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Myocardial Protective Effect of Urethane on Isolated Rat Hearts in Prolonged Hypothermic Preservation

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ABSTRACT

Background: One of the most important factors restricting heart transplantation is the limited myocardial ischemia time. This study investigated the effects of urethane on the hypothermic preservation of donor rat hearts.

Materials and Methods: Hearts isolated from rats were divided into 2 groups (n = 8), a control group with histidine-tryptophan-ketoglutarate (HTK) solution alone and an experimental group with HTK solution plus 30 mM urethane. Hearts were mounted on a Langendorff apparatus to estimate the baseline cardiac function; the hearts were then arrested and stored in one of the 2 solutions for 6 hours and 18 hours at 4°C. After preservation, the hearts were reperfused, and cardiac function was evaluated. Lactate dehydrogenase (LDH) release, adenosine triphosphate (ATP) content, cardiomyocyte apoptosis, and myocardial ultrastructure were examined.

Results: Compared with the control group, the experimental group showed a significantly higher recovery of cardiac function for both 6 hours and 18 hours of preservation and demonstrated a lower rate of cardiomyocyte apoptosis (8.5% ± 1.2% versus 12.2% ± 1.8% for 6 hours; 14.1% ± 2.1% versus 31.4% ± 2.7% for 18 hours). ATP content was significantly higher in the experimental group than in the control group after 18 hours of preservation (229.4 ± 29.7 μg/g versus 153.2 ± 21.1 μg/g). The experimental group also showed lower levels of LDH release after 18 hours of preservation. Electron microscopy studies demonstrated better cardiomyocyte structure in the experimental group for both 6 hours and 18 hours of preservation.

Conclusions: Use of urethane improved cardiac functional recovery and led to significant protective effects on rat hearts placed in a hypothermic preservation solution for a prolonged period.

Zhenxiao Jin, Tiejun Wu, Shenghui Bi, Heping Zhou, and Qin Cui contributed equally to this work.

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INTRODUCTION

Preservation of donor hearts has usually been limited to 4 to 6 hours in a cold ischemic environment. The effects depend mainly on the preservative solution used and its temperature [Chen 2005]. Thus, the protection and preservation of donor hearts is an important and interesting issue in the transplantation field.

Urethane is an anesthetic agent commonly used in animal experimentation because of its long duration of action, skeletal muscle relaxant properties, and its minimal impairment of circulatory and respiratory systems [Koblin 2002]. Urethane has a number of biological effects in addition to anesthesia, analgesia, and sedation. Urethane has been reported to prevent the occurrence of histologically detectable myocardial alterations after treatment with toxic digoxin doses [Princi 2000] and to reduce organ injury and animal mortality after lipopolysaccharide injection in a rat model of sepsis [Kotanidou 1996].

As we know, some anesthetics can precondition hearts against ischemia/reperfusion injury. Unlike pentobarbital, urethane can cause a hyperglycemic response, but the nitric oxide release does not have a major influence with respect to either anesthetic. Ketamine is a nonbarbital anesthetic agent that acts as an α_2-adrenergic agonist and possesses sedative and analgesic effects similar to urethane [Maggi 1984]. The actions of both pentobarbital and ketamine are fast, but their

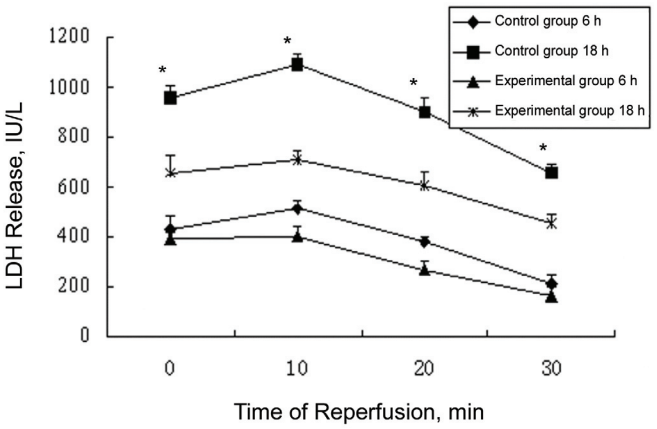

Figure 1. Lactate dehydrogenase (LDH) release after 6 hours and 18 hours of preservation. *P < .05 versus control group after 18 hours of preservation.

Rate of Recovery of Cardiac Function after Preservation*

Variable	6 Hours		18 Hours	
	Control Group	Experimental Group	Control Group	Experimental Group
LVDP, %	79.4 ± 4.3	86.7 ± 4.6†	41.1 ± 3.0	62.8 ± 3.7‡
+*dP/dt*, %	77.2 ± 5.1	88.4 ± 4.9†	39.7 ± 2.9	65.1 ± 5.4‡
–*dP/dt*, %	81.1 ± 6.3	87.5 ± 7.9†	38.2 ± 3.4	63.4 ± 4.9‡

*Data are expressed as the mean ± SD (n = 8 for each group). LVDP indicates left ventricular developed pressure; +*dP/dt*, LV maximal rate of change in pressure; –*dP/dt*, LV minimum rate of change in pressure.

†$P < .05$, versus the control group for 6 hours of preservation.

‡$P < .05$, versus the control group for 18 hours of preservation.

durations are brief. They are not suitable to use as protective agents for preserving donor hearts.

Histidine-tryptophan-ketoglutarate solution (Custodiol HTK®; Dr. Franz Köhler Chemie, Bensheim, Germany) is one of the better protective agents for preserving donor hearts; however, its preservative effect needs further improvement in clinical settings that require a lengthy transportation of a donor heart. The hypothesis of this study is that urethane can improve the protective effects of isolated rat hearts after a long period of preservation in hypothermic HTK solution.

MATERIALS AND METHODS

Experimental Group

Male Sprague Dawley rats (280-300 g) were randomly divided into 2 groups (8 per group) according to the preservation solution used. In the control group, hearts were arrested and stored in HTK solution; in the experimental group, hearts were arrested and stored in HTK solution plus 30 mM urethane. Hearts were arrested and stored in each solution for 6 hours and for 18 hours at 4°C. The investigators measuring the samples were blinded to the group and the treatment. The investigation conformed to the Guide for the Care and Use of Laboratory Animals [Institute of Laboratory Animal Resources 1996].

Isolated Heart Perfusion

Rats were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneally). The hearts were excised, rapidly mounted on a Langendorff perfusion apparatus, and perfused at a pressure of 75 mm Hg with Krebs-Henseleit buffer (KHB) equilibrated with 95% O_2 and 5% CO_2 at 37°C. The KHB contained 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 25.2 mM $NaHCO_3$, and 11 mM glucose. Cardiac function was monitored via a fluid-filled balloon placed into the left ventricle, and the end-diastolic pressure was set to 5 mm Hg by adjusting the balloon volume. The baseline cardiac function was determined after 30 minutes of equilibration. Assessment of cardiac function included measurement of the left ventricular developed pressure (LVDP), the LV maximal rate of change in pressure (+*dP/dt*), and the LV minimum rate of change in pressure (–*dP/dt*). The hearts were then arrested by flushing each with their respective preservation solution and stored in the same solution (100 mL) for 6 hours and 18 hours at 4°C. After preservation, the hearts were reperfused with KHB at 37°C for 30 minutes; cardiac function was then evaluated.

Lactate Dehydrogenase Release Assay

Timed collection of coronary effluent was performed to obtain the baseline coronary flow, and the release of lactate dehydrogenase (LDH) into the coronary effluent was measured with an LDH assay kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturers' instructions. The absorbance was read at 440 nm with a spectrophotometer (Biochrom, Cambridge, UK).

Adenosine Triphosphate Content Assay

Adenosine triphosphate (ATP) content was measured by high-performance liquid chromatography analysis of perchloric acid extracts of tissue samples, as previously described [Pang 2004]. In brief, neutralized acid extracts were loaded onto a Partisil 10 SAX anion-exchange column (250 × 4.6 mm; Whatman/GE Healthcare, Piscataway, NJ, USA) and eluted with a gradient of ammonium dihydrogen phosphate from 15 mM (pH 2.8) to 1 mM (pH 3.7). ATP content was determined by ultraviolet detection.

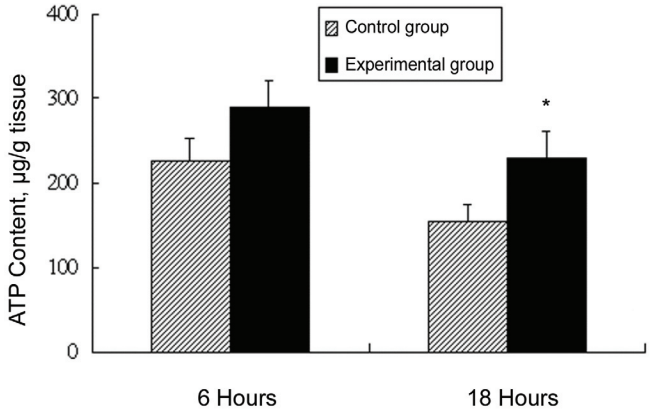

Figure 2. Myocardial adenosine triphosphate (ATP) content after 6 hours and 18 hours of preservation. *$P < .05$ versus control group after 18 hours of preservation.

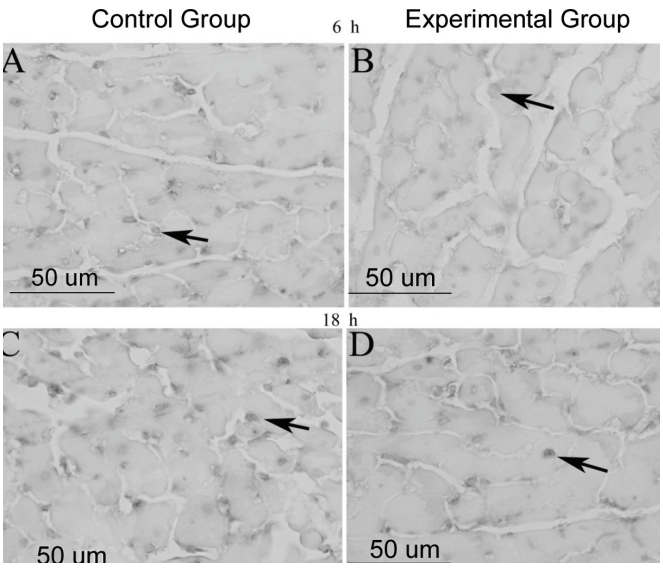

Figure 3. Morphologic apoptosis was determined by terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) staining after 6 hours and 18 hours of preservation (original magnification ×400). Arrows indicate TUNEL-positive cardiomyocyte nuclei.

Cardiomyocyte Apoptosis Assay

Apoptosis was detected by the terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) method with the Apoptosis in situ Detection Kit (B-Bridge International, Mountain View, CA, USA) according to the manufacturer's instructions. Apoptosis in cardiomyocytes was quantified by the number of apoptotic nuclei among the total nuclei in 10 continuous microscopic fields evaluated under 400× magnification.

Electron Microscopy Assay

Specimens were fixed in 2.5% glutaraldehyde at room temperature for 4 hours, postfixed with 1% osmium tetroxide solution at 4°C for 1 hour, dehydrated through a graded ethanol series, and embedded in Araldite. Ultrathin sections were stained with uranyl acetate and lead citrate, and photographed with a JEM-2000EX transmission electron microscope (JEOL, Tokyo, Japan).

Statistical Analysis

Data are expressed as the mean ± SD. The Student *t* test was used for statistical analyses. Differences were considered statistically significant at *P* values <.05.

RESULTS

Recovery of Cardiac Function

There were no significant differences between the 2 groups in baseline cardiac function before preservation (data not shown). The Table shows the recovery rates for cardiac function in each group after 6 hours and 18 hours of preservation. The recovery rates of LVDP, +*dP/dt*, and –*dP/dt* in the experimental group were significantly higher than those in the control group for both 6 hours and 18 hours of

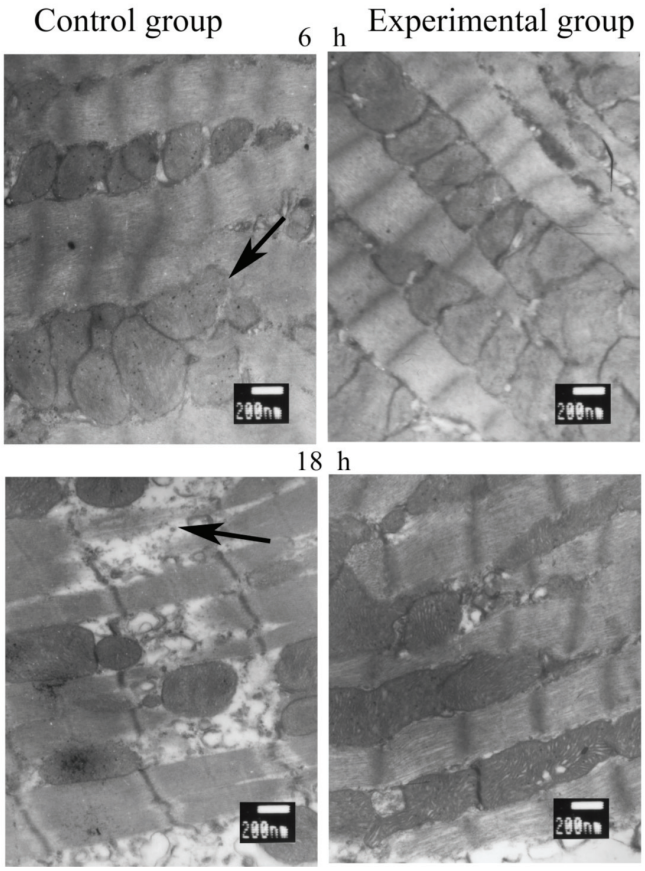

Figure 4. Electron microscopy findings in cardiomyocytes after 6 hours and 18 hours of preservation (original magnification ×20,000). After 6 hours of preservation, the control group showed a decrease in the density of mitochondria (arrow) compared with the experimental group. After 18 hours of preservation, the control group showed an increase in mitochondrial swelling and destruction of myofibrils (arrow), compared with the experimental group.

preservation. Although there was a dramatic decline in cardiac function after 18 hours of preservation in both groups, the recovery of cardiac function was still maintained at almost 64% of the prepreservation value in the experimental group; a progressive decline in the recovery of cardiac function to 40% occurred in the control group.

LDH Release Assay

Figure 1 shows that LDH release significantly increased after 18 hours of preservation in both groups compared with the values measured after 6 hours of preservation. Although the experimental group showed lower levels of LDH release than the control group after 6 hours of preservation, the difference was not statistically significant; however, after 18 hours of preservation, values of LDH release for the 2 groups were significantly different at all time points during reperfusion.

ATP Content Assay

After 6 hours of preservation, there were no significant differences in ATP content between the control and experimental

groups (270.3 ± 28.5 μg/g versus 289.7 ± 23.2 μg/g tissue; $P > .05$). After 18 hours of preservation, however, the mean ATP content was significantly higher in the experimental group than in the control group (229.4 ± 29.7 μg/g versus 153.2 ± 21.1 μg/g tissue; $P < .05$; Figure 2).

Apoptosis Assay

Apoptosis was apparent in both groups after 6 hours and 18 hours of preservation (Figure 3). After 6 hours of preservation, TUNEL staining showed that 12.2% ± 1.8% of the cardiomyocytes in the control group and 8.5% ± 1.2% in the experimental group displayed an apoptotic morphology, which was characterized by a brown staining of the nuclei. Prolonging the preservation time to 18 hours slightly increased the degree of apoptosis to 14.1% ± 2.1% in the experimental group but markedly increased it to 31.4% ± 2.7% in the control group ($P < .05$).

Ultrastructural Morphology Assay

Electron microscopy analysis indicated that cardiomyocyte ultrastructure and mitochondrial integrity were still preserved in both groups after 6 hours of preservation (Figure 4), but there was a decrease in the density of mitochondria in the control group compared with the experimental group. Significant alterations, such as an irregular structure of sarcomeres, destruction of myofibrils, and mitochondrial swelling, were present after 18 hours of preservation (Figure 4). Ultrastructural alterations were more distinctive in the control group than in the experimental group after 18 hours of preservation.

DISCUSSION

Successful organ preservation is an important aspect of transplantation. In this study, we changed urethane from an anesthetic agent to an additive in a heart-preservative solution (Custodiol HTK) to improve the protective effects of the HTK solution in an isolated rat model. This new strategy produced additional protective effects on the isolated rat heart after 6 hours and 18 hours of hypothermic preservation. Compared with the control group (HTK alone), the experimental group (HTK plus urethane) showed a significantly improved recovery of cardiac function, including higher LVDP, $+dP/dt$, and $-dP/dt$ values, lower levels of cardiomyocyte apoptosis and LDH release, a higher ATP content, and a better preserved cardiomyocyte ultrastructure, after both 6 hours and 18 hours of hypothermic preservation.

HTK solution is a high-quality protective agent widely used for hypothermic preservation of donor hearts [Saitoh 2000]. In this study, the recovery of cardiac function (including LVDP, $+dP/dt$, and $-dP/dt$) in the HTK group was satisfactory for a preservation period of 6 hours, and the values for ATP content and LDH release were comparable to those obtained with HTK plus urethane. The efficacy of HTK has been reported to be mostly attributable to the high buffering capacity during prolonged ischemia provided by histidine/histidine hydrochloride, which restricts ischemia-induced tissue acidosis [Yotsumoto 2003].

In the setting of a prolonged preservation time of up to 18 hours, the recovery rate of cardiac function was still maintained at 62.8% to 65.1% in the group treated with HTK plus urethane, whereas it declined to 38.2% to 41.4% in the group treated with HTK alone. The higher ATP level in the group treated with HTK plus urethane was consistent with the better recovery of cardiac function in this group. ATP content has been widely accepted to be one of the best indices for evaluating myocyte recovery [Stoica 2003]. This high-energy phosphate was preserved better in myocardial tissue, a result that might explain the better-preserved myocardial ultrastructure, less cardiomyocyte apoptosis, and less LDH release. It also implies that a urethane-induced hyperglycemic response may be a possible protective mechanism in the isolated heart during a prolonged period of hypothermic preservation, as in this study.

Although the results of this preliminary study were attractive, the study also had some limitations. As we know, urethane is an old anesthetic agent that is degraded to urea in the body, implying that urethane is a safe agent to use; however, its biocompatibility, toxicology, and carcinogenesis potential require further investigation, and a definitive explanation of its cardioprotection mechanism also requires further study.

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