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Subzero Nonfreezing Storage of the Mammalian Cardiac Explant

I. Methanol, Ethanol, Ethylene Glycol, and Propylene Glycol as *Colligative Cryoprotectants*

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We employed hyperosmotic concentrations of penetrating cryoprotective agents (CPA) to store the isolated rat hearts unfrozen at subzero temperatures. The effect of acute exposure to CPA was assessed by flushing the hearts with CP-14, a cardioplegic solution, containing methanol (MeOH), ethanol (EtOH), ethylene glycol (EG), or propylene glycol (PG) for 2 min and reperfusing immediately with Krebs–Henseleit buffer in a working-heart model. The maximal doses that did not cause irreversible suppression of heart function were: MeOH, 1.78 *M*; EtOH, 1.27 *M*; EG, 0.84 *M*; and PG, 0.87 *M*. For nonfreezing storage, the hearts were flushed with CP-14 containing the highest tolerable concentrations of MeOH, EtOH, EG, or PG, stored for 6 h at −3.7, −2.8, and −1.4°C, respectively, and then reperfused. Control cardiac output (CO) was 76.2 ± 1.8 ml/min. Post-reperfusional recovery of CO was 86% in MeOH hearts, 82% in EtOH hearts, 76% in EG hearts, and 79% in PG hearts. Thus MeOH offered not only the least cardiac-suppressing effect but the lowest nonfreezing storage temperature. When storage time was extended, recovery and myocardial ATP level decreased with time in hearts flushed with CP-14 + 1.78 *M* MeOH and stored at −3.7°C. The decay of function was faster than the decay of ATP level, suggesting energy was better preserved than function. The low return of function, however, may be related to CPA toxicity, osmotic stress, and ischemia/reperfusion injury. Nonfreezing storage at subzero temperatures using these CPAs may provide a novel approach to long-term cardiac preservation.

Once removed from the body, the mammalian heart can only survive for a very brief period without implementation of special protective measures. Myocardial energy expenditure quickly exceeds production leading to a decline in cellular energy reserves. Because myocardial ATP levels seem to be intimately related to cardiac viability (9, 20, 29), cardiac preservation conventionally targets conservation of myocardial energy reserve by cardioplegia and hypothermia. Cardioplegia stops contraction and reduces a major portion of energy consumption; hypothermia further retards ATP depletion and other deleterious processes (15). With both measures, myocardial metabolism assessed by oxygen consumption can be reduced to less than 5% of the normal beating heart (6, 8). Nevertheless, the residual metabolic rate can exhaust the energy reserve in a few hours at 4°C, the temperature commonly used in clinical cardiac preservation, thus restricting the storage duration to 4 to 6 h.

Generally for every 10°C decrease in temperature, the rate of cellular metabolism is reduced by two- to threefold; the rate–temperature relationship is described as $Q_{10} = 2$ to 3 (10). Even higher Q_{10} values have been reported at low body temperatures. For example, Krogh found that in several vertebrates the Q_{10} for oxygen consumption varied from 6 between 0 and 10°C to 2 between 20 and 30°C (19). Theoretically, lowering the storage temperature for the cardiac explant by 10°C from +4° to −6°C could reduce the rate of metabolism by up to sixfold and may extend the duration of storage from 4 to 24 h. Yet subzero temper-

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atures pose the hazard of tissue freezing and ice damage. To explore the possibility of circumventing ice formation, we investigated the feasibility of treating the hearts with hyperosmotic solution and holding them at subzero temperatures. While solute toxicity and the osmotic stress are potential limitations to this approach, we recently employed hyperosmotic concentrations of ethanol (EtOH), a known cryoprotectant (CPA) that readily penetrates cells, to colligatively prevent freezing of rat hearts during subzero storage at −1.4°C (32). In the current study, we studied the acute cardiac effect of four small molecular weight fast penetrating CPAs, including methanol (MeOH), EtOH, ethylene glycol (EG), and propylene glycol (PG) as well as their utilization in subzero cardiac storage. Our results showed that MeOH had the least cardiac depressive effect and permitted nonfreezing storage of the isolated hearts at −3.7°C with substantial recovery of function after reperfusion.

MATERIALS AND METHODS

Effect of acute CPA exposure. Male Sprague–Dawley rats (300 to 350 g) were anesthetized with sodium pentobarbital (65 mg/kg, ip) and anticoagulated with heparin (250 units, iv). The heart was excised and immediately immersed in ice-cold Krebs–Henseleit buffer (KHB), which contained (in m*M*): 118 NaCl, 11 glucose, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 0.5 Na_2-EDTA, and 2.5 $CaCl_2$. The aorta was cannulated and the heart was retrograde perfused at 70 mm Hg for 10 min with KHB equilibrated with 95% O_2/5% CO_2 at 36.5°C during which time the pulmonary artery and the left atrium were cannulated. The perfusion was then switched to the working mode with a left atrial perfusion pressure of 15 cm H_2O and an afterload of 70 mm Hg. After a 30-min stabilization period, heart rate (HR, beats/min), aortic and coronary flow (AF and CF, ml/min), cardiac output (CO, the sum of AF and CF), and systolic and diastolic pressures (SP and DP, mm Hg) were recorded as the control function. The heart was then retrograde perfused sequentially with KHB for 2 min, CP-14 + CPA, a cardioplegic solution containing one of the four CPAs for 2 min at 60 mm Hg, and KHB for 2 min. The perfusion was returned to the working mode and the hemodynamic performance was recorded at the end of 20 min reperfusion as after treatment function. The composition of CP-14 + CPA was (in m*M*): 110 NaCl, 14 KCl, 7 glucose, 10 mannitol, 15 $MgSO_4$, 1.2 KH_2PO_4, 5 Na-Hepes, 0.15 $CaCl_2$, various concentrations of CPAs, with a pH of 7.5 (22°C) and saturated with 95% O_2/5% CO_2. The CPA concentrations tested were: MeOH 1.78, 2.78, 3.78 *M;* EtOH 1.27, 2.27, 2.47 *M;* PG 0.87, 1.22 *M;* EG 0.84, 1.22 *M.* Because warming and oxygenation of the flush solution caused the evaporation of MeOH and EtOH, osmolality was maintained by adding MeOH or EtOH to the flush solution just prior to flushing of each heart. Both KHB and CP-14 + CPA were passed through 0.22-μm membrane filter discs after preparation and recirculated during perfusion through 3-μm in-line filter discs. All chemicals were either cell-culture tested grade or American Chemical Society grade from Sigma Chemical Co. (St. Louis, MO). External work (g-m/min) was calculated according to Neely *et al.* (23). Coronary vascular resistance (CVR) (mm Hg-min/ml) was calculated from DP and CF (CVR = DP/CF).

Subzero nonfreezing storage of the cardiac explant. Retrograde perfusion of the isolated rat heart was initially performed at 70 mm Hg for 9 min with KHB and then continued for 2 min at 60 mm Hg with a CP-14 + CPA at 36.5°C. During the second minute of CP flush, the heart was immersed and topically cooled with ice-cold CP-14 + CPA. After cardioplegic flush, the hearts were placed individually in a 50-ml plastic centrifuge tube on CP-14 + CPA-

moistened gauze (5.1 cm × 5.1 cm eight-ply gauze sponge, Johnson and Johnson, New Brunswick, NJ) situated at the bottom of the tube. Temperature inside the tubes was maintained by immersing the tubes in a refrigerated circulating bath. A 1.5 × 3-cm strip of CP-14 + CPA-moistened Whatman No. 1 filter paper was draped over the heart to minimize drying of the surface during storage. Inclusion of CPAs in the flush solution, CP-14, increased its osmolality (280 mOsm/kg) and lowered its freezing point (FP, −0.52°C). Table 1 shows the concentration of CPAs, the osmolality and FP of the flush solution, and the storage temperatures for the different experimental groups. The storage duration was 6 h for CP-14 + EtOH, PG, or EG groups, and were 6, 8, or 9.5 h for CP-14 + MeOH groups. After storage the heart was reperfused retrograde at 70 mm Hg with KHB for 10 min during which the pulmonary artery and the left atrium were cannulated. The perfusion was then switched to the working mode with a left atrial perfusion pressure of 15 cm H_2O and an afterload of 70 mm Hg. The function of the fresh, unstored heart perfused with KHB in working mode for 30 min served as the control. Functional recovery of the stored heart at the end of 30 min working reperfusion was calculated and reported.

End-storage myocardial adenine nucleotide (AN) content determination. Hearts were flushed with CP-14 + 1.78 *M* MeOH, stored at −3.7°C for 6, 8, and 9.5 h, and clamp-frozen in liquid nitrogen. The frozen hearts were dried by lyophilization (27) and myocardial content of ANs including 5′-adenosine triphosphate (ATP), 5′-adenosine diphosphate (ADP), and 5′-adenosine monophosphate (AMP) were determined and expressed as μmol/g dry wt (31). Total AN (TAN) content was the sum of ATP, ADP, and AMP.

TABLE 1
Osmolality and Freezing Point (FP) of the Flush Solutions and the Temperatures at which the Hearts Were Stored

Solution	mOsm/kg	FP, °C	Storage temp, °C
CP-14 + 2.78 *M* MeOH	3350	−6.2	−5.5
CP-14 + 1.78 *M* MeOH	2450	−4.5	−3.7
CP-14 + 1.27 *M* EtOH	1550	−2.9	−2.8
CP-14 + 0.87 *M* PG	1150	−2.2	−1.4
CP-14 + 0.84 *M* EG	1120	−2.1	−1.4

Note. See Material and Methods for description of FP determination.

Osmolality and FP determination. The osmolality of CP-14 + CPA was measured using a freezing point depression osmometer. To estimate FP of CP-14 + CPA, 10 ml CP solution was cooled to its expected FP, calculated as FP (°C) = osmolality (Osm/kg) × −1.86°C/Osm/kg in a refrigerated bath. The solution temperature was monitored with a digital telethermometer and a thermocouple probe. An ice crystal at −20°C was introduced into the solution to initiate freezing, upon which an exotherm would occur. If there was no freezing, the process was repeated at progressively lower temperatures of 0.1°C decrements. The temperature at which freeze occurred was taken as FP of the solution. CP-14 + 1.78 and 2.78 *M* MeOH had FPs similar to the calculated ones (Table 1).

Statistical analysis. For the acute CPA exposure experiment, pre- and post-treatment function was compared using the one tail paired *t* test. For the storage experiments, differences among groups were established using the analysis of variance (ANOVA) and the Fisher's least significant difference test. *P* values less than 0.05 were considered statistically significant.

RESULTS

Effect of CPAs on cardiac function after acute exposure. Flushing the hearts with CP-14 + CPA arrested the beating instantaneously. The hearts were flushed with

CP-14 + CPA for 2 min and then reperfused with KHB. Contraction resumed immediately and forcefully, and function recovered quickly to a steady level after 10 (MeOH- and EtOH-treated hearts) or 20 min (PG- and EG-treated hearts) of reperfusion. Among all functions, heart rate was little affected by CP flush, regardless of the kind and the concentration of CPA used (Table 2). The hearts treated with both 2.78 and 3.78 *M* MeOH showed a small but significant reduction in function after reperfusion. MeOH at 1.78 *M* had no permanent effect on function; all hearts recovered normal function. Treatment with 2.47 *M* EtOH severely impaired the cardiac performance; AF was reduced by 71%, CF by 78%, and CO by 78%. With 2.27 *M* EtOH, the inhibition was less but still significant: 9% for AF, 28% for CF, and 15% for CO. EtOH at 1.27 *M* had insignificant effect; the recovery was 100% for CF and slightly higher than 100% for both AF and CO. Flushing with CP-14 + 1.22 *M* PG irreversibly suppressed AF, CF, and CO all by about 20% whereas 0.87 *M* PG did not cause any inhibition. EG (1.22 *M*) treated hearts showed a 30% reduction in AF, 21% decrease in CF, and 27% loss in CO. EG at 0.84 *M* caused a small but significant inhibition in function: 8% loss in AF and 7% loss in CO. These results established that with acute 2-min exposure, the irreversible cardiac depressing effect of the four CPAs to the rat heart is MeOH < EtOH < PG < EG in increasing order. Moreover, this test indicates that the CPAs were compatible with the other ingredients of the flush solution, especially at the

TABLE 2
Effect of Acute Exposure to Cryoprotectants on Function of the Isolated Rat Heart

CPA	*n*	HR	AF	CF	CO
MeOH					
Control	5	307 ± 17	55.1 ± 2.8	24.1 ± 2.2	79.2 ± 4.5
3.78 *M*		315 ± 15	49.5 ± 1.9*	21.8 ± 2.3*	71.3 ± 3.1*
Control	6	320 ± 28	53.8 ± 1.6	27.5 ± 2.6	81.3 ± 3.3
2.78 *M*		308 ± 29	51.0 ± 1.9*	25.8 ± 2.5*	76.8 ± 3.6*
Control	5	279 ± 14	51.3 ± 1.4	21.8 ± 1.8	73.0 ± 1.7
1.78 *M*		292 ± 14	51.4 ± 1.5	22.5 ± 1.5	74.0 ± 1.2
EtOH					
Control	4	254 ± 7	50.9 ± 4.4	21.9 ± 1.6	72.8 ± 5.0
2.47 *M*		198 ± 24	14.9 ± 4.8*	4.8 ± 1.3*	16.0 ± 6.0*
Control	5	288 ± 7	51.8 ± 2.8	22.2 ± 1.6	74.0 ± 3.6
2.27 *M*		296 ± 9	47.0 ± 3.4*	16.0 ± 0.9*	62.9 ± 3.9*
Control	5	346 ± 21	51.4 ± 0.7	24.7 ± 1.1	76.1 ± 0.8
1.27 *M*		343 ± 19	54.0 ± 0.8*	24.8 ± 0.8	78.8 ± 1.2*
PG					
Control	5	280 ± 4	54.9 ± 1.1	24.4 ± 3.3	79.3 ± 3.9
1.22 *M*		283 ± 14	44.3 ± 0.7*	20.3 ± 2.8*	64.6 ± 3.1*
Control	5	256 ± 19	49.5 ± 2.2	21.8 ± 1.4	71.3 ± 2.4
0.87 *M*		276 ± 14	48.2 ± 2.2	20.8 ± 0.7	69.1 ± 2.9
EG					
Control	5	277 ± 16	54.1 ± 5.9	24.2 ± 1.0	78.3 ± 6.6
1.22 *M*		311 ± 26	38.0 ± 5.2*	19.1 ± 0.8*	57.5 ± 5.2*
Control	5	303 ± 13	52.1 ± 1.7	24.2 ± 2.1	76.3 ± 3.8
0.84 *M*		302 ± 13	47.7 ± 1.7*	23.5 ± 2.3	71.2 ± 4.1*

Note. Means ± SE are presented. Control values represent function before CP flush. Function before and after treatment were compared by paired one-tailed *t* test.

* $P < 0.05$ vs control.

concentrations which caused little or no inhibition of the cardiac function.

Subzero nonfreezing storage experiment. After determining the highest concentrations of CPAs tolerable to the isolated rat heart in the acute exposure experiment, we assessed the effects of these CPAs on cardiac viability after subzero nonfreezing storage. The hearts were flushed with the appropriate solutions and stored unfrozen for 6 h at temperatures slightly above the FP (Table 1). Post-storage recovery in the four groups was statistically similar. For ease of comparison, CO was used as the overall index of cardiac function. The recovery of CO was 86 ± 4% of control in 1.78 *M* MeOH group, 82 ± 5% in 1.27 *M* EtOH group, 76 ± 4% in 0.84 *M* EG group, and 79 ± 4% in 0.87 *M* PG group (Table 3). Considering the concentration of these CPAs and the storage temperatures involved, results of this experiment corroborated with the acute exposure test that MeOH was the most promising CPA among the four, because it offered the lowest storage temperature.

Importantly, CP-14 + 2.78 *M* MeOH, which was slightly cardiac-suppressing as shown by the acute test, was detrimental to the hearts during cold storage; all of the hearts treated with 2.78 *M* MeOH and stored at −5.5°C failed to recover when reperfused with KHB (data not shown). Nevertheless, if these hearts were first reperfused with CP-14 + 1.47 *M* MeOH (1740 mOsm/kg) for 5 min before reperfusion with KHB, there was 19% recovery in CO (Table 3). This observation suggests that nonrecovery in this group was partially attributable to osmotic shock during the initial phase of reperfusion.

Because CP-14 + 1.78 *M* MeOH allowed nonfreezing storage at −3.7°C and offered the best preservation after 6 h, we further studied the effect of prolonging the storage duration on functional preservation in hearts flushed with this solution (Table 4). In the 8-h hearts, except for HR and CVR, the recovery of other hemodynamic parameters was lower than that of the 6-h group. Extending the storage duration to 9.5 h further impaired the hearts; all function recovered to levels significantly lower than those of 6-h hearts and HR, AF, CO, and work were distinctly less than the 8-h hearts (Table 4).

Myocardial AN content. End-storage AN contents were measured in hearts flushed with CP-14 + 1.78 *M* MeOH and stored for 6, 8, and 9.5 h (Table 5). Levels of AMP increased 78 ± 25 and 74 ± 19% over the control in the 6- and 8-h hearts ($P < 0.005$); it went up further to 211 ± 19% of control after 9.5 h storage ($P < 0.05$). ADP content showed a slight but insignificant decrease during storage. ATP levels in 6-h hearts declined to 89 ± 8% of control. The difference was, however, not significant. It further decreased to 78 ± 3 and 76 ± 4% of control levels after 8 and 9.5 h storage ($P <$

TABLE 3
Hemodynamic Performance of the Isolated Rat Heart after 6-h Nonfreezing Storage

CPA	*n*	HR	AF	CF	CO	SP	Work	CVR
1.78 *M* MeOH	8	303 ± 22	47.3 ± 1.5	18.9 ± 1.4	65.4 ± 2.8	125.3 ± 3.5	76.7 ± 3.6	3.8 ± 0.2
1.27 *M* EtOH	5	307 ± 30	46.2 ± 2.8	16.2 ± 1.1	62.4 ± 3.7	125.8 ± 1.3	78.0 ± 7.0	4.6 ± 0.2
0.87 *M* PG	5	288 ± 23	43.4 ± 2.1	16.7 ± 1.6	60.1 ± 3.1	120.4 ± 3.2	66.8 ± 3.9	3.9 ± 0.5
0.84 *M* EG	4	272 ± 6	41.9 ± 2.0	15.9 ± 0.8	57.8 ± 2.7	136.8 ± 3.5	76.8 ± 4.5	4.9 ± 0.3
2.78 *M* MeOH[a]	3	235 ± 22	8.6 ± 2.1	6.8 ± 0.5	15.4 ± 2.8	85.3 ± 2.9	14.1 ± 2.6	8.6 ± 0.7

Note. Means ± SE at the end of 30 min working reperfusion are presented. Hemodynamic function of the unstored control hearts was (n = 24): HR, 294 ± 9 beats/min; AF, 53.0 ± 1.0 ml/min; CF, 23.2 ± 1.0 ml/min; CO, 76.2 ± 1.8 ml/min; SP, 142 ± 2 mm Hg; work, 94.9 ± 2.6 g-m/min; CVR, 3.4 ± 0.2 mm Hg-min/ml.

[a] The hearts were reperfused with CP-14 + 1.47 *M* MeOH for 5 min before reperfusion with plain KHB.

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