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Biochemical and Morphological Changes in Isolated Rabbit Hearts after Prolonged Hypothermic Ischaemia: Comparison of Two Cardioplegic Solutions

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Summary

This work evaluates the myocardial protective potential of potassium cardioplegia on ischaemically arrested and reperfused hearts by two cardioplegic solutions: the University of Wisconsin solution (UW) and the standard crystalloid solution of St. Thomas' Hospital (ST). Evaluation of myocardial preservation was based on creatine kinase and lactate releases and on high-energy phosphate preservation of isolated rabbit hearts after 4 hours' hypothermic ischaemia. A morphometric ultrastructural evaluation of mitochondria in cardiomyocytes was also performed. The hearts of 24 rabbits were normothermally perfused with oxygenated Krebs-Henseleit solution for 30 min (Langendorff preparation), and the baseline contractile performance and biochemical parameters were evaluated. The hearts were then arrested and stored in the cardioplegic solutions (12 UW and 12 ST) at 4 °C for 4 hours. The hearts were then rewarmed and reperfused with oxygenated Krebs-Henseleit solution for further 30 min. At the end of reperfusion, creatine phosphate and high energy phosphates were higher with UW ($p<0.05$); creatine kinase release during reperfusion was significantly lower with UW both at 15 min ($p<0.01$) and at 30 min ($p<0.05$). Lactate release during the first 15 min of reperfusion was about doubled ($p<0.05$) with respect to controls in both groups; at 30 min this increase had almost vanished (+8 %) with UW but not with ST (+30 %). Ultrastructural morphometry did not show any significant difference at the level of mitochondria between the two treatments. The results indicate, for UW, an improved myocardial preservation associated with relative retention of high-energy phosphates and higher recovery of mechanical function, accelerated metabolic recovery and reduced stress of cell membranes.

Key words

Hypothermic cardioplegia – High energy phosphates – Creatine kinase – Lactate – Ultrastructural preservation

Introduction

Hypothermic potassium cardioplegia, routinely employed to reduce myocardial ischaemic damage during some thoracic surgical procedures, has also been widely used for the preservation of explanted hearts for human transplantation (Swanson *et al.* 1980, Darracott-Cankovic *et al.* 1987). Attempts to extend the safe period of heart storage have been made by modifying the ionic composition of the Krebs balanced solution used for cardioplegia, from that of the extracellular to that of the intracellular fluid, and by adding membrane stabilizers (Rosenfeldt and Sabiston 1982, Suaudeau *et al.* 1982), metabolic additives (Robinson *et al.* 1984, DeWitt *et al.* 1983), Ca^{2+} channel blockers (du Toit *et al.* 1990), metabolic inhibitors (Myers *et al.* 1986) and sanguineous cardioplegia (Julia *et al.* 1991). Currently, the accepted

safety margin for heart transplantation is up to 4 hours of global myocardial ischaemia.

In the present research we have tested the efficacy of two cardioplegic solutions: the University of Wisconsin solution (UW) and the St. Thomas' Hospital cardioplegic solution (ST). ST is at present the most frequently used solution during cardiac surgery and transplantation, while UW has been used clinically for flushing and storing liver, pancreas and kidney grafts (Jamieson *et al.* 1988, Wahlberg *et al.* 1987, Ploeg *et al.* 1988), but it has also been proposed for heart preservation. The aim of this study was to evaluate the metabolic and morphological aspects of extended preservation of isolated rabbit hearts with either solution. Preliminary results, regarding only cardiac dynamics, were published in a previous paper (Poltronieri *et al.* 1994).

Methods

Experimental preparation

Experiments were performed on isolated perfused rabbit hearts; the details of the preparation have been described in our previous report (Poltronieri *et al.* 1994). Briefly, the heart was rapidly excised through a midline sternotomy, immediately transferred to a non-recirculating Langendorff apparatus and perfused *via* the aorta with the Krebs-Henseleit solution at constant pressure (70–80 mm Hg) for 30 min at 37 °C. The perfusing solution was equilibrated with 95 % O_2 and 5 % CO_2 (pH 7.38–7.42). Heart pacing was performed through a pair of electrodes placed on the ventricular epicardium, at 180 beats/min with pulses of 3 V and 3 ms (Grass, S-48). Pacing was stopped in the period of heart arrest. Coronary perfusion pressure was continuously monitored from a sidearm just above the aortic cannula, which was connected to a pressure transducer (Gould-Statham P23dB). A latex balloon was placed into the left ventricle to evaluate mechanical function.

After completion of a set of control measurements, heart pacing was stopped and the coronary arteries were flushed with a cardioplegic solution and stored in the same solution at 4 °C for 4 hours by topical cooling. At the end of the preservation period, the heart was reperfused by gradually increasing perfusion pressure to the preischaemic level. Reperfusion was protracted for 30 min to observe recovery of function.

Twenty-four rabbits were divided into two equal groups. The hearts of the first group were treated with ST solution (composition in mmol/l: NaCl 110.0, KCl 16.0, $MgCl_2$ 16.0, $CaCl_2$ 1.2, $NaHCO_3$ 10.0, pH 7.8, osmolarity 290 mosm/kg H_2O); the hearts of the second group were treated with UW solution (composition in mmol/l: KH_2PO_4 25.0, $MgSO_4$ 5.0, adenosine 5.0, glutathione 3.0, raffinose 30.0, allopurinol 1.0, K-lactobionate 100.0, pentastarch (%) 5.0, insulin (IU/l) 40, pH 7.4, osmolarity 320 mosm/kg H_2O).

The coronary sinus effluent was collected at the end of the control period and 15 min as well as 30 min after the start of reperfusion. Samples were stored at 4 °C until the determination of creatine kinase (CK) and lactate. Both analyses were performed with standard spectrophotometric techniques. CK activity, measured at 37 °C, was expressed in milli-international units per minute per gram wet weight (mIU/min/g ww) of the heart, while lactate production, measured at 25 °C, was expressed as micromoles per minute per gram wet weight (μmol/min/g ww).

Nucleotide content

Biopsy specimens from the left ventricles were collected at the end of the experiments, frozen in liquid nitrogen and stored for analysis of myocardial high-energy phosphates. In a mortar precooled in liquid nitrogen, preweighed portions of frozen tissue were ground in 0.4 N $HClO_4$ ($\approx$3 ml/500 mg) until complete homogeneity was obtained. It was further homogenized with an Ultra-Turrax and then centrifuged at 4000 x *g* for 10 min at 4 °C. Supernatant pH was adjusted to 6.0–6.5 with 6 N KOH. Following the removal of $KClO_4$, the extract was used for chromatography (HPLC Waters 600 E multisolvent and a model 990 photodiode array detector). The separation and quantification of the metabolites were performed using a reversed-phase 3 μm column (Supelchelm C_{18}). The mobile phase consisting of a gradient of acetonitril (2.5–25 % v/v) in phosphate buffer solution with the addition of tetrabutylammonium hydrogen sulphate as an ion-pair agent. Detection was performed at 205 nm for creatine phosphate and at 260 nm for nucleotides (ATP, ADP and AMP) (Bernocchi *et al.* 1994).

The following metabolites were measured: adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and creatine phosphate (CP). The values obtained were used to calculate two indexes of the myocardial energy status: the high energy phosphates, HEP = 2 x ATP + ADP + CP and the energy charge, EC = (ATP + 0.5 ADP)/(ATP+ADP+AMP). Data for HEP were expressed in micromoles per gram dry weight (μmol/g dw).

Functional and metabolic assessment

Mechanical function at balloon volume of 0.4 ml was assessed before arrest and 30 min after the start of reperfusion, as explained in our previous paper (Poltronieri *et al.* 1994).

To assess the relationships between mechanical performance and energy metabolism, we

plotted the percentage recovery from the control values of the maximum positive derivative of left ventricular pressure (+dP/dt) and minimal derivative of left ventricular pressure (−dP/dt) at a balloon volume of 0.4 ml versus energy charge (EC).

Morphometry

Additional biopsy was obtained for the ultrastructural study which was addressed in particular to mitochondria. Myocardial tissue was sampled from the upper part of the left ventricle, cut into fragments and fixed for 3 hours with glutaraldehyde (2 % in 0.1 M phosphate buffer). Specimens were then post-fixed for 1 hour with osmium tetroxide (1 % in distilled water). All fixation procedures were performed at 4 °C. The tissue fragments were dehydrated through graded concentrations of acetone and embedded in a mixture of Epon/Araldite. Semithin sections (2 μm) stained with toluidine blue were used to select well fixed regions for thin sectioning. Thin sections (about 70 nm) were cut on a Ultracut E ultramicrotome (Reichert), placed on copper/rodium grids and stained with lead citrate. Observations were made with a Zeiss EM10 electron microscope operated at 60 kV. Five areas for each myocardial specimen were photographed at a magnification of 3150x according to a systematic randomized protocol (Weibel 1979). For morphometric analysis, prints at a final magnification of 9000x were prepared and analyzed by a semi-automatic method. The profiles of myocytes, nuclei and mitochondria were traced by hand with the aid of a digitizing tablet and the respective areas were automatically calculated. The mitochondrial surface fraction (MSF) was then calculated as percentage of the sarcoplasmic area (total myocyte area minus total nuclear area). Moreover, the mean sectional mitochondrial surface (MSMS) was calculated by dividing the total mitochondrial area by the total number of mitochondrial profiles and expressed in μm^2.

Statistical analysis

All data are presented as mean ± S.E.M. The unpaired Student's t-test was used for comparison between pairs of the means. When several groups were compared, mixed design analysis of variance (ANOVA) and multiple comparison with Tukey's two-tailed test were performed. A difference was considered statistically significant if $p<0.05$.

Table 1
Levels of creatine kinase activity and lactate in the hearts preserved with ST and UW

	Creatine kinase		Lactate	
	ST (n=12)	UW (n=12)	ST (n=10)	UW (n=10)
Before ischaemia	505±101	357±43	2.04±0.4	2.02±0.5
Reperfusion				
15 min	2510±420[a,b]	774±107	4.08±1.0[a]	3.35±1.2
30 min	1013±208[b]	337±66	2.66±0.5	2.15±0.4

Creatine kinase activity is expressed in mIU/min/g wet tissue and lactate as µmol/min/g wet tissue (means±S.E.M.). ST, St. Thomas' cardioplegic solution; UW, Wisconsin solution. [a] *Significantly different from preischaemic values,* [b] *Significantly different from UW*

Results

The values of CK activity and lactate content for the two groups of hearts are shown in Table 1. Baseline values were comparable. After 15 min of reperfusion, the CK release significantly increased ($p<0.01$) in the hearts preserved with ST, and at 30 min of reperfusion the value was still markedly greater than that observed before ischaemia. In the UW experiments, smaller insignificant increases of CK release were observed. Group comparison between ST and UW indicated significant differences both at 15 min ($p<0.01$) and 30 min of reperfusion ($p<0.05$).

For lactate production, after 15 min of reperfusion, a significant increase from the baseline was observed in both groups ($p<0.05$). However, during the following 15 min, the values of UW group returned almost to preischaemic levels (+8 % of control), while those of ST group remained markedly above the baseline (+30 % of the control); the difference between groups after reperfusion was not significant.

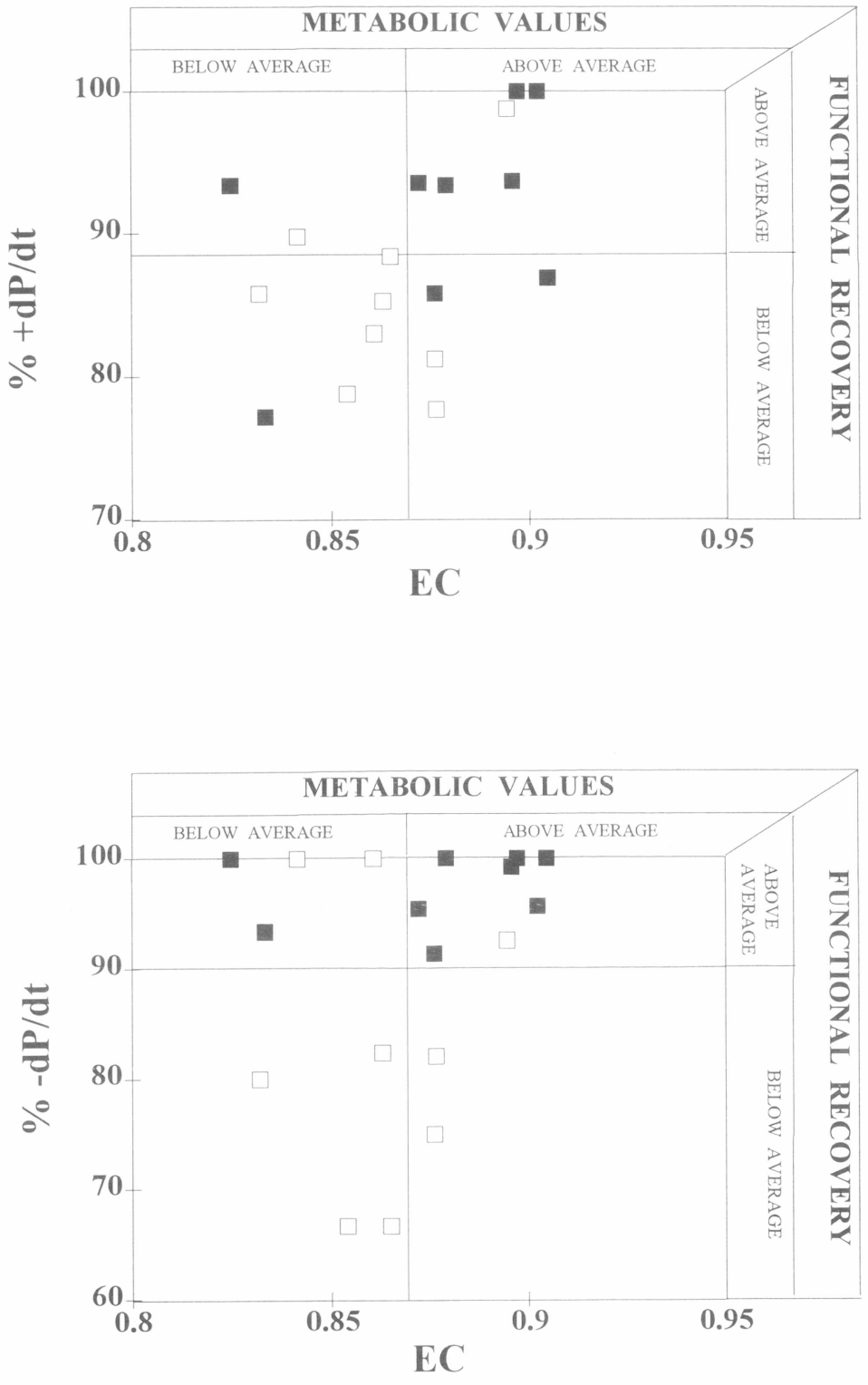

Fig. 1
Correlation of functional and metabolic status in 18 hearts (9 for each treatment). On the vertical axis, +dP/dt (upper panel) and −dP/dt (lower panel) at the end of the experiment are expressed as percentage of the control values. On the horizontal axis, the energy charge at the end of experiment (EC) is reported. Black squares = UW, open squares = ST.

Table 2
High-energy phosphate levels of the hearts preserved with ST and UW

	ATP	ADP	AMP	CP	HEP
ST (n=9)	11.37±0.73	2.84±0.21	0.61±0.08	25.84±3.39	51.44±3.71
UW (n=9)	13.71±1.22	3.00±0.26	0.65±0.10	37.78±3.88[a]	68.23±6.33[a]

Values are expressed as μmol/g dry weight (means±S.E.M.). [a] Significantly different from ST

Tissue adenine nucleotides and CP concentrations at the end of reperfusion are shown in Table 2. In general, the adenine nucleotide levels were slightly higher with UW, while the mean content of CP was significantly higher ($p<0.05$). The levels of HEP, which represent total high-energy phosphate bonds, including creatine phosphate, were markedly higher in UW ($p<0.05$).

The relation between mechanical activity and metabolic values is demonstrated in Fig. 1, which shows the plot of functional recovery against EC for 18 hearts in ST (=9) and UW (=9). The plot is divided into quadrants resulting from the intersection of two perpendicular lines demarcating the average EC value and the average of functional recovery in +dP/dt (upper panel), and −dP/dt (lower panel). In the upper part of Fig. 1, the above-average quadrant contains 5 hearts from UW and one heart from ST and the below-average quadrant contains one heart from UW and 5 hearts from ST. In the lower part of Fig. 1, the above-average quadrant contains 7 hearts from UW and one heart from ST and the below-average quadrant contains no heart from UW and 4 hearts from ST.

Ultrastructural examination revealed that the overall morphology of the myocardium was generally well preserved. However, lesions of the cell membrane, dilated sarcoplasmic reticulum and disorganization of mitochondrial cristae with partial swelling were observed in some cells. These ultrastructural changes were randomly distributed between the two groups. Morphometry indicated that both MSF and MSMS were comparable in hearts arrested with UW and ST (36.0±1.5 % and 0.56±0.05 μm^2 for UW; 34.8±1.5 % and 0.51±0.03 μm^2 for ST).

Discussion

It has been well documented that cardiac preservation in a cold environment allows relatively good myocardial recovery after up to 4 hours' ischaemia (English *et al.* 1984, Molina *et al.* 1985). We have, however, used two different cardioplegic solutions, seeking for improvements of preservation both during the arrest period and at reperfusion. The ischaemic period was within the limit of 4 hours, to conform with the accepted practice.

Previous studies on isolated heart models have identified different factors suitable for inducing cell necrosis on reperfusion, such as depletion of high-energy phosphates (Engelman *et al.* 1979), altered release of intracellular enzymes (Jynge *et al.* 1978), production of oxygen-derived free radicals (Ferrari *et al.* 1985, Simpson and Lucchesi 1987), intracellular calcium accumulation (Nayler *et al.* 1988) or activation of phospholipase and protease (Das *et al.* 1986). These multiple and interdependent factors cause an incomplete recovery of cardiac functions. For the above reasons, identification of the optimal composition of the cardiac storage solution is one of the principal problems in cardiac transplantation.

In a previous report (Poltronieri *et al.* 1994), we examined the effects of hypothermic preservation of the isolated heart with ST and UW on postarrest myocardial performance and oedema formation. It was found that UW allows better recovery of myocardial function by reducing the impairment of left ventricular end-diastolic function and improving relaxation; in addition, significantly lower oedema formation was documented. To study the cellular mechanisms underlying these results, we have now performed measurements of cardiac biochemistry (CK and lactate), energy metabolism (total adenine nucleotides and CP) and a morphometrical evaluation of the mitochondrial population. In summary, we observed that with UW the efflux of CK and lactate on reperfusion is reduced, while high-energy phosphates, mainly in the form of CP, are better preserved; furthermore, higher levels of myocardial function are associated with higher levels of EC. Finally, morphometrical parameters were not significantly different between the two treatments.

As shown previously (Poltronieri *et al.* 1994), changes in the general ultrastructure observed in the hearts treated with either solution were not striking, because the hearts were preserved at low temperature.

We have shown here, by means of ultrastructural morphometry, that both MSF and MSMS are not significantly different in the two groups. Yet, the good high-energy phosphates status in the hearts treated with UW indicates a definitely better functional preservation of mitochondria. It may also be remarked that, during the extended cardiac preservation, mitochondrial function may not be the only limiting factor, while other components of the cardiomyocytes, such as the sarcolemma, may undergo functional changes. Indeed, the better preservation of the functional integrity of cell membranes in UW leads to strongly reduced CK leakage, allowing the rapid utilization of CP to locally regenerate ATP from ADP (Banerjee *et al.* 1991). The better membrane protection is accounted for by the antioxidant action of allopurinol and gluthatione which reduce free radical production at the start of reperfusion. Free radicals may induce a positive feedback mechanism by activating tissue proteases and phospholipases which damage the membrane ultrastructure and increase membrane permeability (Das *et al.* 1986).

The oxidative stress causes a cytosolic calcium overload, which may not be compensated by active sequestration into the sarcoplasmic reticulum, because sarcoplasmic pumps are strongly inhibited by prolonged hypothermia (Fukumoto *et al.* 1991). The greater availability of CP and ATP in UW may account for a rapid restoration of sarcoplasmic and sarcolemmal pumps as well as for active sequestration of Ca^{2+} ions into the mitochondria. Thus, UW helps myocytes to retain high-energy phosphates during ischaemia and reperfusion. This ability of UW may be attributed to the presence of adenosine, which slows high-energy phospate loss during ischaemia by reducing the oxygen consumption (Belardinelli *et al.* 1983). Moreover, it exerts a stabilizing effect on cell membranes which inhibits lipolysis and reduces intracellular lactate (Fredholm 1985), as confirmed by the present results. The better performance of UW in sparing high-energy compounds concerned a higher content of CP rather than that of phosphorylated nucleotides. This observation is in keeping with the notion that CP stores are exhausted more rapidly than ATP stores during ischaemia, since the former is used rapidly and continuously to build up the latter (Bretschneider *et al.* 1975). Therefore, CP concentration is a good index of available chemical energy in the cell. Moreover, the relationship observed between ventricular performance and EC suggests that the contractile function is better preserved by the higher EC retention in UW in comparison to ST. This is probably due to the fast recovery of mitochondrial function which is critical on reperfusion, when oxidative phosphorylation is resumed. The lower production of lactate in UW confirms that mitochondrial function was less severely impaired during sustained ischaemia and oxidative energy production could meet the work demand upon reperfusion.

It may also be mentioned that the two cardioplegic solutions have a different pH: UW has a physiological pH of 7.4, while ST is definitely basic (pH 7.8). The general idea that a basic solution may exert a better protective effect during ischaemia than an acidic one was questioned by Nugent *et al.* (1982) who described progressively impaired functional recovery of dog hearts after 2-hour ischaemic arrest using solutions at pH 7.1, 7.4 and 7.7. They attributed this effect to a more prompt reduction of metabolic activities when using acidic cardioplegia at the start of ischaemia and to a consequent sparing effect on energy stores. It is thus possible that the lower pH of UW contributes to the effects, which we have described, by reducing myocardial oxygen consumption at the moment of heart arrest. The mechanisms of UW positive effects on heart preservation attributable to its richer composition, compared to that of ST, have been discussed in our previous report (Poltronieri *et al.* 1994).

We conclude that improvements observed in heart preservation with the UW solution can be attributed to a better recovery of mitochondrial function, enhancement of high-energy compound storage and preservation of membrane stability. Such processes may well account for the reduction of end-diastolic left ventricular pressure and the improved relaxation on reperfusion described in our previous work (Poltronieri *et al.* 1994). On the basis of the present results, it may be expected that, for ischaemic periods extending beyond the accepted limit of 4 hours, differences between the two cardioplegic solutions could become even more distinct.

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