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Resuscitation of cardiac energy metabolism in the rabbit heart by brief hypothermic reperfusion after preservation studied by ^{31}P NMR spectroscopy

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Abstract Rabbit hearts were subjected to 24-h cold ischaemic storage (at 0 °–2 °C in melting ice) after initial flushing with either St Thomas' cardioplegic solution (STS) or modified lactobionate/raffinose solution (LR), and the status of phosphorylated energy metabolites was measured by ^{31}phosphorus nuclear magnetic resonance (P NMR) spectroscopy. In both groups signals for ATP and phosphocreatine (PCr) were still detectable by ^{31}P NMR after 24 h, and there was significantly more ATP in the LR group ($P < 0.01$). The hearts were then subjected to coronary reperfusion via an aortic cannula using the same storage solution (either STS or LR) at 6 °–8 °C, which was oxygenated. In both groups PCr recovered within 30 min of cold reperfusion, and by 60 min PCr was significantly higher in the LR group ($P < 0.001$). Also, levels of ATP were maintained at higher values during cold reperfusion in the LR group. These studies suggest two important points: (1) the general supply of phosphorylated high-energy intermediates of hearts during cold ischaemic storage is better preserved using LR, and (2) brief cold reperfusion may be used to restore energy metabolism in hearts before re-implantation.

Key words Heart preservation, NMR, rabbit · Hypothermic reperfusion, heart, rabbit · NMR, heart preservation, rabbit

Introduction

One major logistical problem that is an obstacle to the growing application of cardiac transplantation is the relatively short cold preservation time that is considered safe. Most centres consider that the heart is damaged to an unacceptable degree if cold ischaemia extends beyond ± 6 h. Longer periods of cold ischaemia lead to decreased function and increased morbidity [6]. Low temperature itself is not necessarily damaging to the mammalian heart, since it was demonstrated two decades ago that canine hearts could be successfully transplanted after cold perfusion for up to 3 days [12]. However, cold ischaemic storage of the heart results in a rapid depletion of high-energy phosphorylated (HEP) compounds, namely, phosphocreatine (PCr) and ATP, and this has been linked to ischaemic contracture [5]. In addition to a depletion in energy status, there is a decrease in tissue pH with increasing time of preservation, together with additional metabolic abnormalities such as increased susceptibility to oxygen-derived free radical damage [11].

Following re-implantation, the transplanted heart must function immediately upon disconnection from the extracorporeal circulation, and any metabolic insufficiencies must be rapidly corrected, as the heart is very dependent upon an immediate and sustained supply of PCr and ATP to perform synchronised contraction. Other factors, such as free radical generation upon reperfusion [16], may impose additional damaging sequelae, but at a basic level regeneration of HEP is fundamental for survival of the rewarmed heart. One reason for failure to regain normal HEP levels may be the unavailability of sufficient precursors for resynthesis. Recently, there have been several reports on the effects of

different preservation solutions for minimising cold ischaemic damage in stored hearts, including the recently developed University of Wisconsin (UW) solution [13, 14, 17], though in all cases the energy status of the heart is depleted to a lesser or greater extent during cold ischaemic preservation.

We have recently demonstrated that cold (4 °C) reperfusion, using the same solution as for storage, is capable of regenerating ATP in preserved rat livers [3] after a period of cold ischaemia. Cold reperfusion may be clinically advantageous, as it could be performed using simple equipment and sterile solutions already available, while avoiding risks of infection from perfusion at body temperatures. In those studies ^{31}phosphorus nuclear magnetic resonance (^{31}P NMR) spectroscopy was used for non-invasive assessment of high-energy phosphorylated intermediates. We have now extended these studies to the rabbit heart to establish whether cold reperfusion can resuscitate cardiac energy status after 24-h cold ischaemic storage. This preservation period was chosen to address the logistical problems encountered in clinical cardiac transplantation. We also chose to study two preservation solutions for storage and reperfusion. Hearts were stored and reperfused after 24 h using either St Thomas' cardioplegic solution (STS), which, in many centres, constitutes the basic clinical heart preservation solution, or a lactobionate/raffinose (LR) solution based on UW solution, which has previously been shown experimentally to be suitable for cold storage of hearts [14]. Our aim was to test the hypothesis that a short cold reperfusion following storage could be developed as a method to resuscitate cardiac metabolism, which may have beneficial effects on immediate graft function following re-implantation, and as a first step to investigate the possibility of applying ^{31}P NMR spectroscopy to the heart during hypothermic perfusion.

Materials and methods

Isolation and storage of the rabbit heart

In these studies male New Zealand white rabbits were used as organ donors. The animals were maintained and treated in accordance with the guidelines set out under "Principles for Laboratory Animal Care" (NIH Publication No. 85–23) and the Animals (Scientific Procedures) Act (1986). The rabbits (2–3 kg) were anaesthetised by intravenous injection of fentanyl citrate/fluanisone and diazepam, as previously described [15], and maintained with oxygen via an open face mask at a gas flow of 1 l/min. The chest was opened via a midline incision and the heart removed by a single cut through the major vessels. The heart was then placed in a beaker of ice-cold preservation solution, the vessels trimmed, and the aortic stump cannulated with a nylon cannula.

The coronary vasculature of each heart was then flushed via retrograde perfusion through the aorta with 100 ml of sterile, filtered (0.22-μm pore size) perfusate (either STS, group 1, or LR, group 2, $n = 5$ for both groups) at a hydrostatic pressure of 50 cm. The hearts were then placed in 50 ml of fresh solution at 4 °C and packed in ice for 24 h. The aortic cannula was left in place to allow connection to the perfusion circuit.

Two solutions were chosen for investigation in this study. One was standard STS [2], and the other was a modified version of UW solution (LR), which was simplified principally by the omission of adenosine to allow direct comparison of both solutions with respect to their ability to maintain tissue adenine nucleotides.

St Thomas' solution contained: Ca^{2+}, 1.2 mM; K^+, 16 mM; Na^+, 120 mM; Mg^{2+}, 16 mM; Cl^-; 160 mM and HCO_3^-, 10 mM. It had a pH of 7.8. LR solution contained: K^+, 125 mM; Na^+, 20 mM; Mg^{2+}, 5 mM; SO_4^{2-}, 5 mM; $H_2PO_4^-$, 25 mM; lactobionate, 100 mM; raffinose, 30 mM; glutathione, 3 mM and allopurinol, 1 mM. It had a pH of 7.35.

Cold reperfusion

After 24 h of cold storage the hearts were placed in a home-built NMR probe and reperfused with oxygenated solution at 5 °C in the bore of an 8.5 tesla vertical magnet, as previously described [9], at a hydrostatic pressure of 70 cm. Oxygenated perfusate (oxygenated by bubbling with 100 % O_2 at 100 ml/min) was pumped through a circuit of nylon tubing from an external reservoir maintained in ice. This reservoir contained approximately 500 ml of the perfusate and was surrounded by an ice/water jacket to maintain the solution at 5 °C. A peristaltic pump was positioned at a safe distance away from the magnet and provided recirculating perfusion through an 8.0-μm filter. Immediately before the perfusate entered the NMR probe in the magnet it passed through a large coil of nylon tubing within the ice/water jacket to ensure that the perfusate was cooled to 6 °–8 °C. The cold perfusate passed through a bubble trap immediately prior to entering the heart (positioned in a chamber constructed from a 50-ml syringe) via retrograde perfusion. By means of an external tap, perfusate could be directed into the aortic cannula (cold reperfusion) or externally around the heart in the chamber (perifusion) to maintain a cold, unperfused organ. At the end of the preservation period, hearts were attached to the circuit via the indwelling aortic cannula and placed in the bore of the magnet. The heart was maintained at hypothermia by perifusion whilst initial spectra (heart unperfused) were acquired. After this, cold perfusate was directed into the heart to achieve reperfusion. Effluent from the coronary bed returned by gravity to the reservoir. Group 1 hearts were reperfused with STS and group 2 hearts with UW solution.

^{31}P NMR spectroscopy

A saddle coil tuned to the frequency of phosphorus (145.8 MHz) surrounded the chamber containing the heart so that all of the heart tissue lay within the coil. Initial shimming was performed on the total proton signal within the sensitive volume of the coil. Linewidths of 40–60 Hz were typically obtained. For ^{31}P NMR spectroscopy, a total of 20 radiofrequency pulses were applied (40 μs produced an approximate 45 ° flip angle for the ^{31}P nuclei). A delay of 20 s between each pulse ensured that the spectra acquired were fully relaxed, determined from separate experiments. Spectra were acquired on a Bruker AM 360 spectrometer. Spectra were produced by Fourier transformation of the summed free induction decay (FID), with a line broadening equivalent to 5 Hz being applied. Baseline correction was carried out and peak areas were calculated using the integration software of the spectrometer. Peak areas are expressed with respect to a reference peak arising from 1.0 M methylene diphosphonate contained within a capillary and situated within the RF coil. Tissue pH was measured from the chemical shift

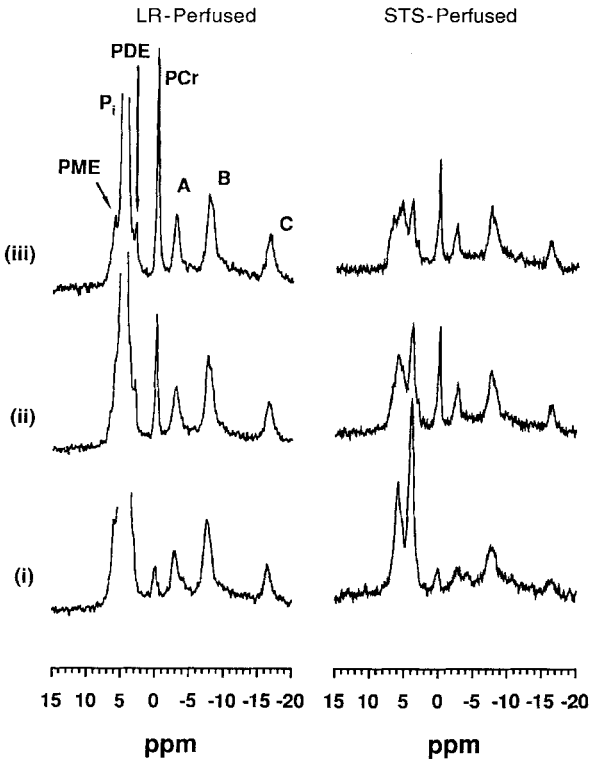

Fig. 1 Three spectra from a rabbit heart flushed and stored with 4 °C modified LR or STS solutions, followed by cold perfusion with the same solution for 60 min. Spectrum (i) is after storage for 24 h on ice, following removal and flushing. Spectra (ii) and (iii) are from the same stored heart after 15 and 60 min of cold reperfusion with LR or STS solutions, respectively. The peaks are assigned as shown. The peaks labelled A, B and C arise from ADP + ATP, NADP, and β-ATP, respectively. The inorganic phosphate *(P_i)* peak (at ± 5.0 ppm) in the LR-perfused hearts is very large as the perfusate contains 25 mM phosphate. For clarity, the phosphate peak has been removed from the LR spectra. After 24-h cold ischaemia in STS, peaks from phosphomonoesters and inorganic phosphate (± 7.0–5.0 ppm) are most prominent. The mean pH, calculated from the chemical shift of the inorganic phosphate peak, is 6.3 ± 0.1 (SD) for the group of five STS rabbit hearts. A small phosphocreatine *(PCr)* peak at 0.0 ppm is present even after 24 h of cold storage in both preservation solutions, and small peaks from ADP and ATP are just visible in the STS hearts [spectrum (i)]

of the inorganic phosphate peak with respect to PCr and calculated as described previously [2], using the relationship:

$$pH = 6.88 + \log_{10} \frac{\sigma\text{-}3.35}{5.6\text{-}\sigma}$$

where σ is the chemical shift difference between PCr and P_i.

All values are expressed as means ± standard deviations. Statistical evaluations were made between results from the two groups using a 1-way ANOVA and the Dunnett's test.

Results

Figure 1 shows representative ^{31}P NMR spectra from two rabbit hearts obtained after 24-h cold ischaemic storage using either LR or STS. Spectra labelled (i) in each case are from ischaemic hearts maintained at 6 °–8 °C in the probe by perifusion after 24-h storage. In both cases, ATP and PCr were still present after 24 h of cold ischaemia. Higher levels of ATP and ADP had been maintained in the heart stored in LR solution (Fig. 1). In both hearts, the PCr levels were similar. Spectra labelled (ii) and (iii) were obtained during 6 °–8 °C reperfusion for 15 and 60 min, respectively. The signals from PCr increased markedly in both hearts. Some resynthesis of ATP and ADP during the first 15 min of reperfusion was observed in the STS-perfused heart. Signal from inorganic phosphate decreased demonstrably in the heart reperfused with STS, but comparable observations for hearts stored and reperfused in LR could not be made because the tissue inorganic phosphate peak from hearts in this group was obscured by the large peak from phosphate contained in the LR solution.

Peak areas of PCr and β-ATP in both groups were calculated as a percentage of the reference peak area, and the mean values were computed. Figure 2 shows the values for PCr with time of cold reperfusion. Hearts stored in LR solution for 24 h contained significantly more PCr than those stored in STS solution ($P < 0.01$). In both cases PCr increased during 30 min of cold reperfusion. In the STS group, PCr values then remained constant, whilst in the LR group PCr increased further, such that by 60 min of reperfusion the values were significantly higher ($P < 0.01$) than those in the STS group.

ATP levels (assessed from the β-ATP peak) were evaluated. Significantly more ATP was present in the LR group ($P < 0.01$) at the end of the cold ischaemic period before cold reperfusion (Fig. 3). This level was maintained during cold reperfusion but there was no further increase in the level of ATP after 60 min. The STS group of hearts tended to show a slight increase (not significant) in ATP level during the first 20 min of cold reperfusion, but the final level of ATP at the end of 60 min was not significantly different from the level of ATP after 24 h of cold ischaemia.

The mean pH values were calculated from the chemical shift of the P_i peak, and changes in tissue pH were measured during reperfusion. The initial mean pH of group 1 hearts, stored in STS, was pH 6.3 ± 0.1, compared to pH 6.5 ± 0.2 for group 2 hearts, stored in LR solution. The tissue pH increased during cold reperfusion with STS, and by 60 min the tissue pH was 7.6 ± 0.2. The LR solution contained 25 mM inorganic phosphate buffered at pH 7.35, which produced a large, inorganic phosphate peak that obscured the signal arising from inorganic phosphate within the tissue, and thus pH could not be determined in these hearts. However, before reperfu-

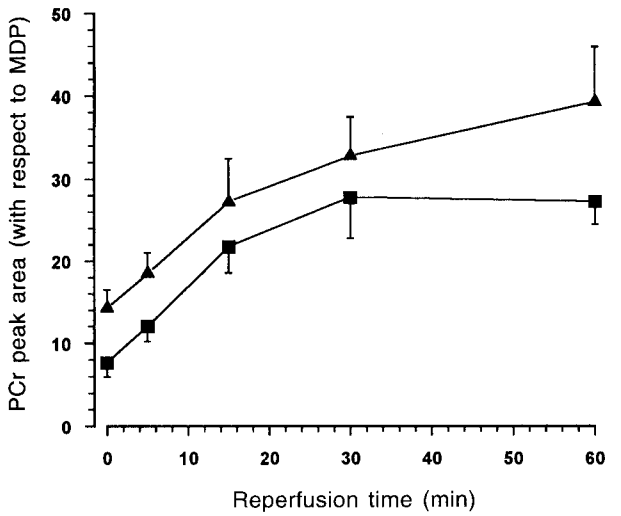

Fig. 2 Comparison of mean % peak area of phosphocreatine *(PCr)* in the two groups of hearts. After 24-h cold ischaemic storage, the level of PCr was significantly higher in the group of hearts stored in LR solution (▲) than in those stored in STS (■). In both groups the PCr level increased rapidly during the first 15 min of perfusion. The group of hearts perfused with STS reached a maximum mean PCr after 30 min of perfusion, which was not significantly different following a further 30-min perfusion. The mean PCr level of hearts perfused with LR solution reached a maximum level after 1 h of perfusion and was greater than the levels in STS hearts ($P < 0.001$)

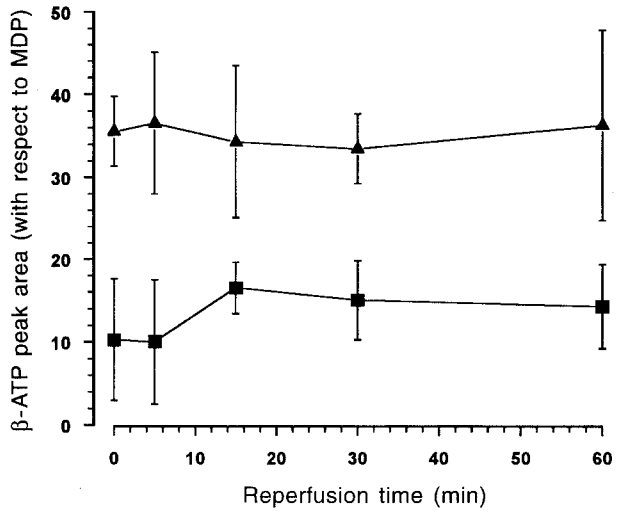

Fig. 3 Comparison of the level of the energy-rich adenine nucleotide, ATP, from the two groups of hearts. The ATP level is expressed as a mean % peak area with respect to the MDP (methylene diphosphonate) reference peak (not shown). After 24-h storage, the levels of adenine nucleotides in the two groups of hearts were significantly different. Hearts flushed and then stored in LR solution (▲) showed a mean ATP peak intensity that was three times greater than the hearts flushed and stored in STS (■), indicating that a higher level of ATP was present in hearts preserved in LR solution. In both cases the β-ATP peak area did not increase significantly during the 1 h of reperfusion following the 24 h of cold storage

sion, the inorganic phosphate in the tissue appeared as a separate peak on the side of the solution peak, and tissue pH was calculated as 6.5 ± 0.2. STS contained no inorganic phosphate, and so the P_i peak only arose from inorganic phosphate within the tissue, allowing accurate determination of tissue pH from the chemical shift.

Discussion

The results presented have demonstrated that cold reperfusion is capable of regenerating cardiac stores of high-energy phosphorylated intermediates. Both ATP and PCr were present in all hearts reperfused after 24-h cold ischaemia, indicating that the energy status of the heart had been maintained to some extent. PCr is the first high-energy intermediate to be lost in ischaemia and disappears before ATP stores are consumed [4]. Previous studies have shown that signals from PCr and ATP, although clearly demonstrable after cold flushing, disappeared within approximately 12 h of cold ischaemia [2]. A recent report [7] showed that oxygenated STS was more effective than LR in preserving tissue ATP during short periods of cold ischaemia, which conflicts with our data demonstrating that LR preserves cardiac ATP better than STS over 24 h. (This previous report did show, however, that normothermic reperfusion promoted greater resynthesis of ATP following storage in UW solution than with STS storage [7].) Our findings are consistent with other studies in which UW-type solutions have been used over a shorter period of cold ischaemia [10]. Clinical studies on the use of UW solution in cardiac transplantation show a similar trend [1]. Hypothermic reperfusion with LR solution after 24-h cold ischaemia increased the level of PCr and reversed acidosis. Although reperfusion with STS had a similar effect, the levels of PCr and ATP present after hypothermic reperfusion were lower than in the LR group. This may reflect a greater degree of damage to the cardiomyocytes during 24 h of cold ischaemia using STS preservation solution. LR solution would, therefore, appear to have a beneficial protective effect during cold ischaemic storage. On the basis of these findings of good preservation of energy status using LR solution, we suggest that cardiac viability and immediate function upon rewarming and transplant would be better preserved using a UW-type solution for cold ischaemic storage. Recovery of normal cardiac ATP stores following ischaemia has been reported to be associated with a parallel recovery of contractile function, while diminished ATP recovery resulted in abnormalities of time-differentiated left ventricular pressure [8]. Further work will be required to evaluate the effects of replenishment of cardiac energy stores in the cold on subsequent physiological function.

Cold perfusion has been known for many years to provide the best method of preservation for hearts stored

for transplantation. Over 20 years ago it was demonstrated that canine hearts could be successfully transplanted after 72–96 h of continuous cold perfusion [12]. The complicated nature of perfusion, along with the need for skilled technical back-up, has limited the use of perfusion in clinical transplantation. This study has shown that it is possible to combine cold ischaemic preservation with a brief resuscitation, either by cold perfusion or with a prolonged cold flush. We suggest that by using oxygenated perfusate to promote aerobic metabolism, it should be possible to improve the energetic status of the heart and to enhance early graft function. Resuscitation perfusion could be performed using a simple system at the transplanting hospital. The time required to regenerate heart high-energy stores is relatively short (< 1 h) and could be performed whilst the recipient was being prepared. Finally, resuscitation perfusion could be investigated as a means of assessing cardiac viability following storage or of reversing metabolic deficiencies arising from harveting hearts from unstable or hypotensive donors. For all of these reasons, further studies on cold resuscitation perfusion as an adjunct to cardiac preservation, together with the search for improved flushing solutions to protect against specific aspects of ischaemic damage, may prove worthwhile in the quest for prolonged heart preservation.

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