SDC, Materials and Methods

Tissue water content

The cardiac grafts were harvested and weighed at post-operation hour (POH) 2 after 24-h preservation to measure the wet weight. The dry weight was determined by desiccation to constant a weight at 80°C for up to 48-h. The tissue water content was calculated by subtracting the dry weight from the wet weight and then dividing the result by the wet weight.

RNA preparation and quantitative reverse transcriptase polymerase chain reaction

Heart grafts were harvested at POH6 after 24-h preservation, and immediately submerged in RNAlater® stabilization solution (Life Technologies, Carlsbad, CA) for freezing. Total RNA was extracted using Sepasol®-RNA I Super G (Nacalai Tesque, Kyoto, Japan) and simultaneously treated with a DNA-free kit (Ambion, Life Technologies). The complementary DNA was reverse-transcribed from total RNA using a PrimeScript® RT Reagent Kit (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. A qRT-PCR was performed using a TaqMan system (Takara Bio) on an Applied Biosystem PRISM7700 instrument (Applied Biosystems, Foster City, CA). The threshold cycle (Ct) values of the target genes were normalized to the Ct value of 18S rRNA. Relative gene expression levels were calculated by Delta-Delta Ct calculation method[1].

Immunohistochemistry

Formalin-fixed, paraffin-embedded heart graft tissues at POH24 after 24-h preservation were

cut into 4μm sections for the immunohistochemical staining using anti-caspase-3 (sc-7148, Santa Cruz Biotechnology, Dallas, TX) and anti-8-OHdG (JAICA, NIKKEN SEIL Co., Shizuoka, Japan). Antigen revival was performed in sodium citrate buffer (pH = 6.0) using a microwave oven. For caspase-3 staining, slides were blocked in PBS containing 10% normal goat serum (Sigma) for 1 h and incubated with the anti-caspase-3 antibody at 1:200 dilution overnight at 4°C. Incubation with the secondary antibody (biotinylated goat anti-rabbit IgG) at 1:200 dilution and Vector ABC reagent (VECTOR Laboratories, Burlingame, UK) was performed in accordance with the manufacturer's instructions. Since the anti-8-OHdG antibody was produced in mice, a Vector® M.O.M.™ Immune Detection Kit (VECTOR Laboratories) was used according to the manufacturer's instructions in order to prevent non-specific staining. Bound antibodies were visualized using DAB (3,3′ diaminobenzidine) chromogenic substrate (Vector Laboratories). Positive cells were counted from nine random 400× high power fields (HPF) on each slide.

Myocardial cell apoptosis detection

Formalin-fixed, paraffin-embedded heart graft tissues at POH24 after 24-h preservation were cut into 4-μm thick sections. Apoptosis was detected by a TUNEL assay using an Apoptosis *in situ* Detection Kit (WAKO). The numbers of TUNEL-positive cardiac myocyte nuclei were quantified as the percentages of total nuclei from nine random HPF each section.

Lipid oxidation assay

TBARS were commonly used to detect lipid peroxidation. Serum was collected at POH6

after 24-h preservation, and the TBARS levels were measured using a Lipid Peroxidation Assay Kit (ab118970; abcam), according to the manufacturer's instructions. The samples were then read on a FlexStation® 3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA).

Tissue infiltrating neutrophil staining

Formalin-fixed, paraffin-embedded heart graft tissues at POH24 after 24-h preservation were cut into 4-μm sections. Tissue infiltrating neutrophils were stained with a Naphthol AS-D chloroacetate esterase kit (Muto Pure Chemicals, Tokyo, Japan) according to the manufacturer's instructions. Neutrophils were counted from nine random HPFs on each slide.

Myocardial glycogen staining

Formalin-fixed, paraffin-embedded heart graft tissues harvested at the end of 24-h preservation without transplantation were cut into 4-μm-thick sections. Myocardial glycogen was stained with a PAS Kit (Sigma) according to the manufacturer's instructions. PAS-positive substances were stained pink to red and nuclei were stained blue.

Transmission electron microscopy

For transmission electron microscopy, the cardiac grafts were harvested at the end of 24-h preservation without transplantation and at POH2, cut into small pieces (3×3×3 mm), fixed in 2.5% glutaraldehyde/0.05 M phosphate buffer for an additional 48 h, and then post-fixed in 1% osmium tetroxide/0.05 M phosphate buffer at 4°C for 2 h. The pieces were dehydrated with a

graded ethanol series, substituted with 100% acetone, and then embedded in epoxy resin (Quetol 812; Nissin EM). Semi-thin sections were cut, stained with 1% toluidine blue, and then observed under a light microscope to reveal the myocardium. Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and then examined under a JEM-1400 transmission electron microscope (JEOL, Tokyo, Japan). From the electron microscopic images, we assessed the grade of cellular damage by mitochondria score and sarcomere score according to Amir et al.[2] For mitochondria and sarcomere scoring, we examined twenty randomly selected sarcomeres mitochondria from images of each of 5 sections for every sample (100 sarcomeres and mitochondria in total).

Reference:

1. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25(4):402-408. doi:10.1006/meth.2001.1262

2. Amir G, Rubinsky B, Basheer SY, et al. Improved viability and reduced apoptosis in sub-zero 21-hour preservation of transplanted rat hearts using anti-freeze proteins. *J Heart Lung Transplant*. 2005;24(11):1915-1929. doi:10.1016/j.healun.2004.11.003

SDC, Figure S1.

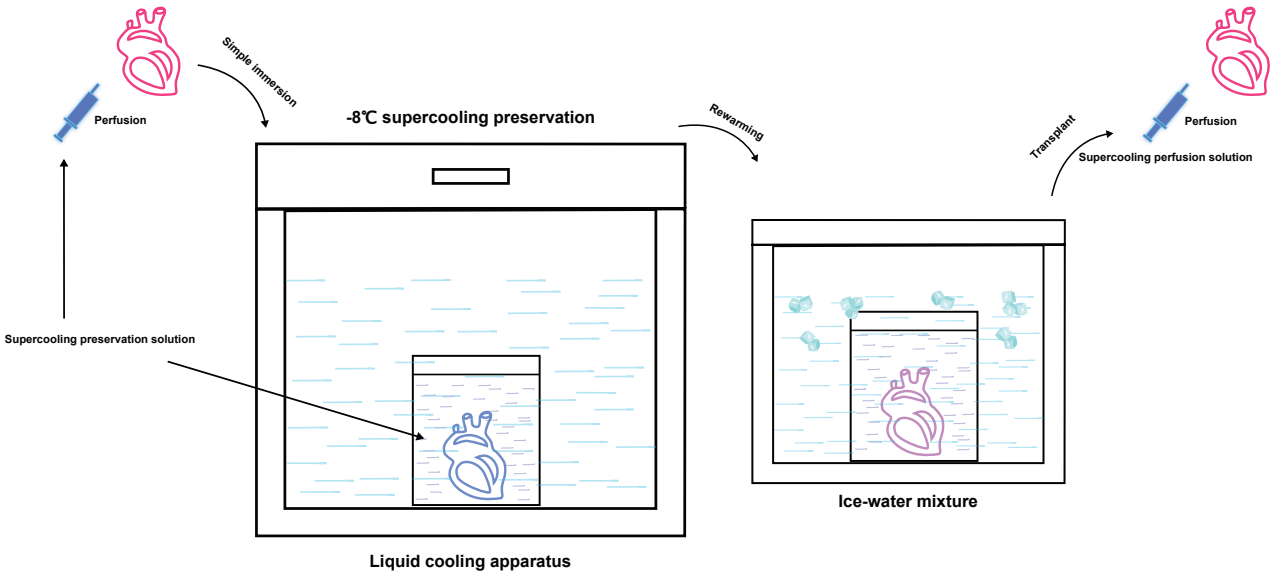

Figure S1. Supercooling preservation scheme. The supercooling preservation was achieved using a liquid cooling apparatus, novel preservation and perfusion solutions.

SDC, Figure S2.

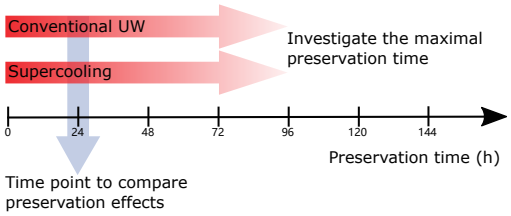

Figure S2. Experimental design. The maximal preservation time was investigated. Twenty-four-hour sample data were collected and analyzed to compare the heart graft protection effect of supercooling preservation at -8°C and conventional UW preservation at 4°C.

SDC, Table S1

Table S1. The primers and probes used in this study

Genes	Forward (5'-3')	Reverse (5'-3')	Probe
18S	ATGAGTCCACTTTAAATCCTTTAACGA	CTTTAATATACGCTATTGGAGCTGGAA	ATCCATTGGAGGGCAAGTCTGGTGC
TNF-α	TGTCTACTGAACTTCGGGGTGAT	AACTGATGAGAGGGAGGCCAT	TCCCCAAAGGGATGAGAAGTTCCCAA
iNOS	CAGTGGAGAGATTTTGCATGACA	CCCCAAGCAAGACTTGGACTT	CCACAAGGCCACATCGGATTTCACTT
HO-1	CAGGGTGACAGAAGAGGCTAAGAC	TTGTGTTCCTCTGTCAGCATCAC	TCCTGCTCAACATTGAGCTGTTTGAGGA
IL-1β	ACCCCAAAAGATGAAGGGCTG	GTGCTGCTGCGAGATTTGAAG	TCTCATCAGGACAGCCCAGGTCAAAGGT
IL-6	CTGCAAGTGCATCATCGTTGT	TGTCTATACCACTTCACAAGTCGGA	CAGAATTGCCATTGCACAACTCTTTTCTCA
CCL-2	GTTGGCTCAGCCAGATGCAG	GTAGCTCTCCAGCCTACTCATTG	CCCACTCACCTGCTGCTACTCATTCACC
HIF-1α	CCATGAGGAAATGAGAGAAATGC	GGCTTGTTAGGGTGCACTTCA	CACAGAAATGGCCCAGTGAGAAAAGGG
IFN-γ	AAGCGTCATTGAATCACACCTGA	ACCTGTGGGTTGTTGACCTCAA	ACTACCTTCTTCAGCAACAGCAAGGCGA