK for 18 hr, while no cardiac wall movement was seen in grafts stored in either saline or CEL (Fig 1, C). Strikingly, the recipients with HTK-treated cardiac grafts exhibited lower serum CPK levels and less macroscopic deterioration of the graft than recipients of CEL-treated cardiac grafts. This suggests superior protective properties of HTK over CEL for cardiac grafts with an extremely extended cold ischemic period (Fig 1, A–D).
HTK preserves the microscopic morphology of grafts subjected to 18-hr ischemia. To further examine the detailed morphologic changes following prolonged cold storage, transmission electron microscopy was performed on heart grafts taken 30 min after reperfusion. In saline-treated grafts, swelling of the mitochondria and irregular muscle fibers were noted. CEL-treated cardiac grafts exhibited myocardial edema, accompanied by damage to the myocardial membrane. The myocardial filaments were arranged irregularly and were partially ruptured with thickened and distorted Z lines. The mitochondria in the CEL-treated exhibited light to moderate swelling, with ruptured, lysed, and vacuolated cristae. On the other hand, the ultrastructural analysis of the myocardium of HTK-treated grafts showed minimal abnormalities; the myocardial filaments were arrayed linearly with vivid Z lines. The mitochondria were enlarged but retained a normal shape and compact cristae (Fig 2).

Cold storage in HTK reduces I/R-induced apoptosis in cardiac grafts. The tissue response to myocardial injury and subsequent apoptosis are critical events during early graft failure. TUNEL assay was performed to detect apoptosis in the
heart grafts after 6- and 18-hr cold ischemia. While TUNEL-positive cells were scarcely seen in the cardiac grafts with 6 hr cold preservation, regardless of preservation solution (data not shown), prolonged cold ischemia for 18 hr resulted in a remarkable increase in apoptosis 3 hr after reperfusion (Fig 3, A). Caspase-3 has been identified as a key mediator of apoptosis of mammalian cells. There was less cleaved caspase-3 in HTK-treated grafts compared to saline or CEL-treated grafts (Fig 3, B). Preservation with HTK solution significantly reduced graft apoptosis as compared with saline or CEL (Fig 3, A and B).

**HTK inhibits lipid peroxidation in cardiac grafts.** The antioxidant capabilities of CEL and HTK were evaluated by measuring tissue MDA levels, a marker of lipid peroxidation. Tissue MDA levels markedly increased 3 hr after reperfusion in the cardiac grafts stored in saline for 18 hr. Both CEL and HTK had antioxidant effects and reduced tissue MDA levels 3 hr after reperfusion. Of interest, the grafts stored in HTK contained significantly less MDA compared with those in CEL, suggesting that HTK may prevent graft oxidative injuries better than CEL (Fig 4).

**Preservation in HTK inhibits upregulation of inflammatory mRNAs.** Quantitative RT-PCR for inflammatory mediators was conducted using heart graft samples taken 3 hr after reperfusion. A robust inflammatory response, with upregulation of IL-6, ICAM-1, and iNOS, occurs during cold I/R even in the absence of alloimmunity. Intra-graft mRNA levels for these proinflammatory mediators were significantly increased after 18 hr of cold ischemia and 3 hr of reperfusion in the grafts stored in saline. Expression of these proinflammatory mediators was efficiently inhibited by preservation with CEL or HTK. IL-6 and iNOS mRNAs were significantly decreased in HTK-treated grafts compared with CEL-treated grafts (Fig 5, A–C). Correlating with mRNA levels, serum IL-6 levels were significantly decreased after 18 hr of cold ischemia and 3 hr of reperfusion in the grafts stored in saline. Expression of these proinflammatory mediators was efficiently inhibited by preservation with CEL or HTK. IL-6 and iNOS mRNAs were significantly decreased in HTK-treated grafts compared with CEL-treated grafts (Fig 5, A–C). Correlating with mRNA levels, serum IL-6 levels were significantly decreased after 18 hr of cold ischemia and 3 hr of reperfusion in the grafts stored in HTK compared with those in saline or CEL (Fig 5, D). The plasma levels of nitrite and nitrate (NO\(_2^-\)/NO\(_3^-\)) were also measured to determine NO production by iNOS. Plasma nitrite levels in the recipients grafted with HTK-treated cardiac grafts were significantly lower than those with CEL or saline (Fig 5, E and F).

**Cold storage in HTK reduces I/R-induced neutrophil infiltration in cardiac grafts.** Neutrophil infiltration plays critical roles in the pathogenesis of cardiac I/R injury. While naphthol-positive cells were scarcely seen in the cardiac grafts with 18 hr of cold preservation and 3 hr of reperfusion, regardless of preservation solution (data not shown), there were marked infiltrating neutrophils in the saline-control grafts after 6 hr of cold ischemia and 12 hr of reperfusion. Preservation with HTK solution significantly reduced neutrophil infiltration of the grafts as compared with CEL (Fig 6).

**Immunoblotting of graft AMP-activated protein kinase.** Because the AMPK system acts as a sensor of cellular energy status, we examined the phosphorylation of AMPK in the grafts. There were no differences in the expression of total AMPK among the saline, CEL and HTK-treated grafts after 18 hr of cold ischemia and 30 min of reperfusion. However, there was significantly less phosphorylation of AMPK on Thr 172 in HTK-treated grafts than in saline or CEL-treated grafts, suggesting that HTK-treated grafts had less metabolic stress than saline- or CEL-treated grafts (Fig 7).

**HTK solution preserves tissue ATP levels in cardiac grafts.** Next, we investigated if there was any difference in the maintenance of ATP depending on which solution was used during cold storage, because functional recovery of the graft depends on the availability of energy at the beginning of reperfusion. ATP levels in the cardiac grafts gradually reduced with time in all 3 solutions, but ATP concentrations were maintained at significantly higher levels in the grafts treated with HTK, as compared with saline or CEL (Fig 8).
DISCUSSION

In this study, HTK exhibited protective effects against cold ischemia superior to those of CEL during cold storage in a syngeneic rat HTx model. The recipients of grafts stored in HTK for 18 hr had lower levels of serum CPK, less upregulation of the mRNAs for IL-6 and iNOS, less apoptosis, and improved gross morphology, including wall movement, compared to recipients of grafts stored in CEL.

The mechanisms by which HTK prevents cold I/R injury in HTx have not been fully elucidated. One possible mechanism may be a higher level of ATP-producing anaerobic glycolysis. Before reperfusion, the myocardial content of ATP is critically important, because a lack of energy-rich phosphates may reduce the activity of the contractile apparatus. Our results showed that the ATP level before reperfusion was significantly higher in HTK-treated hearts than in CEL-treated hearts after both 6 hr and prolonged ischemia, indicating that the energetic starting point before reperfusion was better in hearts perfused with HTK as compared with hearts perfused with CEL. A possible explanation for these differences is the higher buffering capacity of HTK. Both solutions contain the same buffer, histidine, but its concentration in CEL is approximately 15% of that in HTK, leading to an approximately 7-fold higher buffering capacity of HTK as compared with CEL. In a comparison of differently buffered preservation solutions, increasing buffering capacity enhanced anaerobic energy production by relieving the pH inhibition of key enzymes in the glycolytic pathway and by maintaining the phosphorylation of phosphofructokinase. Due to the higher levels of ATP-producing anaerobic glycolysis in HTK-treated hearts, the glycogen values were lower and the lactate values higher compared with CEL-treated hearts. Acidotic damage was prevented because of the high buffering capacity.

Myocardial ischemia results decreased mitochondrial oxidative metabolism and decreased ATP production. AMPK is a key kinase that can increase energy production in the ischemic heart. During ischemia, a rapid activation of AMPK...
occurs, resulting in the activation of both glucose uptake and glycolysis in the myocardium, as well as an increase in fatty acid oxidation. AMPK is activated during metabolic stress, and not only activates a number of energy-producing metabolic pathways, but also inhibits energy-consuming pathways. As a result, AMPK can be considered a “fuel gauge” in the cell. This role of AMPK as a fuel gauge is particularly relevant in the heart because the heart has a very high energy demand and very little energy reserves. If ATP production ceases in the heart, ATP supply would be depleted within seconds.

During ischemic storage prior to heart transplantation surgery, myocardial protection plays a key role in preserving myocardial function after surgery and the preservation solution provides key protective functions. After procurement, blood supply to the myocardium is stopped. Cells then start to go into a hypoxic state and begin to break down glycogen and ATP. When too much glycogen and ATP are used, the cells become acidic and are severely damaged. There are differences by organ in the “acceptable” period of cold ischemia. Cold ischemia times ranging from 12 to 30 hr can be accepted in a kidney transplantation, but they are not suitable for heart transplantation. However, there are unavoidable situations in which the ischemic time must be extended due to procurement of organs at distant locations. In fact, recent clinical observation have suggested that the ischemic time in heart transplantation can be extended to 6–8 hr without adverse effects. Thus, establishment of satisfactory organ preservation methods is mandatory for successful heart transplantation with prolonged ischemic time and may result in expansion of the donor pool.

In recent years, increasing evidence on the advantages of low potassium “extracellular” solutions has emerged. The ingredients of CEL are supposed to prevent important causes of ischemic cell damage. The impermeants, mannitol and lactobionate, are added to reduce cell swelling.
Reduced glutathione, histidine, and mannitol minimize oxygen derived free radical injury. Glutamate is applied as a substrate to enhance energy production and thus prevent contracture. High magnesium content and a slight degree of acidosis are assumed to prevent calcium overload. HTK was developed in 1975 by Bretschneider, and is being widely used as an organ transplantation storage solution in Europe. In contrast to other solutions, HTK contains low levels of both sodium and potassium levels. The addition of histidine provides excellent buffering capabilities, contributing to suppression of acidosis. Histidine also increases glycolysis and stabilizes ATP and high-energy phosphates—further improving myocardial protection. Tryptophan contributes to the stabilization of the cell membrane, thus suppressing intracellular influx of histidine. In addition, ketoglutarate acts as an intracellular energy source that allows increased ATP production. Mannitol suppresses cellular edema caused by anaerobic cell damage, and increases the removal of free oxygen radicals. HTK is superior to University of Wisconsin solution (ViaSpan; DuPont, Wilmington, DE) as an initial flushing solution, not only for heart, but also for liver and small bowel, mainly due to its lower viscosity of 1.3–1.8 cP.

The clinical studies concerning the recovery of heart function after preservation with HTK are partly contradictory. A multicenter study on a large series of HTx using HTK solution showed good long-term results. The longest ischemic time for donor hearts in recent years is 13 hr with HTK. Garlicki et al. reported, in a trial with 224 patients, a trend toward decreased need for inotropic agents postoperatively in patients receiving hearts stored in HTK compared with patients receiving hearts stored in CEL or University of Wisconsin solution. In contrast, the preliminary data of a randomized prospective study with 48 human heart transplantations indicated that recipients of hearts stored in HTK (7/24) needed more postoperative inotropic support than the recipients of hearts.
stored in CEL (2/24). In animal experiments, there is direct evidence of free radical production proportional to the length of ischemia in ischemic reperfused hearts. Similar to our results, Ackemann et al reported that HTK-preserved hearts contained more ATP than CEL-preserved hearts after 8 and 12 hr of ischemia and showed significantly higher systolic and diastolic function.

Our results explicitly demonstrated that HTK had superior protective effects against cold ischemia in particular for cardiac grafts with prolonged cold storage. The data presented here cannot be immediately extrapolated to clinical conditions, because damaged hearts of human donors and hearts grafts retrieved from healthy rats may not be directly comparable. In the healthy rat heart, resuscitation after cardioplegia with CEL and 6–12 hr of ischemia at 4°C proceeded smoothly; the first disturbances (incidences of not beating) occurred after 18 hr of ischemia. At this ischemic time, near the limit of resuscitability, differences in protective efficacy should be pronounced. Another limitation is that heterotopic heart transplantation model may not be ideally suited to detect subtle functional differences of the cardiac grafts, because the hearts are not loaded. Also, transplantation of an isograft is artificial and it does not happen in the clinical transplantation setting, except for the rare case of identical twins. However, syngeneic transplantation is considered an ideal experimental model to study I/R injury because it allows isolation of factors related to I/R injury from factors involved in alloimmune reactions.

In conclusion, our data clearly demonstrate that HTK exhibits superior protection against cold ischemia during cold storage in a syngeneic rat heart transplantation model. HTK may have more therapeutic value than CEL, particularly for cardiac grafts with prolonged cold storage.

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