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OPTIMIZATION OF TECHNIQUES FOR CARDIAC PRESERVATION
^{31}P NMR Spectroscopic and Functional Studies in Isolated Rat and Pig Hearts

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UNIVERSITÉ D'OTTAWA
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To my wife and my parents who have provided encouragement and inexhaustible support throughout my scholastic endeavors

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STATEMENT

I declare that the work presented in this thesis is original, that this material has not been submitted, either in whole or in part, for a degree at this or any other university.

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ABSTRACT

The effects of cardioplegic and reperfusion conditions on energy metabolites and cardiac function were investigated in order to define better conditions for heart preservation (4 or 8 hrs). Myocardial energy metabolites (ATP, PCr and inorganic phosphate), intracellular pH and contractile function were followed using ^{31}P NMR spectroscopy and left intra-ventricular balloon, respectively, during preservation and reperfusion in isolated pig and rat hearts. These hearts were subjected to various conditions of cardioplegia and reperfusion which involved re-arrest perfusion following ischemic preservation, increased concentrations of buffer and Mg^{++} in cardioplegic and reperfusion solutions, and an intracellular-type cardioplegic solution.

The effect of re-arrest perfusion was tested by comparing the recovery of energy metabolites and contractile function between pig hearts subjected to a secondary cardioplegic solution (S-C-S) prior to Krebs-Henseleit (K-H) solution and those reperfused with K-H solution alone after 8 hours of ischemic preservation at 12°C. The levels of ATP and PCr during reperfusion in both groups of hearts were comparable whereas the left ventricular developed pressure was significantly higher in the hearts reperfused with S-C-S than in those reperfused only with K-H solution. The reperfusion-induced ventricular fibrillation that occurred in K-H reperfused hearts was prevented by re-arrest perfusion. The results suggested that re-arrest perfusion significantly improved postischemic functional recovery, but this may not be directly related to its impact on energy metabolism.

Compared to pig hearts stored in St Thomas' solution #2 (containing only 25 mmol/L $NaHCO_3$ as buffer), a cardioplegic solution containing 150 mmol/L MOPS (high buffer cardioplegic solution) helped to maintain intracellular pH during 8 hours of ischemic preservation at 12°C. However, it did not affect the levels of energy metabolites during preservation and

contractile function during reperfusion, suggesting that increasing buffer capacity in cardioplegic solution may not be able to provide significant advantages for long term heart preservation.

The effects of 16 mmol/L Mg^{++} in cardioplegic and reperfusion solution were evaluated using both rat and pig hearts. In working rat hearts, 16 mmol/L Mg^{++} in cardioplegic solution did not alter myocardial oxygen consumption and contractile function following 30 minutes of normothermic preservation. Moreover, Langendorff pig hearts preserved with either 0 or 16 mmol/L Mg^{++} cardioplegic solution showed similar decrements in energy metabolites during 4 hours of ischemic preservation at 12°C and recovery of contractile function during reperfusion while 16 mmol/L Mg^{++} in K-H solution resulted in a dramatic decline of contractile function. Furthermore, 16 mmol/L Mg^{++} in S-C-S also did not affect the levels of high energy phosphates and contractile function during reperfusion. These results suggested that 16 mmol/L Mg^{++} in primary cardioplegic solution and in S-C-S did not offer an essential protective effect on ischemic myocardium.

At 4°C the pig hearts stored with either University of Wisconsin solution (UW, an intracellular-type cardioplegic solution) or St Thomas' solution (an extracellular-type cardioplegic solution) showed comparable changes in energy metabolites during 8 hours of preservation and a similar recovery of contractile function during reperfusion. However, at 12°C, hearts stored in UW solution showed rapid decrease in ATP and PCr during preservation and significantly poorer functional recovery during reperfusion; four of eight hearts stored in UW solution at 12°C showed the "stone heart" phenomenon with disappearance of PCr and ATP upon reperfusion. The addition of 0.5 mmol/L Ca^{++} to UW solution significantly improved contractile function and prevented the occurrence of the "stone heart" phenomenon with stable levels of high energy phosphates.

In conclusion, re-arrest perfusion following long term cardiac preservation improves postischemic myocardial recovery but may not be correlated with recovery of energy metabolism. Augmented buffer capacity in cardioplegic solution may not be significant for long-term cardiac preservation although it helps to maintain intracellular pH. It does not appear that 16 mmol/L Mg^{++} in cardioplegic solution and re-arrest solution is essential for heart preservation. Intracellular-type cardioplegic solution may not be appropriate for cardiac preservation at 12°C because of rapid decrease of high energy phosphates and the possibility of the calcium paradox.

INTRODUCTION

1. Overview

Heart transplantation is the only currently available alternative in the long-term treatment of some end-stage heart diseases[1-2]. The preservation of donor hearts is a critical step in heart transplantation, especially when it is obtained from a distant location[1-2]. Although current preservation techniques offer a reasonable clinical outcome, it is not uncommon to see mild or moderate functional depression of transplanted hearts[2]. This is at least partly due to sub-optimal preservation and inappropriate post-preservation treatment (reperfusion)[3-4]. Therefore, current preservation techniques may need to be improved.

This study was undertaken to determine whether selected modifications of current techniques could be able to improve cardiac preservation and reduce reperfusion injury. Myocardial energy metabolites and contractile function were followed during preservation and reperfusion in isolated rat and pig hearts that were subjected to various conditions of cardioplegia and reperfusion. The results of the study may provide a better understanding of the physiological principles involved in improving cardiac preservation techniques.

Reperfusion is necessary for recovery of the ischemic myocardium[3-4], but it may also cause additional tissue injury (so called reperfusion injury) and its severity is directly dependent on the conditions of reperfusion as well as the events occurring during the preceding ischemia[3-4]. Myocardial ischemia is characterized by depletion of energy stores, acidosis, loss of ionic gradients and dysfunction of sub-cellular organelles[5-6]. Logically, mechanical work during reperfusion should not be imposed on the heart until its homeostasis is fully recovered because energy metabolites consumed in contraction may be better used for improving the activity of ionic pumps and thereby

accelerating re-establishment of the normal ionic gradients, pH and cell volume as well as the function of the organelles. Thus, this study was undertaken first to determine whether inhibition of cardiac mechanical activity by re-arrest perfusion following prolonged ischemic preservation improved postischemic myocardial recovery in metabolism and function.

Recovery during reperfusion is directly related to the severity of ischemic injury[3-4]. Any intervention which reduces ischemic injury may be expected to improve heart recovery[3-4]. One striking effect of ischemia is intracellular acidosis which is associated with other detrimental cellular changes[5]. Therefore, acidosis plays an important role in myocardial ischemic injury and alleviation of acidosis may be expected to reduce such injury[6]. Using isolated buffer perfused rat hearts, Garlick et al[7] found that hearts perfused with Ringer's solution containing 100 mmol/L HEPES showed a significantly slower decline in ATP, PCr and intracellular pH during 15 minutes of normothermic ischemia and significantly better recovery of ATP and PCr during reperfusion, when compared to those perfused with normal Ringer's solution. Therefore, one would expect that increasing buffer capacity in cardioplegic solution may also be beneficial for long term cardiac preservation at lower temperature. It should be noted that the rat hearts in this work were immersed in the solution, diffusion from both sides of ventricular wall may significantly increased buffer capacity of the rat heart because the latter has relatively thinner ventricular wall. However, diffusion is expected to be negligible in large animal or human heart because of thicker ventricular wall. On the other hand, myocardial ischemic injury involves many cellular deleterious changes and cellular acidosis is only one of the fully documented manifestations of the injury[6]. The salutary effect of 100 mmol/L Hepes for 15 minutes of ischemia at 37°C does not necessarily indicate that high concentration of buffer in

cardioplegic solution would improve long-term cardiac preservation at lower temperature. Therefore, in this study we investigated the effect of increased buffer concentration in cardioplegic solution on long-term cardiac preservation.

In addition, it has been well demonstrated that Mg^{++} involves many cellular processes and plays an important role in the regulation of cardiac activity[8]. A large part of intracellular Mg^{++} is bound to ATP, adenine nucleotides and RNA and other exist as free ions[9]. Myocardial ischemia can cause a significant increase in intracellular Mg^{++} as a result of hydrolysis of ATP[9]. This ischemia-induced increase in intracellular Mg^{++} was thought to result in a loss of intracellular Mg^{++} during reperfusion and thereby impede recovery of myocardial metabolism and function[10]. We reasoned that the use of perfusion medium containing high concentration of Mg^{++} may be expected to prevent the loss of Mg^{++} and improve myocardial recovery during reperfusion. Using isolated buffer perfused rabbit and rat hearts, Bersohn et al[11] found that rat hearts subjected to 15 mmol/L Mg^{++} prior to 25 minutes of ischemia at 37°C showed significantly improved functional recovery. The basis for this salutary effect was thought to be due to prevention of Mg^{++} loss and to decrease of the Ca^{++} inward current[11]. The latter reduces cardiac activity and spares high energy phosphates[11]. It has been demonstrated that the cell membrane has very low permeability to Mg^{++} and lacks specific transporting system for Mg^{++} [12]. It was found that myocardium did not lose its Mg^{++} during reperfusion following 30 minutes of normothermic ischemia[12]. Therefore, any beneficial effect of 15 mmol/L Mg^{++} is probably associated only with reduction of Ca^{++} influx. In the presence of high concentration of K^{+}, such as in cardioplegic solution, Ca^{++} channels are in the inactivated state, high concentration of Mg^{++} may not be significant. On the other hand, beneficial effect of 15 mmol/L Mg^{++} is not observed on rabbit hearts[11], indicating that the effect of

increased concentration of extracellular Mg^{++} may be species dependent[11]. Therefore, this study was performed to investigate the effect of 16 mmol/L Mg^{++} in primary cardioplegic solution and re-arrest perfusion solution on myocardial energy metabolites and function.

Intracellular Na^+ overload is an important factor in ischemic injury. Increased intracellular Na^+ will initiate influx of Ca^{++} via Na^+/Ca^{++} exchange during reperfusion[13]. We anticipated that the prevention of Na^+ overload during ischemia would alleviate ischemic and reperfusion injury. An intracellular-type cardioplegic solution containing little Na^+ and Ca^{++} with high concentration of K^+ may abolish these gradients of Na^+, Ca^{++} and K^+ across cell membrane and prevent the increase of intracellular Na^+ and Ca^{++}. This will also reduce energy consumption for these pump activities and help to preserve energy stores. However, experimental results are controversial and use of it for heart preservation has not been universally accepted. Therefore, we investigated the effect of intracellular-type cardioplegic solution on heart preservation.

2. Literature Review

2.1. Heart Preservation

2.1.1. General

The concept of "heart preservation" may have two substantially different implications: either heart protection during open heart surgery or ex vivo heart storage for subsequent transplantation. These two implications are very different entities and differ in many aspects, such as temperature of the myocardium, non-coronary collateral blood flow, duration, myocardial ischemic changes and method used to reduce myocardial injury. The term "cardiac preservation" in the present study refers only to preservation of the heart prior to transplantation, i.e. "long term" (4-8 hrs) ex vivo cardiac preservation at low temperature with no perfusion.

The goal of donor heart preservation is to maintain the viability of the heart during the time needed for transportation. The methods used presently for ex vivo donor heart preservation have made a significant contribution to heart transplantation by greatly increasing the availability of donor hearts and providing optimal tissue matching that is not constrained by distance to be transported. It is important to recognize that, although preservation techniques currently in use have been successful, once the heart is removed from an intact circulatory system, myocardial ischemic injury ensues. Preservation techniques only reduce the extent of this injury; they do not fully prevent the process. It is hoped that with better understanding of the mechanisms of myocardial injury during preservation and reperfusion, we may be able to develop better techniques to minimize the extent of the myocardial injury and thereby to prolong the safe preservation period.

2.1.2. The Methods of Heart Preservation

There are four methods which have been used clinically for donor heart preservation[2]: hypothermia, hypothermic perfusion, cardioplegia with hypothermia and autoperfusing heart-lung preparation. The latter, however, was considered too complicated to serve as a practical method of preservation[2].

I. *Hypothermia.*

In this technique, the donor heart is rapidly excised and put into ice cold saline solution following cutting the inferior and superior venae cavae. The primary advantage of this technique is simplicity; no cardioplegic solution delivery system and no associated cannulas are used. This technique is safe only for very short periods of preservation. Obviously, the disadvantage of this technique is that the heart continues to beat while it is being

removed from the chest, which will result in a rapid unbalance between myocardial energy consumption and production[14].

II. *Hypothermic Perfusion*

To extend the preservation time, hypothermic perfusion has been used in some clinical settings[1-2]. Hypothermic perfusion has the advantage of providing a continuous supply of oxygen and substrates to the heart and maintaining ATP synthesis and washout of metabolic wastes[1-2]. However, edema formation and rising vascular resistance during extended perfusion time have been major problems with this technique. Manipulations of the perfusion solution and conditions have not solved these problems[2].

III. *Cardioplegia and Hypothermia*

In this method, a cold cardioplegic solution is introduced into the coronary system in vivo to arrest the heart and to lower its temperature rapidly. The heart is then excised. This technique rapidly arrests the heart and helps lower the temperature of heart more homogeneously than hypothermia alone. This is the technique being used by most transplant centers. It is generally accepted that a four hour ischemic period is safe for clinical application[2]. Cardioplegic solutions formulated with an electrolyte composition similar to that of the extracellular fluid have been used worldwide although there are some experimental results suggesting the superiority of intracellular-type cardioplegic solutions[15-16].

2.2. **Cardioplegic Solutions**

2.2.1. General

Cardioplegia (elective cardiac arrest) was introduced by Melrose et al[15-16]. in London in 1955 in order to obtain a bloodless, still operative field for intracardiac surgical procedures. Cardioplegic solutions are the solutions used to

achieve this elective cardiac arrest[15-16]. The solutions have been used worldwide for cardiac surgery and cardiac preservation for heart transplantation since the procedure was re-introduced into clinical practice in the 1970's[17]. These solutions substantially increase the safety of cardiac surgery and significantly prolong the safe preservation time for heart transplantation[15]. Cardioplegic solutions reduce myocardial ischemic injury by inducing rapid diastolic arrest and rapidly lowering the heart temperature as well as by delivering substances essential to the heart's viability[15-16]. Cardioplegic solutions help conserve myocardial energy metabolites, slow down metabolic and degenerative processes and prevent the occurrence of unfavorable cellular changes[15-16]. High concentrations of potassium in cardioplegic solutions are largely responsible for their efficacy[15-16]. The pH, osmolarity and temperature as well as concentrations of other components are important factors contributing to the efficacy of such solutions in protecting the ischemic myocardium[15-16]. In spite of the formulation of a variety of cardioplegic solutions, there is no universally accepted formulation largely because cellular changes occurring during cardioplegia have not yet been fully understood[15-16]. Additionally, the solutions that are good for open heart surgery (generally requiring less than one and half hours of arrest) may not necessarily be optimal for longer term heart preservation[17].

Cardioplegic solutions protect the heart from ischemic injury resulting from aortic cross-clamping during open heart surgery and ex vivo heart preservation for transplantation[15-16]. Through induction of rapid diastolic arrest and lowering of heart temperature, cardioplegic solutions allow high energy metabolites in the myocardium to be conserved for use during the subsequent ischemic period[15-16]. This energy storage is very important in maintaining the viability of myocardial cells[15-16]. Rapid and complete diastolic arrest can be induced by a number of

pharmacological agents such as tetrodotoxin, acetylcholine, local anesthetics (e.g., procaine), or even calcium antagonists or calcium-complexing agents (e.g., EDTA or citrate)[17]. The effects of these pharmacological agents are usually temperature dependent, have narrow therapeutic ranges, and easily accumulate in the myocardium resulting in delay of postischemic myocardial recovery upon reperfusion[18-19]. The formulations of the cardioplegic solutions used clinically are usually based on essential characteristics of the cell resting and action potentials[15-17]. Rapid diastolic arrest is most frequently achieved by high concentrations of potassium ion (16-30 mM) by depolarization of the membrane in both myocytes and pacemaker cells[15-17]. The depolarization inactivates sodium and calcium channels. The intracellular calcium spike cannot be generated[15-17]; calcium-induced calcium release therefore cannot take place. As a result, the heart stops in diastole[15-17]. Cardiac arrest can also be induced by solutions containing no calcium and little or no sodium[15-16]. Calcium-free solutions abolish the pacemaker potential of sinoatrial node cells and uncouple the electrical and mechanical components of myocytes[15-16]. Lack of sodium inhibits the generation of an action potential[15-16]. Increased concentrations of magnesium in cardioplegic solution have also been suggested to be beneficial in inducing diastolic arrest as well as in other aspects related to the viability of myocardium[20-21]. Cardioplegic solutions are usually used in combination with hypothermia to achieve rapid diastolic arrest and efficient conservation of high energy compounds.

It has been shown that high concentrations of potassium may result in unwanted effects which include a rise in intracellular resting calcium, myocardial infarction and damage to the coronary endothelium and alteration of the volume regulation of vascular endothelial cells to the point of occlusion of the capillary bed, leading to the no-reflow

phenomenon upon reperfusion[22]. Therefore, the concentration of potassium in cardioplegic solution should be kept at the minimum level required to maintain the arrested state[15-16,22]. The efficacy of high potassium also depends on accompanying conditions such as temperature and the concentrations of other ions[16, 23-24]. For these reasons, the potassium concentrations of cardioplegic solution used in clinical practice is usually in the 16-30 mM range, and is combined with reduced calcium and sodium and increased magnesium concentrations[21].

Magnesium alone can slowly induce cardiac diastolic arrest at concentrations of 20-25 mM[21-22]. The mechanisms underlying this effect are competitive inhibition of calcium influx through the slow sodium and calcium channels in the sarcolemma[21-22]. Increasing the concentration of intracellular magnesium prevents calcium uptake into mitochondria while enhancing calcium uptake into the sarcoplasmic reticulum[25-26]. In spite of the fact that a high magnesium concentration in cardioplegic solution can effectively replace calcium in outer cell binding sites, it cannot act as a membrane stabilizer in a manner similar to that of calcium[15-16]. Additionally, since an increased concentration of Mg^{++} cannot inhibit the electrical activity of the myocyte efficiently, it takes much longer to induce diastolic arrest by this means[15-16]. Thus, high Mg^{++} concentration is used only as the supplementary component in cardioplegic solutions.

Decreasing of sodium and calcium concentrations in cardioplegic solutions to approximately cytosolic levels can induce myocardial arrest by abolishing action potentials and preventing the spike of intracellular calcium concentration. However, myocardial tone can be increased if the decrease in sodium is not accompanied by a corresponding reduction in calcium[16]. The increase in myocardial tone is exclusively due to an increase in intracellular calcium resulting from calcium influx via Na^{+}/Ca^{++} exchange[27]. According to the

following equation, the Na^+/Ca^{++} exchanger requires that the calcium concentration be reduced to 10^{-2} to 10^{-4} of the physiological value in order to keep intracellular calcium at the resting level, if the sodium concentration is reduced to 10^{-1} of its physiological value[16,27].

$$[Ca^{++}]_i = [Ca^{++}]_o\{\frac{[Na^+]_i}{[Na^+]_o}\}^3 \exp\{\frac{EF}{RT}\}$$

where E is the membrane potential, and R, T, and F are the gas constant, absolute temperature and Faraday constant, respectively. The intra- and extracellular calcium and sodium are represented by $[Ca^{++}]_i$, $[Ca^{++}]_o$, $[Na^+]_i$, and $[Na^+]_o$, respectively.

Although Ca^{++} in cardioplegic solutions may have some detrimental effects on the ischemic myocardium, it helps stabilize and preserve the structural and functional integrity of the sarcolemma[16]. The effect of calcium on the myocardium is also dependent on temperature and other components, such as sodium and potassium[16]. Therefore, the optimal calcium concentration in cardioplegic solutions is very difficult to determine. However, if the calcium concentration is lower than a threshold of 25-50 umol/L, a calcium paradox may result upon replenishment of calcium[28].

2.2.2. Classification of Cardioplegic Solutions

Cardioplegic solutions are generally classified into extracellular and intracellular-type solutions, according to their formulations[15-16]. Those similar in composition to the extracellular fluid are referred to as the former cardioplegic solutions while those similar to intracellular fluid are named intracellular-type cardioplegic solutions.

I. *Extracellular-Type Cardioplegic Solutions*

Many cardioplegic solutions used in clinical practice are of the extracellular-type, for example, St Thomas' solution #2 (Table 1). The important characteristic of this type of solution is that the ionic concentrations are close

to those in the extracellular space with the exception of increased potassium concentrations[15]. Diastolic arrest is mainly due to the latter. The advantage of this type of solution is that a normal sodium gradient across the cell membrane is maintained. This helps maintain the low intracellular calcium level. On the other hand, normal concentration of sodium (140 mmol/L) and calcium (1.2 mmol/L) will stimulate the Na^+/K^+ and Ca^{++} pumps. This may increase the consumption of stored energy metabolites and hasten sodium and calcium overload when the stores are exhausted.

II. *Intracellular-Type Cardioplegic Solutions*

The ionic concentrations in this type of cardioplegic solutions are similar to those of the intracellular milieu, in that they contain little or no sodium and calcium and have high levels of potassium (30-130 mmol/L). They arrest the heart through three mechanisms: hyperkalemia, hypocalcemia and low sodium concentration. These solutions have several advantages[15]. Firstly, the concentrations of sodium, potassium and calcium in the solutions are close to those of intracellular fluid; hence the trans-sarcolemmal ionic gradients are abolished or reduced and the activity of energy consuming ion pumps (Na^+/K^+ and Ca^{++} pumps) is decreased. This helps conserve cellular energy stores which are important in maintaining the cell's structural integrity. Secondly, due to their intrinsically low osmolarity, high concentrations of osmotically active impermeants (such as mannitol, lactobionate, raffinose and dextrose) can be incorporated into the solutions without rendering the solution excessively hyperosmolar[15]. It has been shown that dextrose and mannitol help attenuate myocardial edema during cardioplegia[16]. Mannitol has also been demonstrated to have antioxidant properties[29]. Since it contains very little sodium, this type of cardioplegic solution may attenuate or delay the occurrence of sodium

overload during ischemia. Such highly empirical considerations lead to the expectation of efficacy. In addition, although it contains no calcium, its use usually does not cause a calcium paradox[16]. One of the reasons for this could be the low temperatures at which these solutions are used[30]. Another reason could be that the residual calcium in the solution prevents the calcium paradox. However, this does not mean that the calcium paradox never occurs in hearts arrested with Ca^{++}-free intracellular type cardioplegic solutions[31].

2.2.3. Cardioplegic Solution Vehicles

Cardioplegic solution vehicles usually include crystalloid fluids or blood. The former have been used in conventional cardioplegic solutions since cardioplegic solution was first introduced. They are easy to prepare, inexpensive, while being quite effective. Compared to blood cardioplegic solutions, they are not associated with problems of capillary obstruction, particularly during hypothermic conditions.

Blood cardioplegia has recently become popular, mostly for intracardiac procedures. The advantages of blood cardioplegic preparations are in the intrinsic characteristics of blood, including its higher oxygen carrying capacity, high buffer capacity, appropriate osmotic load, endogenous oxygen free radical scavengers as well as its rheologic effect and its ability to deliver metabolic substrates compared to crystalloid cardioplegic solution[32-33]. However, when blood cardioplegic solutions are used at low temperatures, a number of disadvantages become obvious such as blood sludging, platelet aggregation, rouleaux formation and crenation of red blood cells, which can obstruct capillaries and enhance release of vasoactive substances. Moreover, the high oxygen affinity of hemoglobin prevents its unloading in the tissue at low temperature.

2.2.4. Temperature of Cardioplegic Solutions

Temperature is a very important factor determining the efficacy of cardioplegic solutions. Hypothermia can reduce myocardial metabolic rate considerably and helps maintain cellular energy stores and reduce ischemia-induced damage. To separate the effects of hypothermia and potassium cardioplegic solution on heart protection, Hearse et al[32] compared the myocardial ATP and PCr levels at the end of 30 minutes of ischemia and recovery of contractile function during reperfusion between the rat hearts subjected to normothermic cardioplegic arrest and those kept at 4°C without cardioplegia. They found that the concentrations of ATP in the former and latter hearts were 11.1 ± 4.2 and 20.5 ± 1.0 μmoles/g dry weight and the concentrations of PCr were 9.4 ± 2.1 and 25.8 ± 1.8 μmoles/g dry weight, respectively. The recovery of contractile function assessed by comparing aortic flow obtained before and after ischemia were 40% and 90% in the normothermic cardioplegic arrested hearts and those preserved only by hypothermia, respectively. Based on these results, they suggested that hypothermia alone can provide twice as much protection against ischemic injury as the use of a cardioplegic solution. However, hypothermia suppresses the activity of Na^+/K^+ ATPase and Ca^{++} ATPase in the myocardial sarcolemma, leading to accumulation of Na^+ and Ca^{++}, causing loss of cell volume regulation and swelling; hypothermia also decreases the fluidity of cell membranes which may affect the integrity of membrane structures and intracellular pH may be altered by precipitation of ionic complexes. Since hypothermia has numerous disadvantages, the optimal temperature for heart protection still remains to be determined. This is the reason that in some clinical settings, rather than 0°C-5°C, 12 °C is used[15]. Furthermore, while in experimental situations, temperature can be rigidly controlled, in the clinical setting this is usually not practicable.

2.3. **Myocardial Ischemia**

2.3.1. General

The word "ischemia" is derived from the combination of the Greek words *ischo* (meaning "to hold back" i.e. "too little") and *aemia* (meaning "related to blood"). Myocardial ischemia means a condition in which coronary blood flow is inadequate to permit the maintenance of a steady-state level of metabolism with the accumulation of metabolic waste in the myocardium. Myocardial ischemia is usually caused by a primary decrease of coronary flow. Myocardial ischemia may be either global or local (regional). The former usually is the case in open heart surgery and cardiac preservation by aortic across-clamping. Coronary artery disease (atherosclerosis, thrombosis, arterial spasm) is the most common cause of regional myocardial ischemia, but its presence affects the distribution of the infused cardioplegic agent, resulting in non-homogeneous arrest and cooling. Although some cellular changes in the ischemic myocardium are very similar to those in the anoxic myocardium, ischemia differs considerably from hypoxia or anoxia. There is a primary decrease in coronary flow in ischemia, whereas there is a reactive increase in coronary flow in hypoxia or anoxia, which may be caused by the release of adenosine. Hypoxia and anoxia are usually caused by a decrease in oxygen content in blood or a relative increase in demand while there is no primary decrease in coronary flow.

Myocardial ischemic injury has been arbitrarily divided into reversible and irreversible, according to the degree of cellular changes[6]. As myocardial ischemia results in a decrease in high energy phosphate compounds, decline of chemical potentials, diffusion gradients and equilibrium potentials, this leads to failure of cellular homeostasis which is summarized below.

2.3.2. Ischemia-Induced Myocardial Changes

I. *Energetic Metabolites*

Although there is an early and progressive fall of cellular ATP level, the decrease of PCr is much more striking, starting within seconds of the onset of ischemia[34]. The reason that creatine phosphate falls more rapidly than ATP is that the specific process transferring ATP in and out of the mitochondria by adenine nucleotide transferase is inhibited by an accumulation of acyl CoA during ischemia. As ATPase in the sarcolemma and contractile filaments continues to use ATP, PCr is used to replenish cytosolic ATP stores. The decrease in PCr is accompanied by a rise in free inorganic phosphate (Pi), an important contributing factor to the early suppression of force development[35]. When the rate of ATP hydrolysis during ischemia exceeds the rate at which ATP is formed from PCr and glycolysis, ATP falls and ADP increases. As a result, ΔG_{ATP} (ΔG_0 + RTln[ADP][Pi]/[ATP]) decreases in magnitude from about -60 Kj/mole to as low as -45 Kj/mole[36].

The degradation of ATP to ADP and AMP, with subsequent deamination to inosine, leads to the conversion of nucleotides to products that are capable of passing through the cell membrane, particularly when the latter is not intact[6,37]. There is also evidence that ATP and its products pass directly through the membrane during ischemia to be deaminated extracellularly, leading to loss of the cell's adenine nucleotide pool[38].

II. *Changes in Ionic Gradients*

Loss of intracellular potassium starts early in ischemia and has been linked to early ventricular arrhythmias[39-45]. With the efflux of intracellular potassium, the gradient across the membrane decreases, leading to depolarization of the cell and to inhibition of sodium channel activity. Therefore, the upstroke and duration of the action potential decrease. Although the

mechanisms of potassium loss are not fully understood, three possible theories have been proposed[46-52]: 1) opening of ATP-inhibited potassium channels as a result of deficiency of ATP, 2) Inhibition of Na^+/K^+ ATPase, 3) efflux of potassium accompanied by negatively charged lactate and phosphate ions.

Since ΔG_{ATP} is normally about 15-20 Kj/mole greater than the energy required to drive the Na^+/K^+ pump, it does not normally limit pump kinetics[53]. However, as ΔG_{ATP} falls during ischemia, the pump will become limited and may even reverse[53]. In addition, accumulation of ADP and inorganic phosphate, together with the lowered temperature and pHi, would be expected to reduce the pump activity. Na^+/H^+ exchange exacerbated by ischemia and cellular acidosis would also promote sodium influx[54]. As a result, intracellular Na^+ rises during ischemia whether or not there is any increase in passive Na^+ influx. This will have important consequences for cellular homeostasis since it is the transmembrane Na^+ gradient that controls other transmembrane ionic gradients such as those of H^+ and Ca^{++} [55-56].

The increase in intracellular Ca^{++} usually occurs after the rise in Na^+ [57-59]. Consequences of the increased calcium level include activation of phospholipases, increased depolarization, ischemic contracture, and mitochondrial damage[60-66]. The mechanisms responsible for the massive accumulation of calcium in the ischemic myocardium are currently not fully understood, but several possible mechanisms have been proposed[57]: (1) increased entry of calcium through slow calcium channels; (2) decreased extrusion of calcium through the sarcolemmal calcium ATPase; (3) reversed Na^+/Ca^{++} exchange; (4) increases in the passive permeability to calcium through damaged cell membranes.

Regarding the increased influx of calcium through slow calcium channels, several studies have demonstrated that slow calcium channel antagonists cannot inhibit the

pathological increase of calcium in a way that is independent of their attendant hemodynamic effect, suggesting that calcium influx through slow channels could not be significant[67-69]. There is some evidence demonstrating that slow calcium channel blockers administered prior to ischemia can protect the ischemic myocardium[70-72]. This may be related to their depressive effect on contractile function and consequent preservation of cellular high energy phosphates[68-69]. In reversibly injured tissue, such as the stunned myocardium, use of a calcium channel antagonist after the induction of ischemia was found to be beneficial[73-75].

With regard to the inhibition of the sarcolemmal calcium pumps, hearts subjected to hypoxia did not show decreased calcium efflux, suggesting that simple inhibition of the calcium pumps may not be a major determinant of calcium entry during ischemia[75-76]. There are a number of studies suggesting that reversed Na^+/Ca^{++} exchange might account for a portion of the increase in calcium content during irreversible myocardial injury[77].

Recent studies in cultured myocardial cells have also suggested that Na^+/Ca^{++} exchange is able to contribute to the massive accumulation of calcium in irreversible cell injury but may not totally account for the magnitude of calcium entry into the cell[67]. However, due to the fact that inhibition of the Na^+/K^+ pump by ouabain does not result in irreversible myocardial injury, Na^+/Ca^{++} exchange may not totally account for the magnitude of calcium entry into the myocardial cell either, although it is an important mechanism responsible for the increase in calcium level.

Lastly, recent studies have found that structural defects in the sarcolemmal membrane may be causally related to the massive influx of calcium into irreversibly injured myocardium during ischemia[78-86].

III. *Accumulation of Lactate*

Rapidly following the decline in PCr, stimulation of glycolysis results in the production of lactate[87]. Due to poor washout of metabolites, lactate accumulates in myocardium. Increased lactate content in the ischemic myocardium may have several effects: decreased contractile activity[88], promotion of mitochondrial damage[89], decreased action potential and inhibition of glycolysis at the level of glyceraldehyde-3-phosphate dehydrogenase[90-92]. The mechanism of these lactate effects may be variable and speculatively, may include extracellular calcium-binding, increased calcium entry into mitochondria and dcreased glycolytic flux. In the connection of glyceraldehyde-3-phosphate dehydrogenase, there is a stereospecific interaction between lactate and the enzyme[92].

IV. *Increased Intracellular Osmolarity*

The combined effects of increasing intracellular lactate, phosphate and Na^+ concentrations, as well as breakdown of some cellular constituents, result in an osmotic increase of 40-60 mOsmol in the cell. In a closed ischemic system, such as the heart preserved in cardioplegic solution, water movement is minimal and the osmotic pressure of the extracellular fluid soon equals the intracellular value following relatively small exchanges of water and solutes. However, in an open system, such as during reperfusion, this would result in a large movement of water into the cells, leading to cell swelling and disruption that occurs in both myocytes and capillary endothelial cells[3]. The latter "external" compression is probably the main cause of increased coronary vascular resistance and inadequate myocardial capillary blood flow during reperfusion.

V. *Proton Accumulation*

One important effect of ischemia is the generation of protons derived from breakdown of ATP and from other

metabolic cycles that form protons[5]. Many enzymes, such phosphofructokinase (PFK), are inhibited by low pH, which compounds the effects of ischemia by slowing the only metabolic reactions available to generate ATP. Protons also inhibit force generation by competing with calcium binding sites on contractile proteins[93-95].

VI. *Loss of Cellular Constituents*

Membrane damage and increased permeability inevitably leads to the loss of soluble cytosolic proteins[96-97]. The leakage of enzymes, such as creatine kinase, is readily demonstrated and has been used as an indicator of myocardial injury[98]. Since damage is not homogeneous, the loss of enzymes may appear graded as the mass of the injured cells increases. Enzymes can be detected long before morphological injury can be clearly seen by currently available technologies[98].

2.3.3. Cardiac Preservation and Myocardial Ischemia

Cardiac preservation is a critical step during the entire heart transplantation procedure. During hypothermic preservation, myocardial ischemic injury occurs although the metabolic and degeneration rates are slowed down in the arrested heart. The severity of ischemic injury is primarily dependent on the preservation temperature and the length of the preservation period. Ischemic injury is also influenced by the cardioplegic solution, (composition, dose, temperature etc)[15-16]. A well formulated cardioplegic solution could reduce myocardial ischemic injury and prolong the safe preservation time (reversible ischemic injury). Using isolated working rat hearts, Choong and co-workers[99] found that hearts arrested with St Thomas' solution #2 containing 20 mmol/L added aspartate showed significantly better functional recovery (aortic flow) after 24 hours of hypothermic preservation at 4°C than hearts arrested with St Thomas' solution #2 alone, leading to the conclusion that

added aspartate can considerably extend the duration of safe ex vivo storage of hearts. This beneficial effect of aspartate was postulated due to the attenuation of mitochondrial injury and improvement of intracellular Na^+ and Ca^{++} regulation[99].

2.4. **Reperfusion Injury**

2.4.1. General

Although reperfusion is an absolute prerequisite for survival of the ischemic myocardium, reperfusion is not without adverse effects[100-105]. Under certain circumstances, reperfusion can result in deleterious effects in the ischemic myocardium and can also lead to lethal myocardial damage. In 1935, Tennant and Wiggers[106] found that hearts subjected to reperfusion showed ventricular fibrillation. In 1960 Jennings et al[107] reported that reperfusion induced adverse structural and electrophysiological changes. Furthermore, Hearse et al[108] in 1978 reported that reperfusion of rat hearts resulted in a sudden and massive release of cellular enzymes, myocardial contracture bands and ultrastructural damage, coining the term "reperfusion injury". More important is that reperfusion injury now is no longer purely a laboratory event; it does occur under three types of clinical conditions: 1) reinstitution of coronary blood flow following heart surgery or heart transplantation, 2) sudden release of a coronary artery after severe coronary spasm, 3) coronary reperfusion by thrombolytic therapy, angioplasty or bypass surgery. Reperfusion injury includes four unfavorable sequelae: 1) reperfusion-induced arrhythmias, 2) myocardial stunning (transient postischemic contractile and metabolic dysfunction), 3) microvascular damage and 4) acceleration of the necrotic process[100].

2.4.2. Manifestations of Reperfusion Injury

I. *Reperfusion-Induced Arrhythmias*

Potentially lethal arrhythmias can be induced not only by an ischemic episode but also by reperfusion of the myocardium[95,109]. Reperfusion-induced arrhythmias include closely coupled activity, tachycardias, and ventricular fibrillation[110]. The major mechanisms underlying ischemia-induced heart arrhythmias are related to re-entrance activity[104-105], while reperfusion-induced arrhythmias have characteristics different from those seen during ischemia[111]. Studies of reperfusion arrhythmias in hearts in situ suggest that reperfusion arrhythmias are generated by more than one mechanism; re-entry and automaticity are likely candidates[112-113]. Studies suggest that re-entrance activity is responsible for more rapid arrhythmic activity, such as ventricular fibrillation, whereas oscillatory after-potentials (OAPs, also referred to as delayed after-depolarizations) underlie slower activity like premature beats, coupled rhythms and tachycardias[112-113]. The cellular bases of these arrhythmias are mainly related to the loss of cellular calcium homeostasis[100], since excess cytosolic calcium levels during reperfusion has been demonstrated, supporting the theory that excess calcium levels with recycling may underlie these arrhythmias[114].

Hearse has proposed tight links between the formation of free radicals and reperfusion ventricular fibrillation with a remarkably consistent effect of free radical scavengers in decreasing the incidence of such arrhythmias[115-117]. Free radicals can induce electrophysiological changes, such as membrane depolarization[117]. Free radicals in sufficiently high concentrations can also modify the gating properties of the sarcoplasmic reticulum calcium release channel in such a way that excess calcium is released, thereby producing calcium-dependent arrhythmias[117].

II. *Myocardial Stunning*

Reperfusion after a short period (about 5-15 minutes) of ischemic insult does not result in rapid and complete recovery of myocardial mechanical function in some situations[100,118-119]. Several days to weeks may elapse before full recovery of contractile function occurs. Because function eventually does recover completely, the myocardium is said to be only stunned under these conditions. Myocardial stunning does not occur in the heart after long term hypothermic preservation; it is relevant only to open heart surgery and a short period of local ischemia caused by coronary thrombosis or spasm. Myocardial stunning is defined physiologically as a reversible defect of myocardial function on reperfusion and is part of the spectrum of reperfusion injury[100]. There have been many theories to explain the development of this phenomenon. Yet the mechanisms responsible for the prolonged depression of contractile function during reperfusion are still debated. An early hypothesis was that resynthesis of ATP is delayed due to the loss of adenosine and related compounds during ischemia[120]. Nevertheless, there is no direct correlation between the tissue content of ATP and recovery of contractile function; furthermore, low levels of ATP do not seem to limit adequate functional recovery during reperfusion. The fact that stunned myocardium can respond well to inotropic interventions by catecholamines, calcium infusion as well as postextrasystolic potentiation, seems to preclude persistent disturbances of energy supply as the underlying mechanism[121]. The potential mechanisms responsible for myocardial stunning may involve calcium overload and generation of free radicals[118-119,121-122], with a subsequent decrease in myofibrillar calcium sensitivity[100]. One hypothesis is that internal cytosolic calcium overload damages the organelles involved in contraction and impairs the normal physiological response to

calcium with the consequent manifestations of mechanical stunning[118,121].

III. *Microvascular Damage*

The fact that reperfusion does not always restore normal coronary flow to ischemia-insulted myocardium is referred to as the no-reflow phenomenon[100,123]. The benefits of reperfusion are certainly lessened by failure of the restoration of normal flow[123]. The mechanisms underlying this pitfall of reperfusion are multiple and probably interactive. Firstly, an ischemic episode can damage microvascular endothelial cells, resulting in swelling of the endothelium which increases coronary resistance and lessens coronary flow[100]. Secondly, myocardial contracture induced by ischemia can compress the coronary microvasculature and prevent re-establishment of normal blood flow[124]. Furthermore, damage to coronary endothelial cells could affect the release of vasodilator substances, such as endothelium-derived relaxation factor (EDRF), and possibly promote the formation of the vasoconstrictor endothelin[100]. In addition, damaged endothelium could remove or inactivate factors inhibiting platelet plugging and neutrophil adherence. As with other manifestations of reperfusion injury, some microvascular damage could be caused by neutrophil-derived free radicals. Undoubtedly, microvascular damage would contribute to non-lethal reperfusion-induced injury and may accelerate expression of necrosis. It is noteworthy that the myocardium might be lethally damaged by sub-optimal reperfusion, however there is no conclusive evidence supporting the existence of this sequela of reperfusion.

IV. *Accelerated Expression of Myocardial Necrosis*

After a prolonged ischemic period, the myocardium has already sustained irreversible injury before reperfusion is initiated. At that time, reperfusion merely accelerates the

process of necrosis[108]. The characteristics of this consequence of reperfusion include sarcolemmal disruption, severe cell swelling, myocardial contracture, and massive leakage of intracellular constituents[100]. The most plausible mechanisms are related to drastic sodium and calcium overload with consequential intensive myocardial contracture and cell swelling[124-125]. Myocardial contracture in turn promotes tearing of the sarcolemmal membrane, exaggerating sodium and calcium influx and the loss of cellular constituents. As with other consequences of reperfusion injury, free radicals may also play an important role in this consequence of reperfusion injury[120,122,126-127].

2.4.3. The Mechanisms of Reperfusion Injury

I. *Calcium Overload*

There is no doubt that cellular calcium overload can occur during reperfusion and that it is deleterious[4,100]. An increase in cytosolic calcium hastens breakdown of any remaining adenosine triphosphate, and activates phospholipases and proteases[63,65-66]. Several mechanisms have been proposed to account for the increase in cytosolic calcium during reperfusion. First, inhibition of the Na^+/K^+ pump leads to elevated levels of intracellular sodium during myocardial ischemia and the sodium gradient across the sarcolemmal membrane decreases[55,77]. The sodium gradient across the membrane is one of the major mechanisms for maintaining cellular calcium homeostasis[55]. As cytosolic sodium increases, activity of Na^+/Ca^{++} exchange decreases and the direction will eventually reverse, i.e. sodium efflux and calcium influx will occur, leading to calcium overload[13]. Secondly, due to intracellular acidosis during ischemia and washout of protons from the extracellular space during reperfusion, Na^+/H^+ exchange is enhanced, leading to cytosolic sodium overload and consequent to calcium overload by Na^+/Ca^{++} exchange. This may explain why the Na^+/H^+ exchange inhibitor amiloride can attenuate calcium

accumulation and act as an antiarrhythmic[13]. Calcium can also enter myocardial cells as a result of catecholamine stimulation[57,128-129]. Since calcium pumps in the sarcolemmal membrane and the sarcoplasmic reticulum are energy limited, calcium extrusion and uptake decrease and the level of intracellular calcium increases[130]. In addition, a small amount of calcium may also enter cells through the voltage-operated calcium channels[61].

It has been demonstrated that calcium might not only be a marker of myocardial reperfusion injury but may also be causally related to the loss of myocardial cell viability in response to a wide variety of pathologic stimuli[131]. The potential detrimental effects of calcium overload are mainly based on calcium activation of degenerative processes, including calcium dependent proteases and calcium dependent phospholipases[61]. An increased intracellular calcium will in turn hasten the breakdown of any remaining adenosine triphosphate, and also cause calcium accumulation in mitochondria and subsequent impairment of mitochondrial function[128]. Even the generation of free radicals is, to some extent, calcium dependent[61].

II. *Generation of Oxygen-Derived Free Radicals*

Free radicals are highly reactive chemical species having unpaired electrons in their outer orbitals. Free radicals, and other reactive oxygen intermediates, may arise from a number of sources at intracellular and extracellular sites[126], including the arachidonic acid pathway, leucocytes, the xanthine oxidase pathway, enzyme-mediated oxidation of catecholamines, haemoglobin and myoglobin. Xanthine oxidase is not found in the human myocardium[132]. Oxygen-derived free radicals include superoxide, hydrogen peroxide and hydroxyl radical. These reactive intermediates are produced under both physiological and pathophysiological conditions, although probably to a greater extent in the latter[126]. Although radicals are highly reactive and

potentially toxic, their formation is, of course, not necessarily detrimental. Thus, leukocytes, for example, fulfill their normal biological function through the production of radicals that are "useful" in the inflammatory response[126].

Free radicals and other highly reactive short-lived intermediates can cause severe cellular injury and undoubtedly contribute to the pathophysiology of a variety of processes injurious to the myocyte, particularly reperfusion injury[4,133-139]. The generation of free radicals is considered to be one of the causes of reperfusion injury[126]. Calcium overload and generation of free radicals are also closely related. However, in very few cases has radical production been demonstrated or quantified directly and any association between radicals and tissue injury is indirect and circumstantial. A variety of agents with, among other properties, the ability to inhibit the production of free radicals or to enhance their elimination, have been shown to reduce tissue injury[126-127]; conversely, agents known to promote radical production have been shown to enhance the injury[126-127]. Recent studies utilizing electron spin resonance and spin trapping techniques have demonstrated a burst of free radical production early in reperfusion[128]. Free radicals and other reactive intermediates can severely injure a variety of cellular structures and some metabolic processes[140]. Radical-induced lipid peroxidation can alter the fluidity and permeability of cellular and sub-cellular membranes; radical-induced changes in redox state can activate or inactivate a variety of membrane pumps and channels, including those involved in Ca^{++} transport, Na^{+}/Ca^{++} exchange and Na^{+}/H^{+} exchange[126-127]. Radicals can also oxidize proteins, thereby inhibiting the activity of enzymes and damage nucleic acids in such a way that long-term survival for replication of the cell may be jeopardized[127]. Hearse and his colleagues[126] have proposed that reactive oxygen intermediates, produced either

intracellularly or extracellularly, may induce damage in membrane proteins and lipids that lead to disturbances in the normal transmembrane ionic balance and specifically to the loss of intracellular K^+ and uptake of extracellular Na^+ and Ca^{++}. In addition to contributing to cell swelling and tissue injury, such changes would be expected to alter the electrophysiological characteristics of the myocytes[127].

III. *Other*

Reperfusion injury is a complex dynamic event. The various mechanisms responsible for injury are interrelated and interactive. Although calcium overload and generation of oxygen-derived free radicals have been considered as major mechanisms involved in reperfusion injury, many other potential factors may also be involved[124-127]. For instance, elevation of cyclic adenosine monophosphate, disturbances of lipid metabolism, formation of lysophosphatides and loss of potassium and sodium gradients also underlie the reperfusion-induced arrhythmia[124-127]. The inability to resynthesize high energy phosphates, abnormalities of excitation-contraction coupling, damage of the myocardial collagen matrix and dysfunction of the sarcoplasmic reticulum have all been shown to be responsible for reperfusion injury[124-127].

2.4.4. Factors Governing the Severity of Reperfusion Injury

I. *Duration of Ischemia*

The incidence of reperfusion-induced arrhythmias is closely related to the duration of ischemia[115]. The delayed after-depolarization, which is responsible for the premature beats, coupled rhythms and tachycardias, is mainly induced by abnormal oscillation of the intracellular calcium level[110]. The latter in turn is due to impairment of the cell's ability to handle calcium[110,130]. The degree of fluctuation in the calcium level is dependent on the severity of ischemic injury. The longer the ischemic period

is, the greater the fluctuation will be, leading to more severe arrhythmia[104,128]. However, if the heart is subjected a prolonged ischemic insult, the myocyte will completely lose its ability to regulate its calcium content and there will be no calcium fluctuation. Therefore, the correlation between the ischemic period and the incidence of arrhythmias is biphasic[141]. Reperfusion arrhythmias cannot take place in dead myocardium, which is in accord with the hypothesis that the presence of sufficient high energy compounds is essential for at least some types of reperfusion arrhythmias because intracellular calcium oscillation requires energy[104]. In addition, the duration of ischemia is closely related to the degree of loss of cellular homeostasis which directly influences the outcome of reperfusion.

II. *Severity of Ischemia*

There is an intuitive correlation between the severity of ischemia and reperfusion injury. Regardless of the type of reperfusion injury, arrhythmia, myocardial stunning, or myocardial necrosis, the severity of events occurring during ischemia is a factor contributing to the severity of reperfusion injury. Because increasing the duration of ischemia increases the severity of ischemic injury, it is likely that a longer period of ischemia will predispose the myocardium to greater reperfusion injury in limited time range.

III. *Conditions of Reperfusion*

It has been widely accepted that reperfusion could induce a certain type of injury that the heart previously did not suffer or is only mild but not detectable by current techniques. Reperfusion-induced injury is also directly related to the conditions and composition of reperfusion as well as to the ischemic episode. It follows that reperfusion injury may be reduced or even prevented if an appropriate reperfusion technique is applied. This has been

partially demonstrated by the work of several groups[140-142], and that of Buckberg in particular[143-147].

2.5. **Models to Study Heart Preservation**

2.5.1. *In Vivo* Preparations

These preparations usually include cardiopulmonary bypass and left coronary occlusion. The two techniques are used only to study short periods of heart protection and can be performed using small animals, such as rats, as well as large animals. The most important advantage of these preparations is that the experimental conditions and procedures are closely comparable to those of clinical situations. However, the models suffer from complexity.

2.5.2. *In Vitro* Preparations with Whole Hearts

Isolated perfused hearts are considered *in vitro* preparations. These preparations offer considerable advantages for studies of heart preservation. The perfusion conditions (pressure, flow, temperature and substrate concentrations) can be controlled precisely and the direct effect of various treatments can be studied without interference from complicating systemic factors. However, the disadvantages of these preparations are that they are no longer innervated and beat spontaneously and are often perfused with crystalloid solutions that can limit oxygen delivery and may lead to metabolic and functional changes. Two models used most frequently are the so-called Langendorff and working models.

I. *Langendorff Model*

After being excised, the heart is cannulated through the aorta, connected to a perfusion apparatus and coronary flow is then resumed. The heart is perfused through the aortic root from either an elevated solution reservoir or a perfusion pump and the aortic valve is kept closed by the aortic pressure. The coronary flow in this model is not

substantially dependent on the contractile function of the heart. Mechanical function of the heart can be readily assessed by measuring the pressure generated by the isovolumically contracting left ventricle. The heart in Langendorff preparation performs no external work. This model has been widely used for the study of cardioplegic solutions and cardiac preservation and applied usually to the dog, rabbit and rat hearts due to its simplicity, stability and reproducibility.

II. *Working Heart Model*

To overcome the deficiencies of the Langendorff preparation (performing no external work), Neely and colleagues developed the isolated working heart preparation[148].

After excision, the heart is mounted on an aortic cannula as for the Langendorff model. The coronary flow then is re-established and the left atrium is cannulated. After a short period (5-10 minutes) of Langendorff perfusion, the preparation is converted to a working or ejecting preparation by opening the aortic outflow tract and the cannula in the left atrium and thereby permitting ventricular filling which can be regulated and clamping the Langendorff aortic inflow cannula. A downstream resistance or fluid column is connected to the aorta to provide controlled afterload. In contrast to the Langendorff preparation, coronary flow is largely determined by the ejection function of heart. Because of this characteristic, the working model heart will not be able to tolerate a significant reduction in oxygen availability or inflow from left atrium, which will result in a severe decrease in cardiac function. The latter leads to a corresponding reduction in coronary flow or no coronary flow and cardiac arrest will ensue rapidly. Therefore, despite the many advantages of this model, it does not replace the Langendorff model.

2.5.3. *In Vitro* Preparations with Heart Fragment

Isolated atria, ventricular strips, septal strips and papillary muscles have been used to study myocardial ischemia and cardioplegic solutions. Lareau and his colleagues[149] developed a human atrial appendage preparation, in which high energy phosphates and intracellular pH can readily be monitored using nuclear magnetic resonance spectroscopy with simultaneous measurement of mechanical function of tissue. Using this preparation, they found that increasing the buffer capacity in the storage solution improves the maintenance of intracellular pH at 12°C but not at 4°C. However, this preparation suffers a major disadvantage in that it is perifused. Therefore, oxygen and substrate delivery and washout of metabolic waste is limited by diffusion characteristics and dependent on the thickness of the preparation. The bath may represent an unrealistically large extracellular space in the conditions of very small preparations.

2.6. Markers to Evaluate the Condition of the Myocardium

2.6.1. Energy Metabolites

The myocardium requires a continuous supply of energy to maintain its cellular integrity as well as to support its contractile function. Although the energy demand is decreased significantly in hearts arrested with a cardioplegic solution, the residual energy metabolism is still an important factor in determining the processes of ischemic and reperfusion injury. Monitoring energy metabolites is very valuable in determining the viability of the preserved myocardium. Using isolated rat hearts, Hearse[32] demonstrated that there is a relationship between the content of energy metabolites (ATP and PCr) at the end of ischemic preservation and extent of functional recovery during reperfusion. These findings supported the view that

there is a critical minimum level of energy metabolites below which irreversible injury will occur and normal cellular function cannot resume upon reperfusion[32]. However, it has been suggested that ATP, PCr and other energetic intermediates are compartmented within the cell[150-152] and present techniques only allow the determination of average tissue content[153]. It is likely that the ATP which determines tissue viability and recovery is in a relatively small cellular compartment and critical changes of ATP in this compartment may be masked by the measured average ATP content[153]. This may account for the apparent uncoupling of contractile function and ATP level[153].

2.6.2. Mechanical Function

Because mechanical function of the heart is directly dependent on myocardial homeostasis (the integrity of myocyte structure and metabolism), assessment of contractile function of the heart is useful in assessing various cardioplegic solutions used for heart protection and heart preservation. For the isolated Langendorff preparation, left ventricular pressure, its derivatives (related to the velocity of contraction and relaxation) and heart rate can be easily and accurately measured, whereas aortic flow and end-systolic pressure-volume relationship can be obtained in the working preparation.

2.6.3. Enzyme Leakage

The analysis of plasma enzyme activity in the effluent from the heart is an alternative and simple (although probably crude) technique to detect tissue injury[154]. A healthy myocyte allows transmembrane movement of only very small amounts of intracellular enzymes. Detection of a significant amount of enzymes in the effluent indicates damage to the cell membrane, which usually occurs in severely damaged myocardium. It has been shown that there

is a close relation between enzyme leakage and degree of ischemic injury[154]. Creatine kinase and lactate dehydrogenase are the preferred marker enzymes[155]. Therefore, this parameter has been used to assess myocardial damage clinically and the effects of various cardioplegic solutions on cardiac preservation experimentally.

2.6.4. Coronary Flow

Coronary flow is determined by many factors, including mechanical function of the heart and endothelial function. Thus, it is an important index of myocardial viability. The dysfunction of endothelial cells will certainly result in a change in coronary flow, which will have some impact on myocardial metabolism and function. Ischemia-induced edema of the endothelium and of the tissue itself reduces coronary flow during reperfusion and is one of the mechanisms underlying reperfusion injury. Using isolated blood perfused lamb hearts, Aoki et al[156] found that the hearts preserved with a crystalloid cardioplegic solution at 2°C showed significantly poorer recovery in coronary flow and decreased response of coronary resistance to the endothelium-dependent vasodilator acetylcholine and correspondingly less recovery of contractile function, compared to the hearts stored at 4°C and 10°C. These findings led them to conclude that excessively cold cardioplegic solution caused endothelial dysfunction which reduced myocardial perfusion. As a result, recovery of contractile function during reperfusion was impaired.

The distribution of coronary flow is another parameter affected by factors such as solution temperature, heart function and perfusion pressure[157]. The myocardium in different parts of heart has varying susceptibility to ischemic injury and changes in coronary flow throughout the ventricular wall and in both ventricles may be different[158-159]. Consequently, the distribution of coronary flow during cardioplegic arrest and reperfusion must have a significant

impact on the myocardium. It has been shown that ventricular fibrillation results in unfavourable redistribution of coronary flow away from the subendocardium, resulting in ischemic injury to this part of the myocardium[160-161].

2.6.5. Electrical Activity

Ischemic injury can cause a shift in the ST segment, development of the Q wave and other portion of the electrocardiogram (ECG) particularly when recorded directly from the heart's surface[162-163]. Ischemic or reperfusion-induced arrhythmias can only be properly defined using ECG[163]. Therefore, ECG monitoring could provide sensitive and reliable data. On the other hand, ischemic and reperfusion injury can also induce changes in the resting potential and action potential which have been monitored for assessment of myocardial damage[39-40].

2.6.6. Morphology

In addition to the changes described above, ischemic and reperfusion injury also cause corresponding structural alterations evident on light and electron microscopic examination[164]. The early changes include tissue edema, mitochondrial swelling and widening of the T-tubules[165]. In severely damaged myocardium, the following structural alterations may be observed: distortion of Z bands, disruption of myofibrils, contracture, loss of staining density and ground substance and loss of glycogen granules[164]. Mitochondrial change may be progressive with loss or vesiculation of cristae and the appearance or disappearance of various granules (amorphous bodies and under some conditions calcium phosphate deposits). Changes in the cell membranes may also occur.

In conclusion, there are a number of experimental models and markers for the study of heart preservation. Differences clearly exist between models and markers. The

results obtained from one model or species may not be the same as those obtained using other models or species. Additionally although different markers all indicate some tissue impairment, they are not directly comparable. Therefore, any investigation with the potential to extrapolate to human use should be carried out in more than one species and with different models and markers.

2.7. **Nuclear Magnetic Resonance Spectroscopy**

2.7.1. Basic Concept

Some atomic nuclei possess a property corresponding to the classical mechanical concept of spin. A spinning charge will have a magnetic dipole moment (Fig.1). In the absence of an external magnetic field, these magnetic dipole moments are randomly oriented and there is no net magnetization (Fig. 2). However, in the presence of a strong external magnetic field, the spinning nuclei will precess about the external magnetic field's axis, either aligning with (Ground state) or against the field (Excited state) (Fig. 3). There is a relatively small difference in the numbers of nuclei between the two states, with a small excess in the ground state. Therefore, a small net magnetization occurs. When a radiofrequency pulse is applied, the small magnetization will be tilted away from the external magnetic field if the pulse frequency corresponds to the frequency of nuclear precession, called the Larmor precession frequency. A 90° radiofrequency pulse tilts the magnetization into the X-Y plane. This flipped magnetization, rotating in the X-Y plane, will result in a radiofrequency signal in the receiving coil (Fig. 4). The signal intensity will decrease as the magnetization returns to the ground state when the pulse ceases. The decreasing signal intensity as a function of time is called free induction decay (FID). Through the mathematical process of Fourier transformation, FID can be converted to signal intensity as a function of frequency. The latter is what we

usually call a nuclear magnetic resonance spectrum (Fig. 4). The integral of the signal is directly related to the number of spinning nuclei in the NMR probe volume.

If all nuclei resonated at the same frequency, NMR spectroscopy would be uninformative. However, the magnetic field experienced by individual nuclei is attenuated by the small magnetic field generated by the surrounding cloud of electrons. Because the micro-environment for each nucleus differs slightly, nuclei resonate at different frequencies according to the effective magnetic field they experience. This is called the chemical shift. Since the chemical shift of inorganic phosphate is also influenced by intracellular pH due to the different chemical shifts for HPO_4^{2-} and $H_2PO_4^-$, this property is useful to estimate intracellular pH.

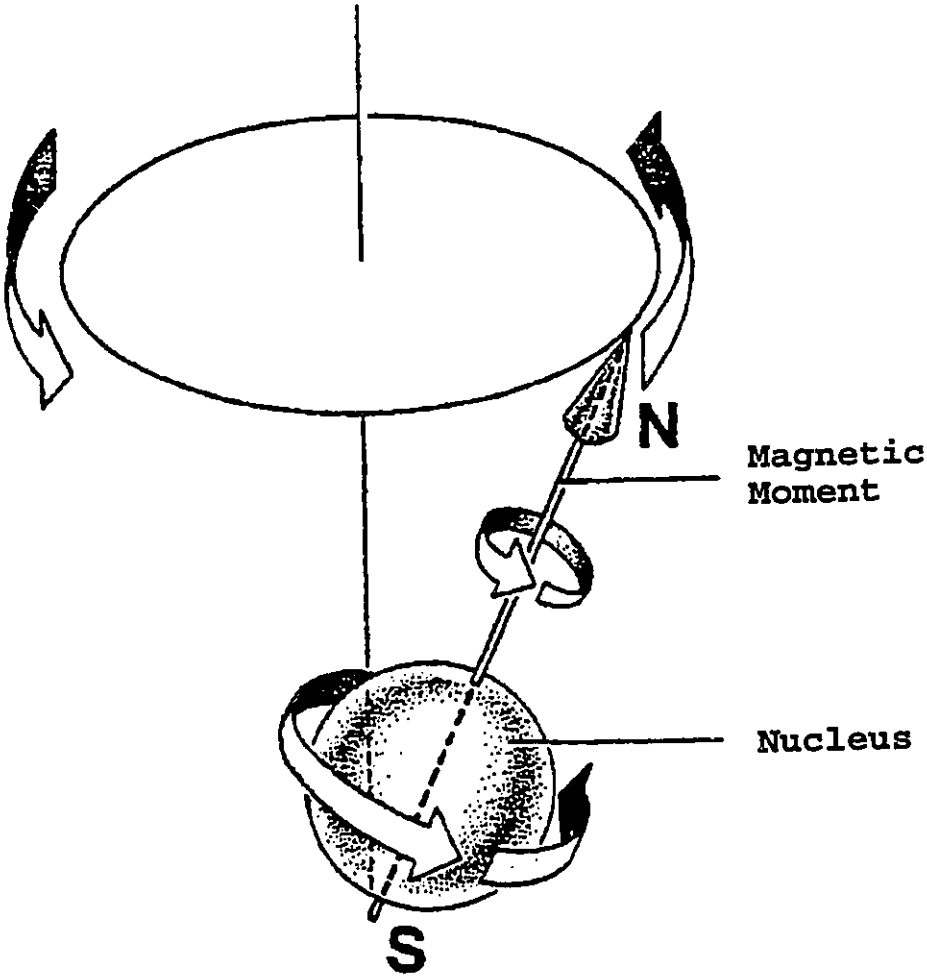


Figure 1. A spinning nucleus precesses about an axis. The spinning nucleus results in a magnetic dipole moment. (Adapted from Harms SE, 1984)

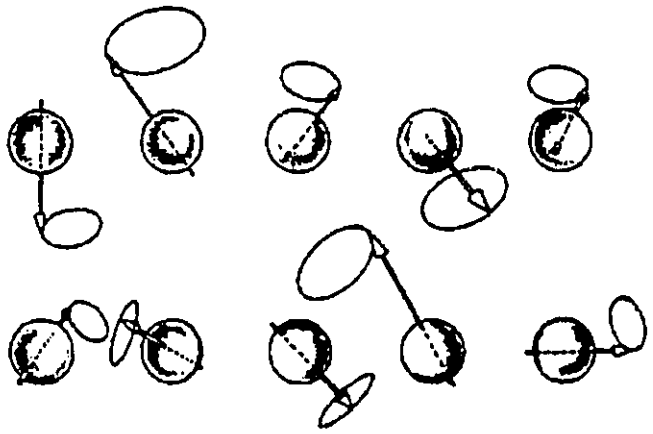


Figure 2. The random orientation of spinning nuclei (i.e. magnetic dipole moments) in the absence of an external magnetic field. (Adapted from Harms SE, 1984)

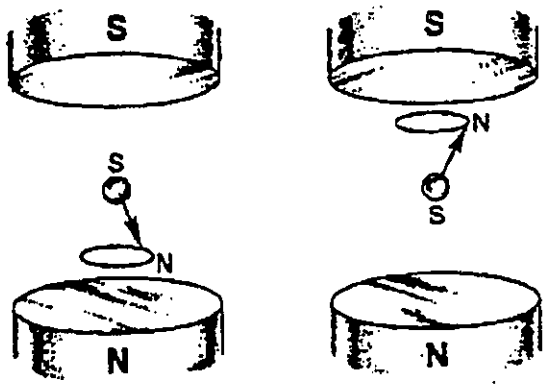


Figure 3. Orientation of the magnetic dipole moments in the presence of an external magnetic field, either aligning with (Ground state) or opposing (Excited state) the applied magnetic field (Adapted from Harms SE, 1984).

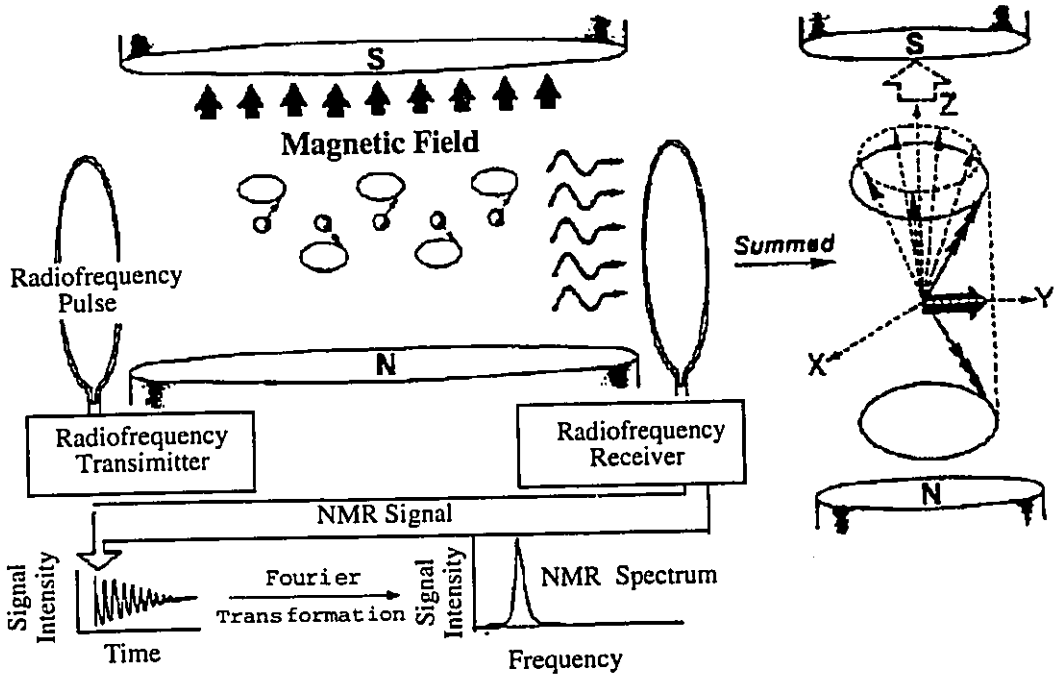


Figure 4. The basic principle of NMR spectroscopy. The radiofrequency pulse generated from the transmitter tips the small magnetization and the receiving coil will detect the magnetization rotating in the X-Y plane. Using Fourier transformation, the signal intensity as a function of time is converted to signal intensity vs frequency. (Adapted from Harms SE, 1984)

2.7.2. Advantages and Disadvantages of NMR Spectroscopy

Because the energy used in NMR is very low and many NMR sensitive nuclei, such ^{1}H, ^{31}P and ^{23}Na, are ubiquitous, NMR technique is non-invasive and non-destructive[33,166]. This makes NMR ideal for serial studies on a single heart. The heart can serve as its own control and the changes in intracellular energy metabolites, enzyme kinetics, ionic gradients and intracellular pH can be quantitatively and repetitively followed throughout an experiment without the need to take tissue samples while physiological parameters, such as cardiac contractile function, myocardial oxygen consumption and coronary flow are measured[167-168]. Therefore, NMR spectroscopy has become of enormous importance in cardiovascular research and its use has led to dramatic progress in studies of cardioplegic solutions, in preservation as well as ischemic and reperfusion injury.

Although NMR has many advantages, it has one serious disadvantage: it is relatively insensitive due to the low energy used in NMR and the significant amount of noise from biological samples (Fig 5)[168-169]. Ideally, NMR samples should be uniform in their magnetic susceptibility, geometry, stationary and be totally enclosed to shield against outside interference. However, the heart does not meet any of these requirements and as a result, obtaining a NMR spectrum from a isolated beating heart often requires a long acquisition time[167]. This reduces the temporal resolution which is very important in some situations. When a relatively short pulse delay is used to increase temporal resolution, the flipped magnetization may not have enough time to fully recover (T_1 relaxation) prior to a subsequent pulse, the next resulting magnetization in the X-Y plane will be smaller than the previous one. This phenomenon is called partial saturation of the NMR signal in a T_1-dependent manner. Temporal resolution can also be improved by averaging fewer FID's, at the expense of a lower signal to noise ratio. Poor spatial localization is another

substantial pitfall of NMR spectroscopy, although there are some complex, time-consuming techniques available currently (such as surface coil and image-selected *in vivo* spectroscopy) which can help define the NMR signal spatially[168]. This disadvantage has particularly important impact on cardiac research because the myocardium in various parts of the heart may differ in blood supply, susceptibility to ischemia and response to treatment. For ^{31}P NMR spectroscopy, the overlap of 2,3-diphosphoglycerate (2,3-DPG) in blood and inorganic phosphate (Pi) peaks further hinders accurate determination of myocardial Pi and pH when the heart is perfused with blood[170]. Moreover, as it is difficult to quantify absolutely the compounds of interest, the addition of absolute measurement of these compounds (such as using High Performance Liquid Chromtography, HPLC) is usually required to calibrate the signal.

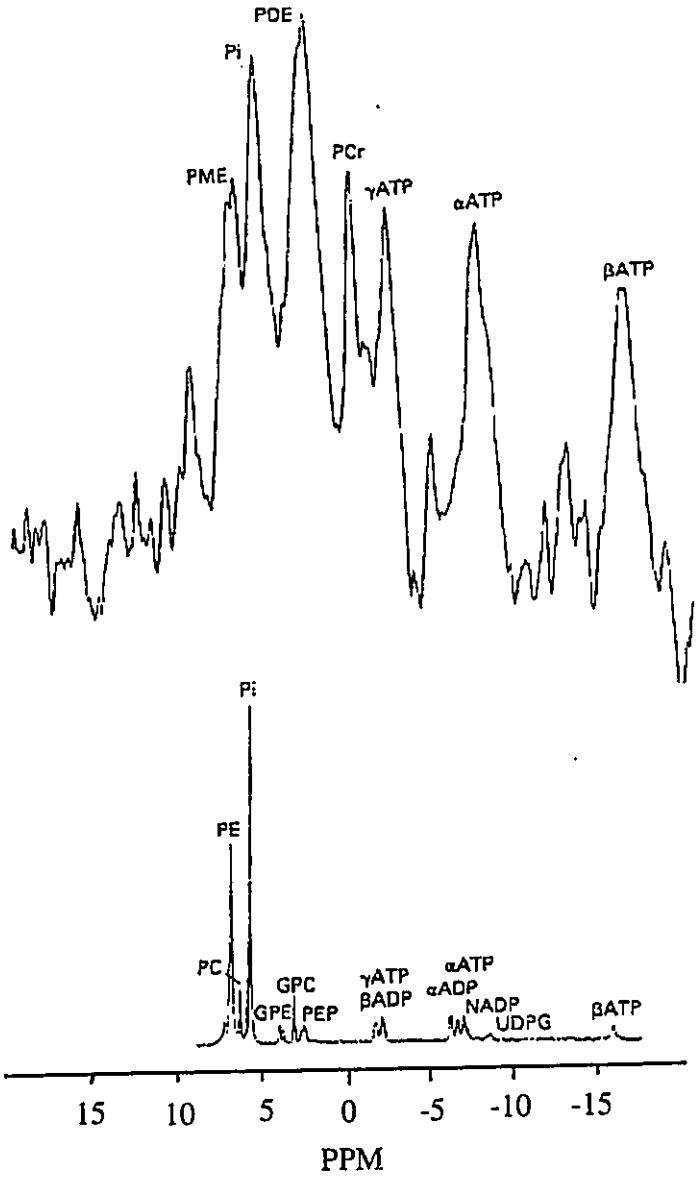


Figure 5. Comparison of spectra obtained from a patient with breast cancer (Top) and from a perchloric acid extract of a sample of breast cancer from the same patient (Bottom). PE = phosphoethanolamine, PC = phosphocholine, GPE = glycerophethanolamine, GPC = glycerophosphocholine (Reproduced from R Kalra, 1993).

2.7.3. NMR Study of Cardiac Preservation

Cardiac preservation is an area where NMR spectroscopy has made a substantial contribution because high energy phosphates, intracellular pH and ions can be readily followed throughout the experiment by NMR spectroscopy without interrupting the metabolism of the hearts and function[166]. Using ^{31}P NMR spectroscopy in the rat heart, Karck et al[171] found that adenosine triphosphate depletion during hypothermic preservation was slower in the hearts stored at 4°C than in those stored at 15°C. The fall in intracellular pH during preservation was also significantly less pronounced in hearts preserved at 4°C as compared with those at 15°C. The postischemic recovery of both the left ventricular peak systolic pressure and the maximum rate of increase of left ventricular pressure (+dp/dt) was enhanced in hearts stored at 4°C, suggesting that hypothermia at this low temperature appears to be preferable over a higher preservation temperature (15°C) for prolonged protection with respect to ATP preservation, prevention of intracellular acidosis and functional recovery.

In a study of the effects of an intracellular-type cardioplegic solution on cardiac preservation, Horska et al[172], using isolated dog hearts, found that ATP and phosphocreatine degraded more slowly in the hearts stored at 9°C with Bretschneider solution than with St Thomas' solution #2. PCr depletion occurred within 7.9 hours in hearts stored in Bretschneider solution and within 6.2 hours in hearts stored in St Thomas' solution #2. Interestingly, they also found that the intracellular pH decreased more rapidly in the hearts stored in Bretschneider solution than in those stored in St Thomas' solution #2 although the former solution contains a higher concentration of buffer than does St Thomas' solution #2. This observation probably resulted from the low concentration of sodium in Bretschneider solution. The findings led to the conclusion

that, although an intracellular-type cardioplegic solution may help maintain high energy phosphates, its sodium concentration should be increased.

3. Objectives

Heart preservation is considered a very important step in heart transplantation. Although currently used techniques have provided reasonable protection to the ischemic donor heart, these techniques of cardiac preservation still need to be improved to reduce myocardial injury and prolong the safe preservation time. In order to do this, we used ^{31}P NMR spectroscopy to study myocardial metabolism and mechanical performance in isolated rat and pig hearts subjected to various conditions of cardiac preservation. The work may provide some information on the physiological impact of various techniques and may help suggest better methods for heart preservation. The specific aims of the present study were as follows:

(1). To monitor the changes in contractile function, energy metabolites and intracellular pH when the heart is subjected to various conditions of preservation and postischemic treatments.

(2). To determine conditions for improved cardiac preservation.

4. Statement of the Questions

The techniques for cardiac preservation cover broad topics and involve a number of parameters, such as components, dose and temperature of cardioplegic and reperfusion solutions[1-2]. It would be an immense task to undertake detailed studies of all combinations of these parameters. Consequently, the present work has addressed specific points of practical importance in the preservation

process using selected parameters that were expected to have an impact from our current knowledge. These include

A. The effect of re-arrest perfusion on postischemic myocardial recovery.
B. The effect of buffer capacity in a cardioplegic solution on protection of the ischemic myocardium.
C. The role of high concentration of magnesium in cardioplegic and reperfusion solution on heart preservation and postischemic recovery.
D. The value of an intracellular-type cardioplegic solution for heart preservation

4.1. **The Effect of Re-arrest Perfusion on Postischemic Myocardial Recovery.**

It is well known that the fate of the post-ischemic myocardium is not solely determined by the events occurring during ischemia[3], but the conditions of reperfusion and the composition of the reperfusate influence substantially the its functional recovery[3-4]. Protective intervention at the time of reperfusion could reverse ischemic damage, reduce reperfusion injury and permit improved myocardial recovery[60-61,142,173]. Inappropriate reperfusion can cause myocardial functional abnormalities, such as water flux into hypertonic cells and organelles, intracellular calcium and sodium overload and aggravate breakdown of energy stores as well as lead to the generation of oxygen free radicals. Therefore, reperfusion techniques play an important role in postischemic recovery[60-61,142,173].

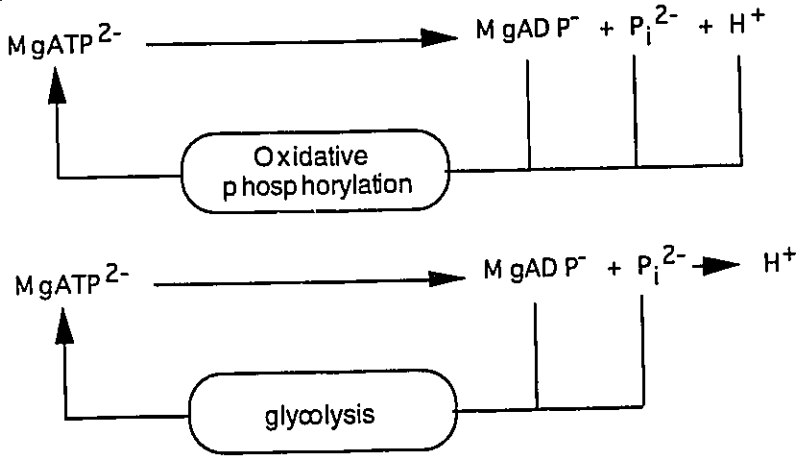
Following the decline in intracellular K^+ there is a progressive increase in intracellular Na^+ [65]. Since ΔG_{ATP} is normally 15-20 Kj/mol greater than the energy needed to drive the Na^+/K^+ pump[53], it does not normally limit pump kinetics. However, as ΔG_{ATP} falls the pump will become limited and may even reverse. Ischemia-induced acidosis would also be expected to reduce pump acitivty, leading to an increase in intracellular Na^+ [53]. In addition, it has

been shown that Ca^{++} pump in sarcoplasmic reticulum work close to thermodynamic equilibrium with ΔG_{ATP}[174-175]. A decrease in ΔG_{ATP} and intracellular pH will result in redistribution of Na^+, K^+ and Ca^{++} [53]. Accumulation of Na^+ and Ca^{++} in the myocardium plays a major role in reperfusion injury[57,62,118-119]. Therefore, it is reasonable to expect that reperfusion should first re-establish myocardial homeostasis, including Na^+ and Ca^{++} gradients, energy potential, intracellular pH as well as cell volume before imposing external work on the heart. One strategy to achieve this may be re-arrest perfusion, i.e. re-introduction of a specially designed cardioplegic solution (so called "secondary cardioplegic solution", S-C-S) for a short period in the early phase of reperfusion. This re-arrest perfusion may allow resumption of myocardial oxidative phosphorylation and work potential of ATP (ΔG_{ATP}) while no energy consumption by mechanical work is taking place. As a result, the resynthesized energy would be used exclusively for recovery of cellular integrity. Such S-C-S should of course contain an increased concentration of potassium to maintain diastolic cardiac arrest. In the present study 16 mmol/L K^+ was used. In addition to increasing the concentration of potassium, the calcium concentration in S-C-S should be reduced (compared to the physiologic extracellular calcium concentration) to minimize calcium influx and to prompt calcium efflux[60-61,142]. We used 0.7 mmol/L Ca^{++} in S-C-S. Additional buffer (30 mmol/L MOPS) was added to S-C-S (relative to Krebs-Henseleit solution) in an attempt to bring the intracellular pH back to a more physiological level (6.9-7.1) to allow cellular homeostatic mechanisms and metabolism to proceed under optimal conditions. Another component of S-C-S is hypertonicity to reduce ischemia-induced cell swelling and to restore normal cellular volumes. It was first intended to determine whether this specially designed S-C-S improved

myocardial recovery after long-term hypothermic preservation.

4.2. **The Effect of Buffer Capacity in a Cardioplegic Solution on Protection of the Ischemic Myocardium.**

During ischemia, myocardial energy production is largely dependent on anaerobic glycolysis rather than aerobic oxidation of substrates[176]. The decrease in mitochondrial ATP production in relation to glycolytic ATP formation results in metabolic generation of protons[5], represented schematically by:



Some protons might also be generated by an increased turnover of glycogen and calcium accumulation intracellularly[5]. Because proton buffering in the extracellular space is limited, outward transmembrane proton movement will be inhibited during ischemia, leading to proton accumulation inside the myocyte and severe acidosis. The decrease in intracellular pH inhibits the activity of the rate-limiting enzymes of anaerobic glycolysis such as phosphofructokinase (PFK)[6]. The limitation of ATP production by the anaerobic path will inhibit activity of Na^+/K^+-ATPase, leading to an increase in intracellular sodium and cell swelling as well as to an increase of intracellular calcium due to the reversal of Na^+/Ca^{++} exchange and impairment of the Ca^{++} pump[37]. On the other hand, the accumulation of protons itself induces sodium and

calcium overload during reperfusion via acceleration of the Na^+/H^+ exchange[67]. The increase in intracellular protons, sodium and calcium will shift the membrane potential to a more positive level than the normal K^+ equilibrium potential and may cause slow loss of intracellular K^+ [39-42]. Accumulation of proton and lactate could cause the damage of lysosomal membrane and release of the acid hydrolases into the cytoplasm, and activation of the enzymes leads to proteolysis[177]. Increase in intracellular Ca^{++} could result in the damage on sarcolemmal membrane[177]. On the other hand, increase of intracellular Ca^{++} accelerates its transport across mitochondrial membrane[128,177], which requires considerable energy. As a consequence, cellular energy depletion is enhanced[177]. In addition, increase of intracellular Ca^{++} causes the damage of mitochondrial membrane by activating phospholipase[128,177], and it could also cause calcium precipitation in mitochondria[177]. Thus, these abnormalities of ionic gradients will result in sub-cellular organelle injury[62]. Thus, the decrease of intracellular pH during ischemia plays an important role in ischemic injury. It would be expected that the attenuation of acidosis during ischemic preservation may reduce ischemic injury and increase myocardial tolerance to ischemia and ultimately may extend the safe preservation time of the hearts destined for transplantation[15].

Myocardial buffer can be divided compartmently into three components[5]: intracellular, interstitial and the blood buffers. For the preserved donor heart, proton can be buffered only by former two components[5]. It is well known that proton released from ATP hydrolysis during ischemia is first buffered by intracellular protein histidine residues and by inorganic phosphate (HPO_4^{2-}) because intracellular buffer has much greater capacity than that of expracellular buffer[5]. Afterwards, some of the protons buffered by HPO_4^{2-} will move out of the cell via Na^+-$H_2PO_4^{2-}$ symport[5]. Unbuffered intracellular protons may also leave the cell

through Na^+/H^+ exchange and H^+-lactate$^-$ co-transport[5]. Since buffer capacity in extracellular space is very limit, these proton transporting systems will soon be inactivated, resulting in accumulation of protons in both intra- and extracellular space[5]. Therefore, increasing buffer capacity in extracellular space may be expected to promote these proton transporting systems and reduce the fall of intracellular pH while allowing increased glycolytic flux and attenuating the detrimental effects of intracellular acidosis. This may help maintain ATP and ΔG_{ATP} at higher levels for a relatively longer ischemic period. The efflux of lactate through a proton-linked transport mechanism will balance the osmotic gradient and reduce cell swelling. Increasing buffer capacity extracellularly may be achieved by increasing the buffer concentration in the cardioplegic solution. Thus, the second purpose of this study was to determine whether increasing the buffer capacity in cardioplegic solutions (high buffer cardioplegic solution) could attenuate the pH decrease during ischemia and improve myocardial postischemic recovery. The organic buffer morpholino propane sulfonic acid (MOPS) appears to be a good candidate for this purpose since it has a pK (7.2, at 20°C, 0.1 mole ionic strength) close to the intracellular pH and a low temperature coefficient (-0.011). This would give it a significantly higher buffer capacity than histidine (which has been used in cardioplegic solutions by other investigators) over the desired extracellular range of 7.4-6.8[178]. In order to achieve the maximum buffer capacity without a significant increase in osmolarity, 150 mmol/L MOPS was used while NaCl was replaced by 92 mmol/L NaOH to neutralize MOPS. In consideration of the events occurring during ischemic preservation, the concentration of calcium in high buffer cardioplegic solutions should be lowered relative to physiological conditions to reduce Ca^{++} influx to a minimum. Thus, 0.7 mmol/L Ca^{++} was used in the high buffer cardioplegic solution.

4.3. The Role of High Concentration of Magnesium in Cardioplegic and Reperfusion Solutions on Heart Preservation and Postischemic Myocardial Recovery.

For about two decades potassium has been widely used as a major component of cardioplegic solutions for heart preservation and open heart surgery, even though it is known that it may impose some side effects on the ischemic myocardium, particularly at very high concentrations[15-16]. It has been shown that intracellular magnesium is involved in many cellular processes and plays an important role in the regulation of myocardial metabolism and mechanical function[8-9]. Using myocytes isolated from rat, rabbit and guinea pig hearts, Hall and co-workers[179] demonstrated that increasing the extracellular magnesium concentration (15 mmol/L) reduced myocardial excitability and conductivity as well as the inward calcium current. Therefore, it may be rational and possibly beneficial to add a high concentration of magnesium to potassium cardioplegic solutions to improve cardiac preservation. However, experimental and clinical results are conflicting, depending partially on the animal species and experimental conditions used. Using isolated rat hearts, Hearse[10] and Geffin[180] found that cardioplegic solutions with a high concentration of magnesium (15 mmol/L) attenuated the increase in intracellular calcium during arrest and improved postischemic recovery when compared to solutions containing little or no magnesium. Conversely, Engelman[181] and Takemoto[182] showed that cardioplegic solutions containing 0 or 16 mmol/L Mg^{++} provided similar degrees of cardiac protection. It should be noted that most of the comparisons were made under varying concentrations of Na^{+}, Ca^{++} and K^{+},[10,180-182]. The transmembrane gradients of these ions have a very important impact on myocardial function[55,63,183]. Changes in these ions in conjunction with variations of Mg^{++} may also mask the beneficial effect of

magnesium if it exists and will complicate the interpretation of results.

The incorporation of high concentrations of magnesium in cardioplegic solutions has not been universally accepted and at the present, it is not clear whether Mg^{++} plays a significant role in cardioplegic solutions[184]. Therefore, the third purpose of the present study was to determine whether a high concentration of Mg^{++} (16 mmol/L) in potassium cardioplegic solutions will reduce myocardial ischemic injury and improve postischemic recovery. In order to achieve this, we compared the energetic metabolites during 4 hours of hypothermic preservation and functional recovery during reperfusion in hearts stored in St Thomas' solution #2 containing either 0 or 16 mmol/L Mg^{++} The concentrations of Na^{+}, Ca^{++} and K^{+} in both solutions were kept identical in order to determine unequivocally the effects of the high concentration of magnesium. St Thomas' solution was used because it is being applied worldwide for heart preservation.

A large proportion of magnesium in cells is combined with ATP and membrane proteins[185-186]. The concentration of intracellular free Mg^{++} can be expected to increase during myocardial ischemia due to hydrolysis of ATP[187-188]. The increased intracellular free Mg^{++} may facilitate Mg^{++} efflux from the cell and recovery of cellular metabolism and function may be inhibited by the reduced concentration of intracellular Mg^{++} during subsequent reperfusion[12,185]. In addition, extracellular Mg^{++} can reduce calcium influx by competitively binding to calcium channels[9]. Increasing the extracellular concentration of Mg^{++} during reperfusion may also be beneficial to postischemic myocardial recovery by preventing the loss of intracellular magnesium and the calcium overload. It has been reported by several laboratories that a relatively higher concentration of magnesium (15 mM) in the early period of reperfusion improved myocardial recovery[11,189-190]. However, it was

found that this protective effect of high Mg^{++} concentration applies to the rat, but not to the rabbit heart[11], suggesting that the effect of Mg^{++} is highly species-dependent and the protective effect of increased magnesium during reperfusion may result mainly from decreased calcium influx since in rat myocardium, Ca^{++} entry during action potential would occur only via Ca^{++} channels; whereas in rabbit heart, the Ca^{++} entry is through Na^{+}/Ca^{++} exchange as well as Ca^{++} channels[191]. It is not known whether Mg^{++} decreases Ca^{++} influx in other species. Furthermore, it has been demonstrated that the cell membrane has very low permeability to Mg^{++} [192-195]. These findings strongly support the view that any beneficial effect of increased Mg^{++} concentration in the reperfusate may occur by reducing the Ca^{++} influx at the outer surface of the membrane rather than from a change in the intracellular free Mg^{++} concentration or prevention of Mg^{++} loss from the cell. Accordingly, in the presence of high concentrations of potassium, such as in S-C-S, Mg^{++} in the reperfusate may not be particularly significant because the increased extracellular potassium concentration can keep the Ca^{++} channels closed through membrane depolarization. The protective effect of a high Mg^{++} concentration may also be achieved by increasing the concentration of potassium. The present study was undertaken to determine whether increased $[Mg^{++}]$ in the presence of a high concentration of potassium during early reperfusion could improve postischemic recovery. To achieve this, hearts were subjected to a short period of reperfusion with S-C-S containing either 0 mmol/L or 16 mmol/L Mg^{++} following 4 hours of hypothermic preservation. To eliminate the possibility that other components in S-C-S conceal the beneficial effect of Mg^{++}, another group of hearts were reperfused with K-H solution containing 16 mmol/L K^{+}.

4.4. **The Value of an Intracellular-Type Cardioplegic Solution for Heart Preservation.**

Cardioplegic solutions are generally divided into two categories according to their formulation. Those similar in ionic composition to the cytosol are called intracellular-type while those similar to the extracellular medium are called extracellular-type[15-16]. These two types of solutions differ mainly in the concentrations of sodium, potassium and calcium. Intracellular-type cardioplegic solutions contain little or no sodium and calcium with very high concentration of potassium. The advantage of this type of solution is that energy consumption for sodium and calcium pumps will be decreased because the gradients are reduced significantly. Furthermore, low concentrations of sodium and calcium ions will help prevent their overload during ischemic preservation[56,62,118,196-198]. Since the accumulation of sodium and calcium plays an important role in myocardial ischemic injury and reperfusion injury, alleviating the Na^+ and Ca^{++} overload would be expected to reduce ischemic injury and improve recovery[4,100]. In the present work we investigated whether intracellular-type cardioplegic solutions confer superior protection for long-term cardiac preservation by comparing the changes in energy metabolites and functional recovery during preservation and reperfusion between the hearts stored in St Thomas' solution #2 and University of Wisconsin (UW) solution. St Thomas' solution #2 was used as the reference extracellular-type cardioplegic solution. UW solution was chosen for the following reasons: 1) In addition to its intracellular formulation, UW solution also contains many other putative protective components, such as osmotically active impermeants, lactobionate (MW, 358) and raffinose (MW, 594), to suppress hypothermically induced cell swelling; the basement membrane interstice blocker hydroxyethyl starch to prevent expansion of interstitial space; allopurinol to suppress the generation of superoxide radicals; glutathione

to chemically reduce cytotoxic oxidants and to suppress lipid peroxidation; adenosine to facilitate regeneration of ATP. 2) It has been demonstrated that UW solution significantly prolongs the safe preservation time for liver and kidney. 3) Experimental studies on heart preservation using the UW solution have produced controversial results. Some showed the solution to confer superior protection, others have shown similar or worse protection when compared with extracellular-type solutions[199-200]. However, it is noteworthy that because the composition of the solutions were not standardized, their comparison is of limited value.

After a short period of Ca^{++} depletion of the extracellular space, calcium replenishment in the myocardium may cause myocardial hypercontracture, massive release of cellular constituents, and finally cell death. This phenomenon is called the "calcium paradox"[63]. The crucial event in the calcium paradox is a period of calcium-free perfusion. There is a potential hazard of calcium paradox when an intracellular-type cardioplegic solution is used to arrest the heart, particularly when it is used for normothermic myocardial protection during open heart surgery as it is difficult to control heart temperature. It has been shown that 0.5 mmol/L Ca^{++} in solution can prevent the calcium paradox. Therefore, the experiments were performed to test whether adding Ca^{++} to UW solution would improve heart preservation.

MATERIALS AND METHODS

1. Animals

Domestic pigs and Sprague-Dawley rats were used in the present study. All animals used in this study received humane care in compliance with the "Guide to the Care and Use of Experimental Animals" (1st edition) formulated by the Canadian Council on Animal Care and the protocols were approved by the National Research Council of Canada Animal Care Committee.

1.1. Isolated Pig Heart Preparation

The pig heart was chosen as the experimental model because its major coronary arterial distribution, collateral circulation, enzyme spectrum and conduction system are similar to those of the human heart[201-203]. Anesthesia was induced in domestic pigs of either sex, weighing 10-30 kg, by intramuscular injection of diazepam (1 mg/kg body wt) and ketamine (25 mg/kg body wt). Halothane, 3% initially, was given by mask and maintained at 1% following tracheotomy. Respiration was maintained using a Penlon AV500 ventilator with a 1:1 mixture of nitrous oxide and oxygen. The rate and volume of positive pressure ventilation were adjusted to keep the arterial blood gases within the normal physiological range.

The brachiocephalic artery was cannulated at the level of the common carotid artery for arterial pressure monitoring, blood sampling and infusion of cardioplegic solution to arrest the heart. A sternotomy was performed. The brachiocephalic and subclavian arteries were dissected. The pericardium was opened longitudinally along the midline and the incision was extended to the right and left in cruciform fashion. The ascending aorta and the main pulmonary artery were isolated by threading umbilical tape around the origin of the descending aorta. Anticoagulation was provided by injection of heparin (3000 IU) into the

superior vena cava. A cannula was inserted centrally in the brachiocephalic artery. The brachiocephalic artery, subclavian artery, superior and inferior venae cavae were then clamped in succession. The descending aorta was clamped, and heparinized cold cardioplegic solution at the designated temperature (either 4°C or 12°C) was infused (10 mL/kg body wt) into the aortic root via the brachiocephalic arterial cannula. The right and left atria were cut to allow drainage of the cardioplegic solution and to prevent the warm blood in the lungs from returning to the heart. Cold saline (4°C or 12°C according to the protocol) was used for topical hypothermia in the chest cavity.

The heart was excised and immersed in cardioplegic solution containing heparin (1000 IU/100 mL). The brachiocephalic artery was joined to a cannula to be connected to the Langendorff perfusion apparatus. A thermocouple was placed in the right ventricle to monitor temperature throughout the experiment. A latex balloon was fixed in the left ventricle with a purse-string suture placed in the mitral valve and tied around the balloon mounting plug in order to measure left ventricular pressure and control preload during the functional recovery tests after reperfusion. Accumulation of blood in the ventricles from Thebesian flow and coronary sinus flow was prevented by use of a small length of polyethylene tubing pierced through the apex of the left ventricle and the free wall of right ventricle. A small glass ball filled with phenyl phosphonic acid (PPA) was inserted into the right ventricle as a reference for the ^{31}P NMR signal intensities.

The Langendorff perfusion apparatus was built with non-magnetic materials to avoid interference with the NMR apparatus.

For the experiments in which hearts were perfused with a mixture of blood and Krebs-Henseleit solution, the technique to establish the isolated heart preparation was somewhat different from the one described above. Both

carotid arteries were cannulated for monitoring aortic pressure (left) and for heart perfusion (right). The right atrium was cannulated for blood drainage to prime the perfusion system. The perfusion system was filled with heparinized K-H solution equal in amount to the volume of blood expected from the weight of the animal (approximate 5% of body weight). After clamping both subclavian arteries, blood was withdrawn using a cannula placed in the right atrial appendage. The aorta was slowly ligated once the aortic systolic pressure fell below 60-70 mmHg. Extracorporeal perfusion of the heart with the mixture of blood and K-H solution was started only after the aortic systolic pressure dropped to 80 mmHg. The flow of perfusate was adjusted to maintain an aortic systolic pressure at about 90 mmHg. The heart was then removed and placed in the NMR probe while being continuously perfused. The major advantage of this procedure is that there is no significant interruption of perfusion during the preparation period.

1.2. **Isolated Rat Heart Preparation**

Hearts were obtained from 250-400g male rats. Animals were anesthetized with an injection of sodium pentobarbital intraperitoneally (65 mg/kg). The hearts were rapidly excised and placed in iced K-H solution. The hearts were then mounted on a Langendorff perfusion apparatus and perfused with K-H solution through the root of aorta from a reservoir 100 cm above the heart for 10 minutes to wash out all blood and to allow the heart to recover from the period of ischemia. During this Langendorff perfusion period, the left atrium was cannulated through a pulmonary vein. A working heart preparation was then established by clamping the tubing from the Langendorff reservoir and perfusing the heart through the left atrium at a pressure of 20 cm H_2O. The left ventricle ejected against a pressure of 70 cm H_2O into a compliance chamber containing 3 mL air connected to the aortic stump. Aortic and coronary flows were measured

by timed volumetric collection of the solution draining from the aortic tubing and the heart, respectively. The reservoirs, tubing of the perfusion apparatus and a chamber surrounding the heart were water-jacketed for temperature control and maintained at 37°C.

2. Perfusion Solutions

In most experiments, the hearts were perfused with K-H solution (Table 1) at a perfusion pressure of 80 or 100 mmHg. Twenty g of Dextran (SIGMA, St Louis, MO) and 5 g of bovine serum albumin (SIGMA, St Louis, MO) were added to 1 L of K-H solution to provide oncotic pressure and thereby reduce edema formation. The solution was bubbled vigorously with a 95% O_2 : 5% CO_2 gas mixture to a final pH of 7.4-7.5. The mixture of pig autogenous blood and K-H solution (1:1, v/v) was used to perfuse pig hearts in the study of effect of adding Ca^{++} to University of Wisconsin (UW) solution on myocardial preservation. Using this mixture to perfuse heart was to hope that it might minimize the unwanted changes of myocardium before infusion of UW solution as stated above. Because this technique was recently introduced to our laboratory from University of Toronto, the mixture of blood and K-H solution was used only in the last part of this study. All of cardioplegic solutions used in this study were not oxygenated.

3. Calculations

3.1. Myocardial Oxygen Consumption (MVO_2)[148]:

$$MVO_2(ml/min/100g) = \frac{pO_2(Aterial\text{-}Venous)}{760} \cdot 2.35 \cdot CF/Heart\ weight\ (g)$$

where CF is coronary flow (mL/min), 2.35 is coefficient for O_2 solubility (O_2 mL/100 mL/atm) at 37°C

3.2. **External Working Efficiency (EWE)**[148,204]:

$$EWE(\%) = \frac{\text{Energy Output (J/100g/min)}}{\text{Energy Input (J/100g/min)}} \bullet 100$$

where Energy Output (J/100g/min) = Cardiac Output (m^3/100g/min) • Average Aortic Pressure (N/m^2)

and energy Input (J/100g/min) = MVO_2 (mL/100g/min) • 20 (J/mL)

(J, N, m are joule, newton and meter, respectively).

4. **Assessment of Contractile Function**

For the pig heart preparation, heart rate (HR), left ventricular developed pressure (LVDP=left ventricular peak pressure minus end-diastolic pressure), and maximal rate of pressure increase (+dp/dt) and decrease (-dp/dt) were continuously recorded from a left ventricular balloon by means of a pressure transducer (Model P23XL, Spectramed Inc, Oxnard, CA) during control perfusion and reperfusion using an EEG & Polygraph Data Recording System (Grass Model 79E, Grass Instrument Co, Quincy, Mass). Contractile function was also assessed by calculating the product of developed pressure and heart rate, to give the rate-pressure product. Electrocardiograms were recorded with the same system to monitor ventricular fibrillation.

For the isolated working rat heart, myocardial mechanical performance was evaluated with cardiac output, external work and working efficiency.

5. **Nuclear Magnetic Resonance Spectroscopy**

^{31}P NMR spectroscopy was performed at 4.7 Tesla on a Bruker Biospec spectrometer with a 30 cm horizontal bore magnet operating at a phosphorus frequency of 81.03 MHz. Magnetic field homogeneity in the sample region was optimized by shimming on the sodium signal of the sample. Free induction decay (FID) signals were obtained using 4 K data points, 40-90° radio frequency pulses and a pulse

repetition time of 4-5 s. 80-120 FIDs were accumulated for each spectrum according to protocol. Thus, each NMR spectrum was acquired over an average over a 5-10 min sampling period according to the protocol. Accumulated free induction decays were exponentially multiplied, resulting in 20-30 Hz line broadening, to improve the signal-to-noise ratio.

The observed phosphorus compounds included Pi, PCr and three peaks of ATP (α, β and γ peaks). The β-peak was used for quantifying ATP. The integrals of the peak areas were measured after removal of the broad baseline component.

Since absolute quantification of metabolites observed by NMR in living tissue and a perfused organ is difficult, spectral data usually were expressed as the percentage of the initial ATP value which was set at 100 %. An alternative method of quantification involves the chemical determination (by HPLC) of high energy phosphates in tissue quickly frozen immediately after NMR measurements. The HPLC data is then correlated with NMR spectral areas measured at the end of experiments yielding "calibration" of the NMR signal. The intracellular pH was calculated from the chemical shift of Pi[33].

6. High Performance Liquid Chromtography

In order to calibrate the NMR signal and to obtain absolute values for high energy compounds, needle biopsy samples from both ventricles were taken at the end of ischemia or/and end of reperfusion, while hearts were still being perfused, for high performance liquid chromtography (HPLC). In all cases the samples were quickly dropped into liquid nitrogen. Samples were later freeze-dried and weighed. Nucleotides and phosphocreatine are stable at room temperature in dried samples. However, as a precaution, they were kept in a dessicator at -30°C. Before extraction, samples were ground in the presence of 10 mg of silica gel (100-200 mesh). The powder was extracted in an ice bath

with 150 ml of 7% perchloric acid. After 5 minutes of extraction, the samples were centrifuged and 10 ml of supernatant was transferred into an Eppendorff tube containing 100 ml of 1.0 mol/L KOH, 25 ml 1.0 mol/L K_3PO_4 and 10 mmole/L EDTA for neutralization. The final pH of the extract after centrifugation was 7.

HPLC assays of the extracts were performed with a Waters instrument equipped with an M680 controller, two M510 pumps, a U6K injector with a 500 ml injecting loop and a M900 photodiode array detector. Nucleotides were analyzed on a Supelco LC18 column, 5 mm particle size (4.6 mm x 25 cm) using reverse phase chromatography and a binary gradient. Measurements were taken at 260 nm. The separation was based on the method of Stocchi et al with minor modifications. Buffer A was composed of (mmole/L) 100 KH_2PO_4, 0.2 Na_4EDTA in 0.1% methanol, pH 5.6. Buffer B was composed of a mixture of buffer A and methanol (85:15). Gradient characteristics were as follows: 0% to 6% of buffer B in 10 minutes, followed by increasing buffer B to 100% after 30 minutes. The gradient was then returned to the initial condition in 3 minutes and re-equilibrated for 7 minutes. The flow rate was 0.9 mL/min. Quantification was carried out by comparing sample peak areas with those of a standard solution containing known nucleotide concentrations.

The analysis of creatine compounds was performed on fresh extracts using a Supelco LC18 column. The separation was performed using the ion-pairing technique in the isocratic mode, with a buffer composed of (mmol/L) 70 KH_2PO_4, 14 EDTA, 5 tetrabutylammonium dihydrogen phosphate in 0.1% methanol, pH 7.2. The flow was 0.9 ml/min, analysis time was 10 minutes, and the injection interval 20 minutes. Creatine and PCr were easily detected at 205 nm. Creatinine was only present in low concentrations and could be detected more specifically at 235 nm.

7. Protocols

7.1. Investigation of Re-arrest Perfusion Following Long-Term Heart Preservation

Pig hearts were divided into two groups, as summarized in Table 2. Both groups of hearts were arrested with St Thomas' solution #2 and then kept ischemic at 12°C for 8 hours. After ischemic preservation, the hearts in group 1 were reperfused with K-H solution for 75 minutes. The hearts were rewarmed to 37°C during first 15 minutes and the functional parameters were recorded during following 60 minutes. The hearts from group 2 were subjected to secondary cardioplegic solution (S-C-S) during 15 minutes of rewarming period and then perfused with K-H solution for 60 minutes at 37°C. NMR measurements were undertaken throughout the protocol.

TABLE 1. Solutions used to preserve and perfuse hearts (mmol/L).

	K-H	St Thomas	S-C-S
NaCl	118	100	90
KCl	3.5	26	16
$MgSO_4$	1.2		
Glucose	11		11
$NaHCO_3$	25	25	25
$CaCl_2$	1.75	1.2	0.7
EDTA	0.5		
KH_2PO_4	1.2		
BSA	0.5%		0.5%
Dextran	2%		2%
MOPS			30
NaOH			18.3
$MgCl_2$		16	16
Osmolality	295	320	338
pH	7.4	7.5	7.4

K-H, modified Krebs-Henseleit solution; St Thomas, St Thomas' solution #2; S-C-S, secondary cardioplegic solution; BSA, bovine serum albumin; MOPS, morpholino-propane-sulphonic acid.

TABLE 2. Groups and protocol for study of re-arrest perfusion

Group	Arrest	Preservation	Reperfusion
1	St Thomas	8 hrs, 12°C	K-H (75 mins)
2	St Thomas	8 hrs, 12°C	S-C-S(15 mins), K-H (60 mins)

St Thomas, St Thomas' solution #2; S-C-S, secondary cardioplegic solution; K-H, modified Krebs-Henseleit solution.

7.2. Study of Buffer Capacity in Cardioplegic Solution for Heart Preservation

In order to evaluate the effect of buffer capacity in cardioplegic solution on long-term cardiac preservation, we designed a special cardioplegic solution according to the principles described earlier (Introduction, 4.2). This cardioplegic solution contained 150 mmol/L MOPS (high buffer cardioplegic solution, Table 3). Pig hearts were divided into two groups. High buffer cardioplegic solution was used to arrest the hearts in group 1, whereas St Thomas' solution #2 was used to arrest the hearts from group 2, to serve as reference. The hearts in both groups then were placed in the NMR probe and subjected to 8 hours of ischemic preservation at 12°C. Reperfusion in both groups was performed with K-H solution at 37°C. Needle biopsy samples were taken at the end of reperfusion from both ventricles for the measurements of high energetic metabolites with HPLC. ^{31}P NMR spectroscopy was performed during preservation and reperfusion.

TABLE 3. Composition of the high buffer cardioplegic solution (mmol/L)

KCl	26
$CaCl_2$	0.7
MOPS	150
NaOH	92
$MgCl_2$	16
Osmolality	326
pH	7.5

MOPS, morpholino-propane-sulphonic acid.

7.3. **Determination of the Role of a High Concentration of Magnesium in Cardioplegic and Reperfusion Solutions**

7.3.1. The Effect of 16 mmol/L Mg^{++} in Cardioplegic Solution

I. *Rat Heart Experiment*

As mentioned earlier, most work on Mg^{++} in cardioplegic solutions has been performed with rat heart preparations and under conditions of varying ionic concentrations (Na^+, K^+, etc). Therefore, the working rat heart preparation was used to confirm the results obtained by other investigators. This work was done without NMR.

Following 30 minutes of control perfusion (control perfusion is usually refereed to the perfusion before ischemia) at 37°C in the working mode, the left atrial and the aortic tubings were clamped and the hearts were perfused with St Thomas' solution #2 containing either 0 (group 1, n=7) or 16 mmol/L Mg (group 2, n=7) for 2 minutes, and then subjected to 30 minutes of normothermic (37°C) ischemia (Table 4). Forty-eight mmole/L sucrose was used to compensate for the osmolarity changes caused by the removal of magnesium. After ischemia, the hearts were reperfused with K-H solution in the Langendorff mode for 5 minutes and the working mode was resumed for a further 30 minutes at 37°C. Aortic flow, coronary flow and oxygen partial pressure at the left atrial inflow and coronary effluent were measured at 10 minute intervals during the working period.

TABLE 4. Protocol for study of magnesium in cardioplegic solution with rat heart preparation

Group	1	2
Langendorff	5 minutes	5 minutes
Working	30 minutes	30 minutes
Arrest	0 mM Mg^{++} St Th	16 mM Mg^{++} St Th
Ischemia	37°C, 30 minutes	37°C, 30 minutes
Langendorff	5 minutes	5 minutes
Working	30 minutes	30 minutes

St Th, St Thomas' solution #2.

II. *Pig Heart Experiment*

Two experimental series were conducted. The first was intended to determine whether Mg^{++} has a negative inotropic effect on the pig myocardium since this has been considered as one of the mechanisms underlying myocardial protection by increased Mg^{++} concentration (Introduction, 4.3). Five hearts were perfused with K-H solution at 37°C in the Langendorff mode. After 20 minutes of perfusion, the magnesium concentration in the perfusing K-H solution was increased in stepwise increments from 1.2 mmol/L to 5 to 10 and to 16 mmol/L. Each level of magnesium concentration was maintained for 5 minutes. HR, LVDP, +dp/dt and -dp/dt were continuously recorded.

The second series of experiments was intended to evaluate the role of high concentration Mg^{++} in St Thomas' solution #2. Twelve pig hearts were divided into two groups (n=6 per group). Following 30 minutes of control perfusion at 37°C, hearts were rendered ischemic for 4 hours at 12°C, initiated by a single infusion of St Thomas' solution #2 (10 mL/kg body weight) containing either 0 (group 1) or 16 mmol/L Mg (group 2). After ischemia, the hearts were reperfused at 37°C with K-H solution for 75 minutes to assess the recovery of contractile function (Table 5). The levels of ATP, PCr and Pi were monitored throughout the protocol with ^{31}P NMR spectroscopy as described above. The functional parameters obtained immediately prior to the induction of ischemia were used as controls, while the highest values obtained during reperfusion were used to calculate the maximal recovery of contractile function.

TABLE 5. Protocol for studying the effect of magnesium in cardioplegic solution with a pig heart preparation

Group	Control	Arrest	Preservation	Reperfusion
1	K-H	0 mM Mg^{++} St Th	4 hrs,12°C	K-H
2	K-H	16 mM Mg^{++} St Th	4 hrs,12°C	K-H

St Th, St Thomas' solution #2; K-H, Krebs-Henseleit solution.

7.3.2. The Effect of 16 mmol/L Mg^{++} in Reperfusion Solution

After excision, the pig hearts were perfused for 30 minutes with K-H solution at 37°C in Langendorff mode. The hearts were then arrested with St. Thomas' solution (12°C, 10 mL/kg body weight) and kept ischemic at 12°C for 4 hours followed by reperfusion which comprised two periods: rewarming (20 min) and beating (30 min). During the rewarming period, the hearts in group 1 were perfused with K-H solution, while the hearts in groups 2 and 3 with S-C-S containing either 16 mmol/L or 0 mmol/L magnesium, respectively, and the hearts in groups 4 were reperfused with K-H containing 16 mM potassium. During the beating period of reperfusion, all hearts were reperfused with K-H solution and maintained at 37°C. The experimental protocol is summarized in table 6. Sucrose was used to compensate for the difference in osmolarity resulting from the removal of magnesium. The sodium concentration in the K-H solution was reduced by 10 mmol/L when the potassium concentration was increased from 4.7 to 16 mmol/L for rewarming perfusion in group 4.

TABLE 6. Groups and protocol to study the effect of magnesium in reperfusion solution

Group	Perfusion (30 min)	Ischemia (4 hrs, 12°C)	Reperfusion Rewarming	Reperfusion Beating
1	K-H	St Th	K-H	K-H
2	K-H	St Th	16 mM Mg^{++} S-C-S	K-H
3	K-H	St Th	0 mM Mg^{++} S-C-S	K-H
4	K-H	St Th	16 mM K^{+} K-H	K-H

K-H, Krebs-Henseleit solution; St Th, St Thomas' solution #2; S-C-S, secondary cardioplegic solution.

7.4. **Evaluation of an Intracellular-Type Cardioplegic Solution**

7.4.1. Comparison of University of Wisconsin Solution and St Thomas' Solution #2 for Heart Preservation

Twenty-eight pig hearts were divided into four groups (Table 7). The hearts from groups 1 and 3 were arrested with St Thomas' solution #2 and were then kept ischemic for 8 hours at 4°C and 12°C, respectively. The hearts in groups 2 and 4 were arrested with UW solution (Table 8) and stored for 8 hours at 4°C and 12°C, respectively. These two temperature points were used to determine the temperature dependency of preservation with UW solution. Following preservation, all hearts were reperfused with K-H solution at 37°C for 75 minutes. The recovery of cardiac performance was assessed by measuring the rate-pressure product (RPP) during the reperfusion period and from Starling curves constructed from measurement of left ventricular developed pressure (LVDP) and diastolic pressure. ^{31}P NMR spectra were obtained during preservation and reperfusion.

TABLE 7. Groups and protocol to study the effect of an intracellular-type cardioplegic solution

Group	Cardioplegia	Preservation	Reperfusion
1	St Th	4°C, 8 hrs	K-H, 75 mins
2	UW	4°C, 8 hrs	K-H, 75 mins
3	St Th	12°C, 8 hrs	K-H, 75 mins
4	UW	12°C, 8 hrs	K-H, 75 mins

St Th, St Thomas' solution #2; UW, University of Wisconsin solution; K-H, Krebs-Henseleit solution.

TABLE 8. Composition of the University of Wisconsin solution (mmol/L)

$MgSO_4$	5.0
Adenosine	5.0
Glutathione	3.0
Raffinose	30
KH_2PO_4	25
NaOH	20
Allopurinol	1.0
K Lactobionate	100
Pentastarch	5%
Insulin	40IU
Osmolarity	310mOsm
pH	7.4

7.4.2. The Effect of Adding Ca^{++} to University of Wisconsin Solution on Heart Preservation

To demonstrate whether adding Ca^{++} to UW solution improves cardiac preservation, fifteen pig hearts were subjected to 8 hours of ischemia at 12°C induced with either unmodified UW solution (n=8 in group 1) or Ca^{++}-containing UW solution (0.5 mmol/L $CaCl_2$, n=7 in group 2). After preservation, all hearts were reperfused with K-H at 37°C for 75 minutes. The left ventricular developed pressure was measured during reperfusion in order to assess the recovery of contractile function. The changes in high energy phosphates were followed throughout the protocol using ^{31}P NMR spectroscopy.

To investigate the effect of Ca^{++}-containing UW solution on diastolic pressure, another protocol was carried out in which thirteen pig hearts were subjected to 30 minutes of control perfusion at 37°C with a mixture of blood and K-H solution (1:1, V/V), followed by 30 minutes of ischemia at 12°C induced with either unmodified UW solution (n=5 in group 1) or Ca^{++}-containing UW solution (n=8 in group 2). After ischemia, the hearts were reperfused at 37°C with the blood/K-H solution for 75 minutes. Contractile function was monitored during control perfusion and reperfusion. Diastolic pressure in the left ventricle was monitored during ischemia because its change is closely related to the change in the intracellular calcium level in the condition of constant ventricular volume. Changes in high energy phosphates were continuously followed using ^{31}P NMR. The protocol is summarized in Table 9.

TABLE 9. Groups and protocols to study the effect of adding Ca^{++} to University of Wisconsin solution

Protocol A

Group	Ischema (8 hrs, 12°C)	Reperfusion
1	UW	K-H
2	Ca^{++}-UW	K-H

Protocol B

Group	perfusion	Ischemia (30 min, 12°C)	Reperfusion
1	Blood/K-H	UW	Blood/K-H
2	Blood/K-H	Ca^{++}-UW	Blood/K-H

UW, UW solution; Ca^{++}-UW, UW solution containing 0.5 mmol/L $CaCl_2$; K-H, Krebs-Henseleit solution; Blood/K-H, mixture of blood/Krebs-Henseleit solution in 1:1 (v/v) ratio.

8. Statistical Analysis

Statistical analyses were carried out using STATGRAPHIC version 5 (STSC, Inc., Rockville, MD). Most values are expressed as mean ± S.E. The precision with which peak areas in the ^{31}P NMR spectrum can be quantitated depends on the signal to noise ratio which in turn is a function of the number of FIDs added together to enhance coherent signal and reduce random noise. The comparison of NMR and functional data obtained during control perfusion, ischemic preservation and reperfusion was carried out by Analysis of Variance with repeated measures. The two-way Analysis of Variance was used to compare some functional parameters obtained under various heart rate and left ventricular end diastolic pressure. The Student's *t*-test (two tailed) was used to compare the high energy phosphates measured by HPLC at the end of reperfusion between the two groups. The incidence of ventricular fibrillation occurring upon reperfusion in the various groups was analyzed with a Chi Square analysis. A statistically significant difference was said to exist at a probability value of less than 0.05.

RESULTS

1. The Effect of Re-arrest Perfusion on Myocardial Energy Metabolites and Functional Recovery during Reperfusion

Figure 6 shows a representative ^{31}P NMR spectrum of an isolated pig heart perfused with K-H solution after equilibration for about 20 minutes. Using PCr as a reference (-2.52 ppm), chemical shift values for the phosphate resonances of the pig heart were [α-P]ATP, ~-10.05; [β-P]ATP, ~-18.54; [γ-P]ATP, ~-4.93; inorganic phosphate, ~+2.86 ppm. Cytosolic pH was estimated from the chemical shift of the inorganic phosphate peak, relative to the resonance of the PCr peak. Under our experimental conditions, the initial levels of energy metabolites could be maintained for about 2 hours with slow decline of mechanical performance (Fig. 7).

Hearts subjected to S-C-S following 8 hours of ischemic preservation remained quiescent. In contrast, hearts reperfused directly with K-H solution always showed reperfusion-induced ventricular fibrillation when the temperature of the heart approached 25°C (Table 10). The relationship of left ventricular developed pressure with reperfusion time, heart rate and left ventricular end diastolic pressure are shown in Figures 8, 9 and 10. The data demonstrate that postischemic re-arrest perfusion results in significantly improved mechanical performance, compared to the perfusion with K-H solution alone. However, this may be not related to any improvement in metabolic recovery, since there was no significant difference in ATP and PCr levels between the two groups of hearts (Fig. 11).

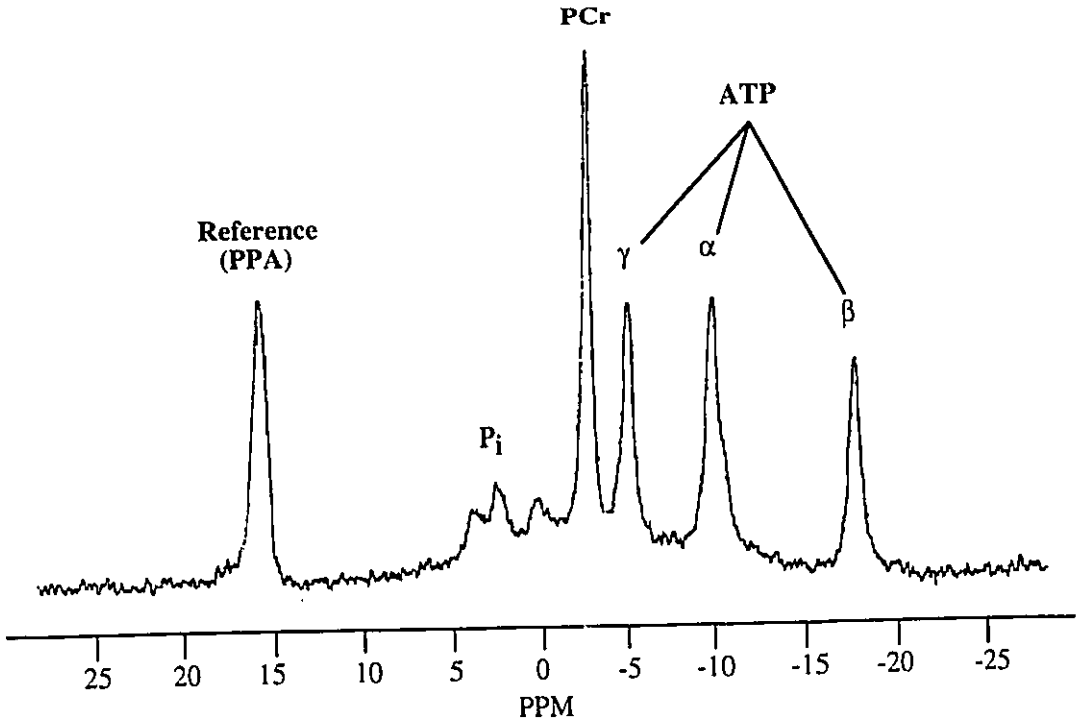


Figure 6. ^{31}P NMR spectrum obtained from an isolated K-H perfused Langendorff pig heart at 37°C.

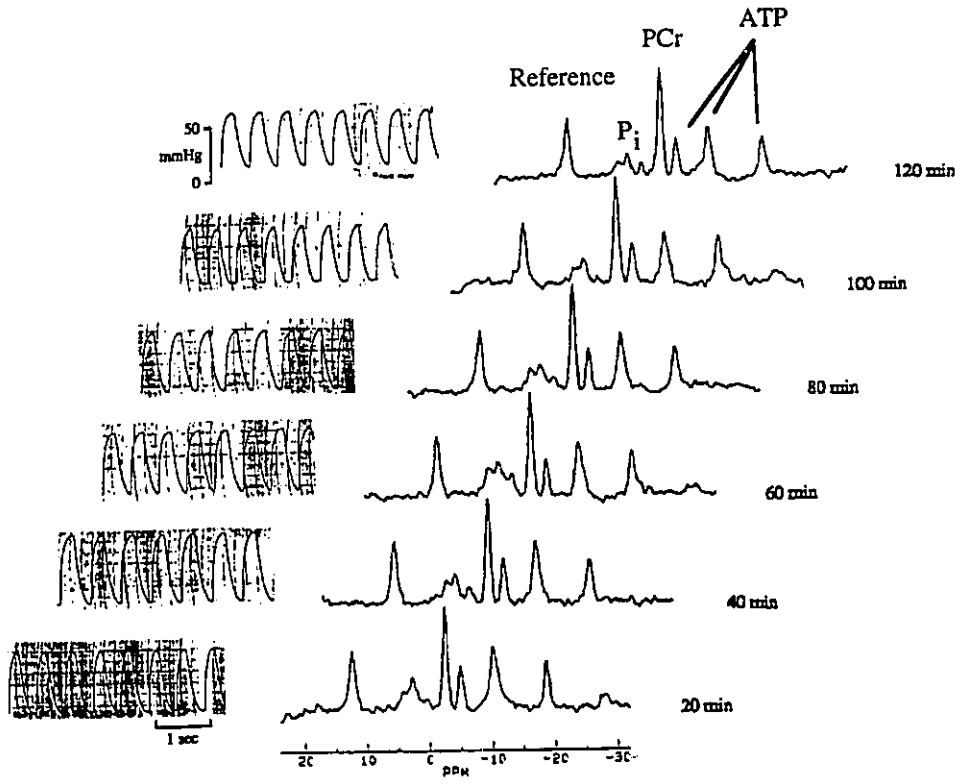


Figure 7. Representative spectra and left ventricular pressure records obtained periodically during 2 hours of control perfusion with K-H solution at 37°C in a pig heart.

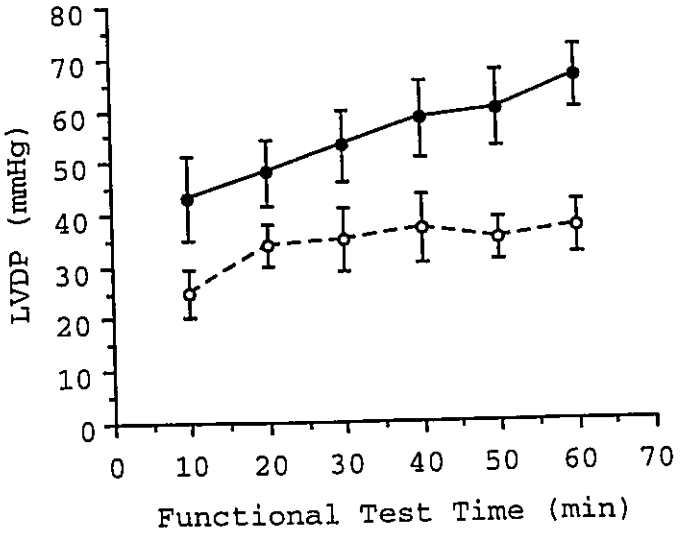


Figure 8. Comparison of left ventricular developed pressure (LVDP) during reperfusion between hearts reperfused with Krebs-Henseleit solution (open circles) and with secondary cardioplegic solution prior to K-H solution (solid circles). Functional test started at the 15 minute of reperfusion. Data are presented as mean ± S.E. The differences between two function curves were statistically significant ($p<0.05$).

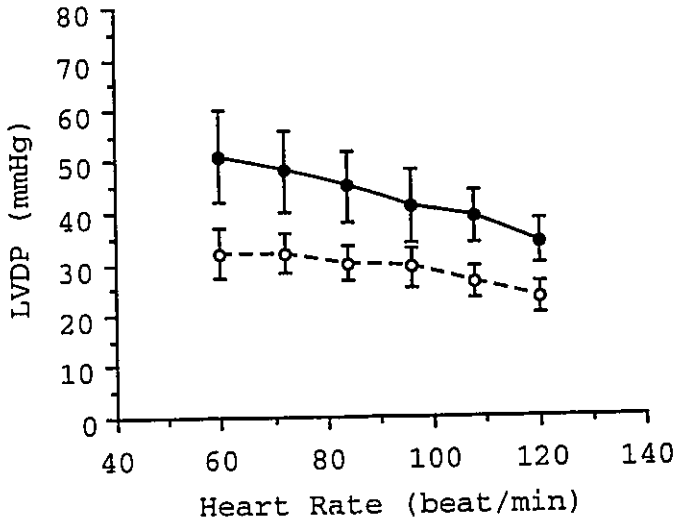


Figure 9. Comparison of left ventricular developed pressure (LVDP) obtained at various heart rates in hearts reperfused with K-H solution (open circles) and with S-C-S prior to K-H solution (solid circles, $p<0.05$). Functional test started at the 15 minute of reperfusion.

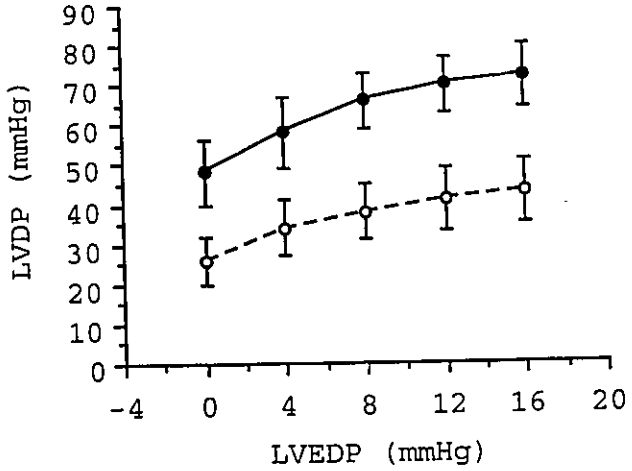


Figure 10. Comparison of left ventricular developed pressure (LVDP) obtained at various left ventricular end diastolic pressures (LVEDP) in hearts reperfused with K-H solution (open circles) or with S-C-S prior to K-H solution (solid circles). Data are presented as mean ± standard error ($p<0.05$).

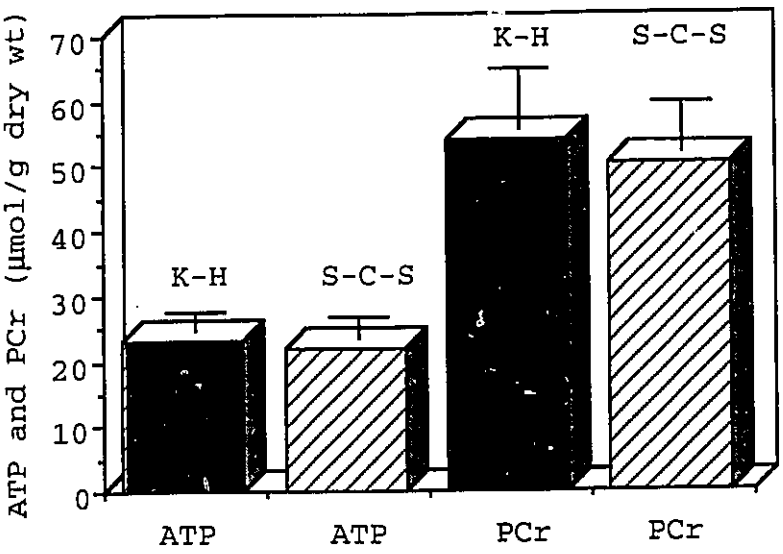


Figure 11. Concentration of ATP and PCr measured with HPLC at the end of reperfusion from hearts reperfused with Krebs-Henseleit solution alone (K-H) or with secondary cardioplegic solution (S-C-S) prior to K-H solution following 8 hours of ischemic preservation. There were no significant differences in these metabolites.

TABLE 10. Incidence of ventricular fibrillation upon reperfusion in hearts subjected to K-H solution alone or to S-C-S prior to K-H solution

Group	Protocol	VF Incidence
1	-S-C-S	4/4
2	+S-C-S	0/5

-S-C-S, no secondary cardioplegic solution; +S-C-S, secondary cardioplegic solution prior to Krebs-Henseleit solution; VF, ventricular fibrillation.

2. Energy Metabolites and Mechanical Performance during Hypothermic Preservation and Reperfusion in Hearts Stored in either High Buffer Cardioplegic Solution or St Thomas' Solution #2

Figure 12 shows the intracellular pH from hearts stored for 8 hours at 12°C in either high buffer cardioplegic solution (HBC) or St Thomas' solution #2. The intracellular pH obtained from the hearts stored in HBC at the end of ischemic preservation was significantly higher than that of those stored in St Thomas' solution #2. Figure 13 shows representative NMR spectra from hearts in each group during ischemic preservation and reperfusion. It is clear that with time, inorganic phosphate increased and phosphocreatine decreased rapidly while adenosine triphosphate showed very slow decline during preservation (Fig.14,15,16). However, there were no significant differences in the three parameters, indicating that increasing the buffer capacity of a cardioplegic solution did not improve the preservation of energy metabolites while it maintained intracellular pH higher than did St Thomas' solution #2. Furthermore, there were no significant differences in LVDP between the two groups during reperfusion (Fig. 17, 18, 19).

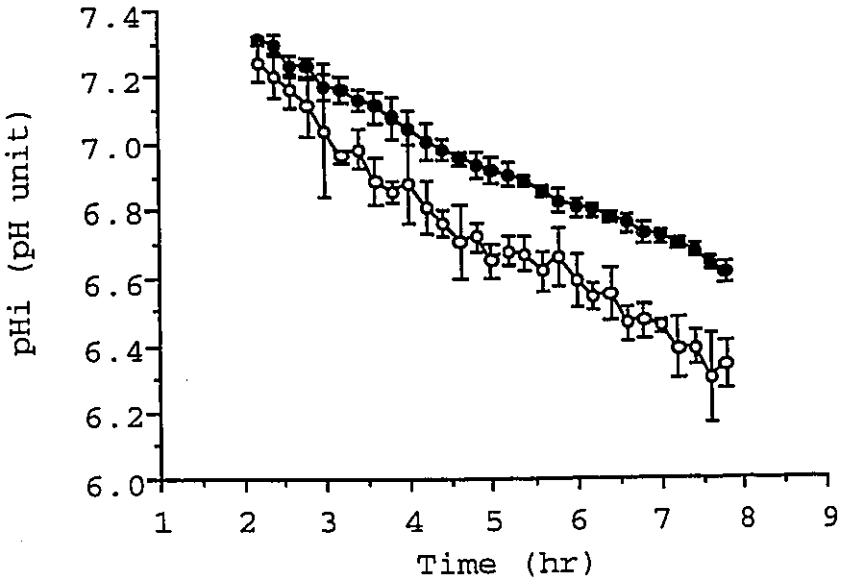


Figure 12. Time course of changes in intracellular pH during 8 hours of ischemic preservation. Hearts stored with high buffer cardioplegic solution (solid circles) show a significantly ($p<0.01$) higher intracellular pH than those arrested and stored in St Thomas' solution #2 (open circles). Data are presented as mean ± S.D.

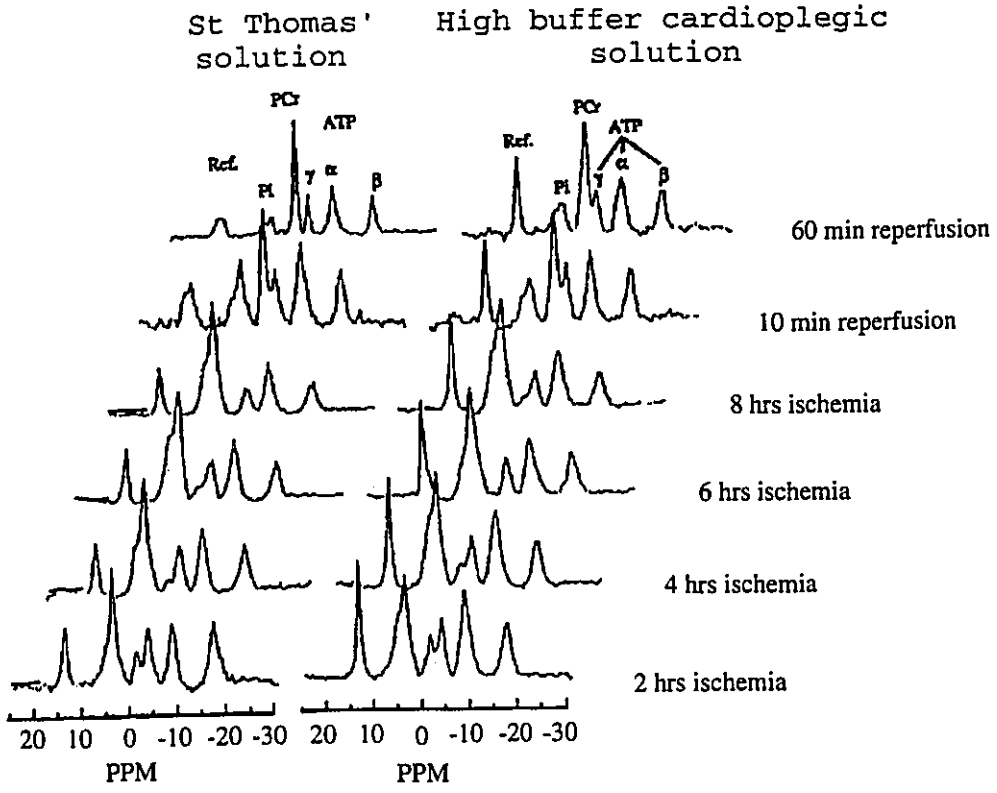


Figure 13. Representative spectra obtained throughout the protocol from the hearts stored in St Thomas' solution #2 and high buffer cardioplegic solution.

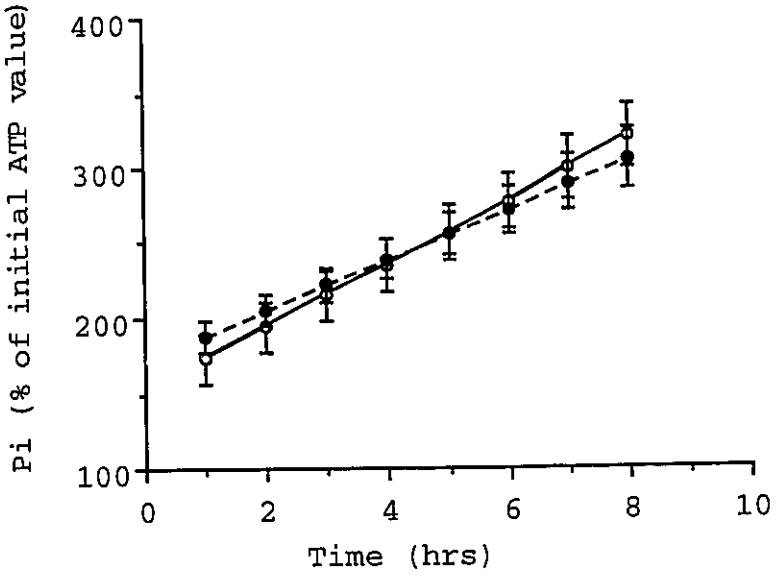


Figure 14. Time course of inorganic phosphate (Pi) levels measured by ^{31}P NMR spectroscopy during 8 hours of preservation in St Thomas' solution #2 (open circles) or high buffer cardioplegic solution (solid circles).

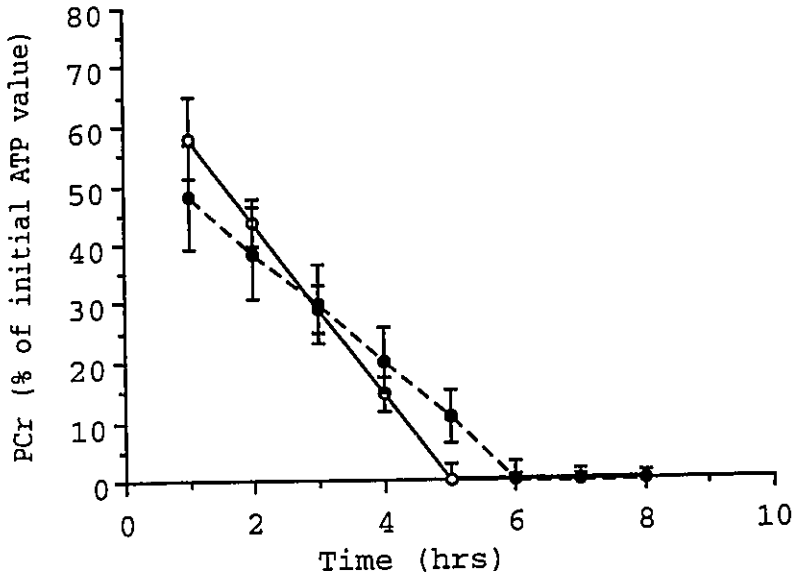


Figure 15. Time course of phosphocreatine (PCr) levels measured with ^{31}P NMR spectroscopy in hearts stored in St Thomas' solution #2 (solid circles) or high buffer cardioplegic solution (open circles) during 8 hours of ischemic preservation at 12°C.

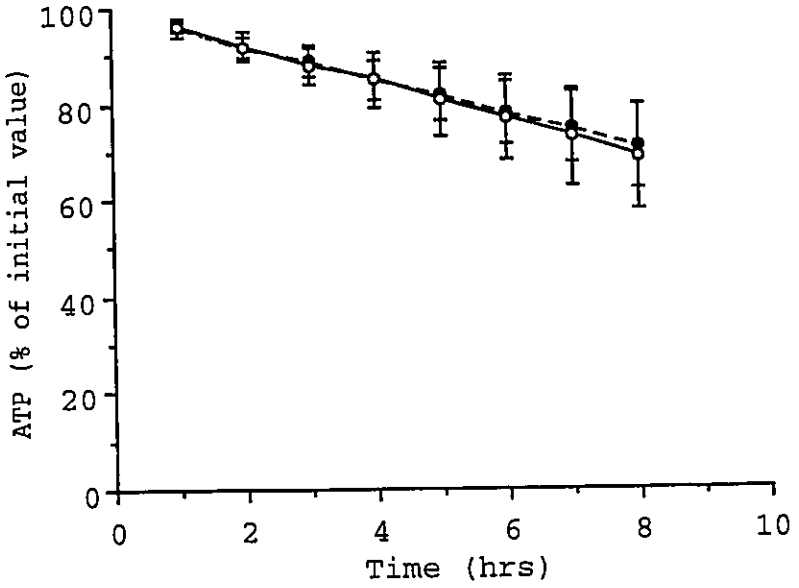


Figure 16. Time course of ATP levels obtained during 8 hours of ischemic preservation at 12°C with St Thomas' solution #2 (solid circles) or high buffer cardioplegic solution (open circles).

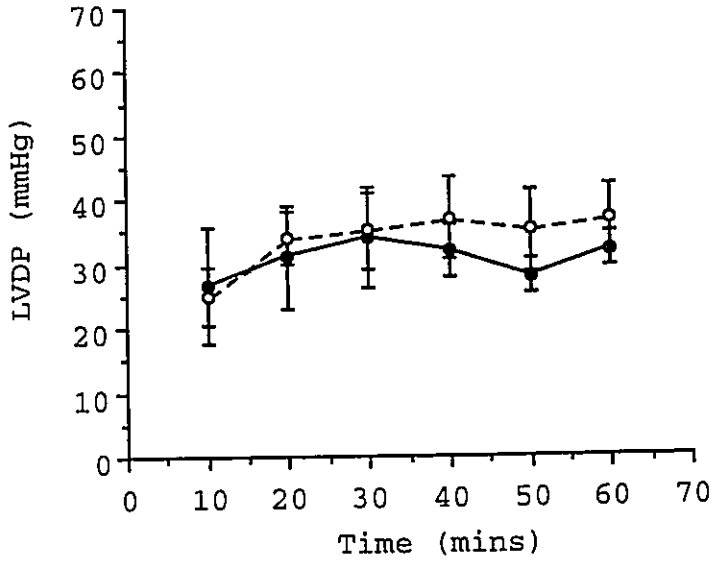


Figure 17. Comparison of left ventricular developed pressure (LVDP) during reperfusion of hearts stored in either St Thomas' solution #2 (solid circles) or high buffer cardioplegic solution (open circles). Data are presented as mean ± S.E (no significant difference).

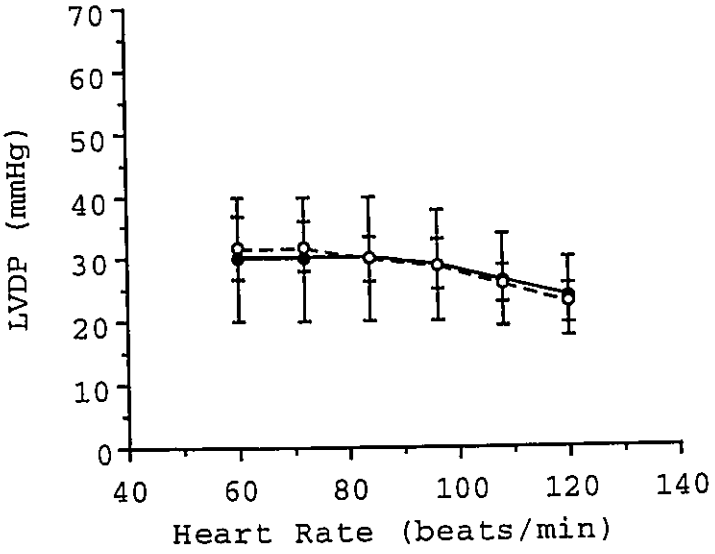


Figure 18. Comparison of left ventricular developed pressure (LVDP) obtained during reperfusion at different heart rates in hearts stored in St Thomas' solution #2 (solid circles) or high buffer cardioplegic solution (open circles) (no significant difference).

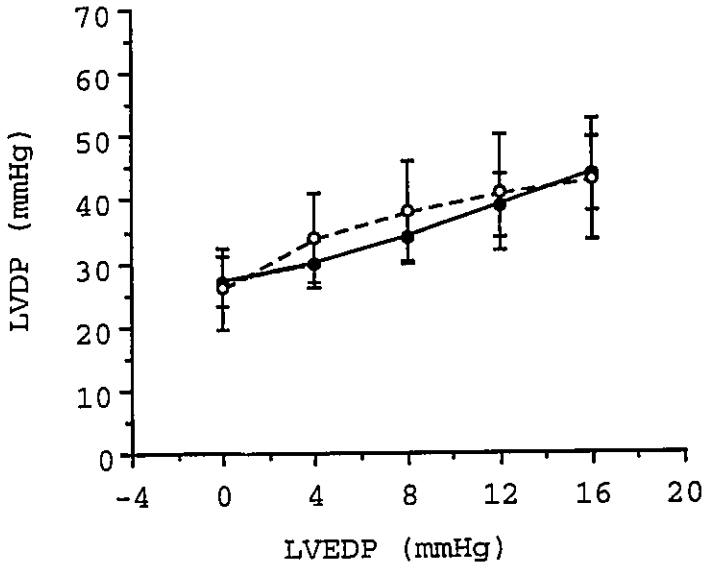


Figure 19. Comparison of left ventricular developed pressure (LVDP) obtained during reperfusion at various end diastolic pressures (LVEDP) in hearts stored in St Thomas' solution #2 (solid circles) or high buffer cardioplegic solution (open circles) (no significant difference).

3. The Effect of a High Concentration of Magnesium in Solution Used for Heart Preservation

3.1. The Effect of 16 mmol/L Mg^{++} in Cardioplegic Solution on Myocardial Energy Metabolites and Contractile Function

3.1.1. The Effect of 16 mmol/L Mg^{++} in St Thomas' Solution #2 on the Postischemic Functional Recovery of the Rat Heart

Cardiac output, oxygen consumption, external pressure work and external working efficiency during control perfusion and reperfusion in the rat hearts arrested with either 0 mmol/L or 16 mmol/L Mg^{++} St Thomas' solution #2 are shown in Figures 20, 21, 22 and 23. All hearts showed a significant decrease in these parameters following 30 minutes of normothermic ischemia. However, there were no significant differences on recovery of function and metabolism between the hearts arrested with either solution.

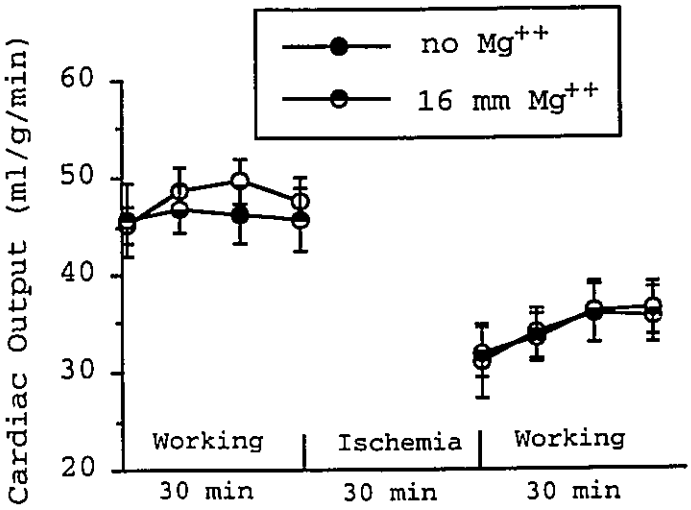


Figure 20. Comparison of cardiac output during control perfusion and reperfusion between rat hearts arrested with St Thomas' solution #2 containing either 0 or 16 mmol/L $MgCl_2$.

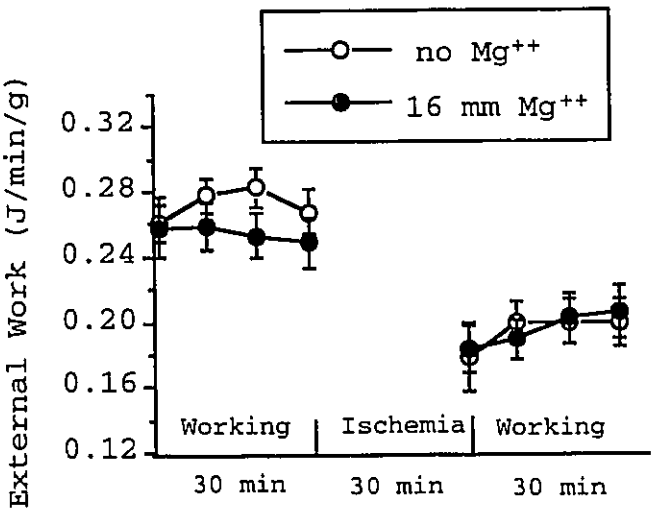


Figure 21. Time course of external work performed during control perfusion and reperfusion by hearts arrested with St Thomas' solution containing 0 or 16 mmol/L $MgCl_2$. Data are expressed as mean ± S.E.

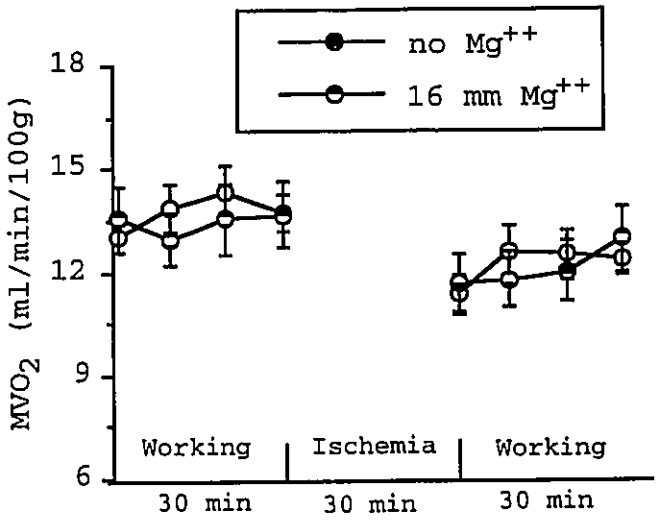


Figure 22. Comparison of myocardial oxygen consumption (MVO_2) during control perfusion and reperfusion in rat hearts arrested with St Thomas' solution #2 containing either 0 or 16 mmol/L $MgCl_2$. Data are presented as mean ± S.E.

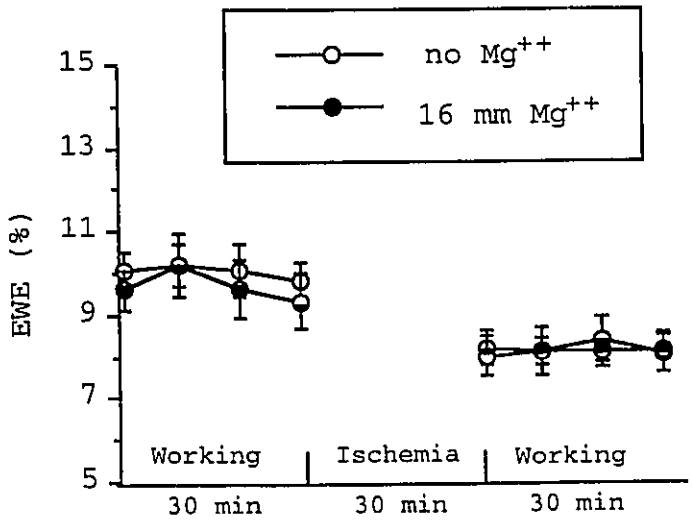


Figure 23. Comparison of external working efficiency (EWE) of the rat heart arrested with St Thomas' solution #2 containing either 0 or 16 mmol/L $MgCl_2$. Data are expressed as mean ± S.E.

3.1.2. The Effect of Mg^{++} on Contractile Function in the Pig Heart

The relationship between the Mg^{++} concentration in K-H solution and heart rate (HR), left ventricular developed pressure (LVDP) and maximal rates of pressure increase and decrease (±dp/dt) is shown in Figure 24, indicating concentration-dependent depressions in all these functions. Increasing the concentration of Mg^{++} in the perfusate resulted in a significant progressive decline in mechanical performance of the isolated pig heart.

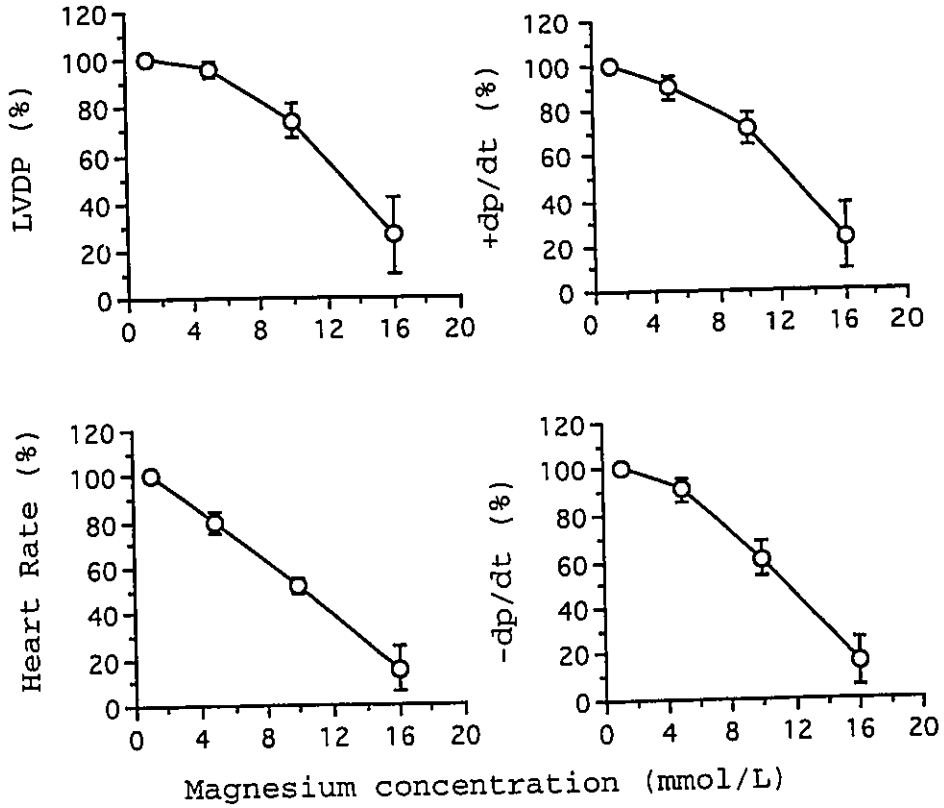


Figure 24. Relationship between the magnesium concentration in Krebs-Henseleit solution and mechanical performance of pig hearts (left ventricular developed pressure, LVDP; heart rate, HR; derivatives of left ventricular pressure, ±dp/dt). Data are expressed as mean ± S.E (n=5).

3.1.3. The Effect of 16 mmol/L Mg^{++} in St Thomas' Solution #2 on Myocardial Energy Metabolites and Mechanical Function of the Pig Heart

The changes of ATP, PCr and Pi levels during 4 hours of ischemic preservation and reperfusion in pig hearts stored in St Thomas' solution #2 containing either 0 or 16 mmol/L $MgCl_2$ are shown in Figures 25, 26 and 27. Both groups of hearts showed a significant increase in Pi and decrease in PCr during preservation. The preischemic levels of the two metabolites were reestablished during reperfusion while the β-ATP peak showed a small decrease during ischemic preservation. The differences in ATP, PCr and Pi levels between the two groups throughout the protocol were not significant. The functional recovery of the hearts during reperfusion is presented in Figure 28. There were no significant differences between the two groups of hearts in HR, LVDP or ± dp/dt.

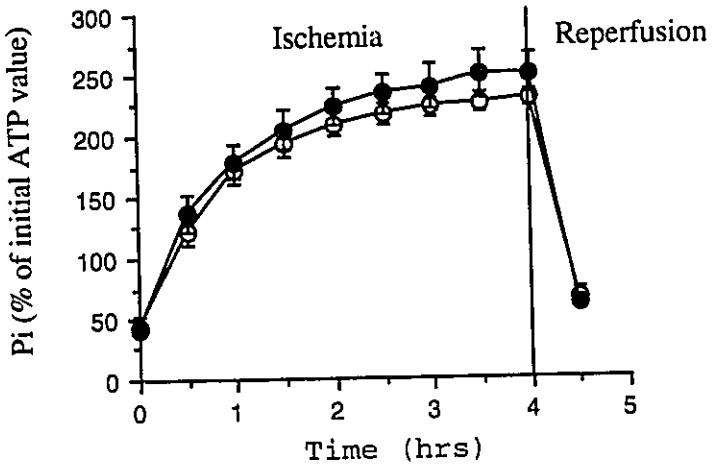


Figure 25. Time course of inorganic phosphate (Pi) levels during ischemic preservation at 12°C and reperfusion. Pig hearts were preserved with St Thomas' solution #2 containing 0 (open circles) or 16 mmol/L Mg^{++} (solid circles). Data are presented as mean ± S.E.

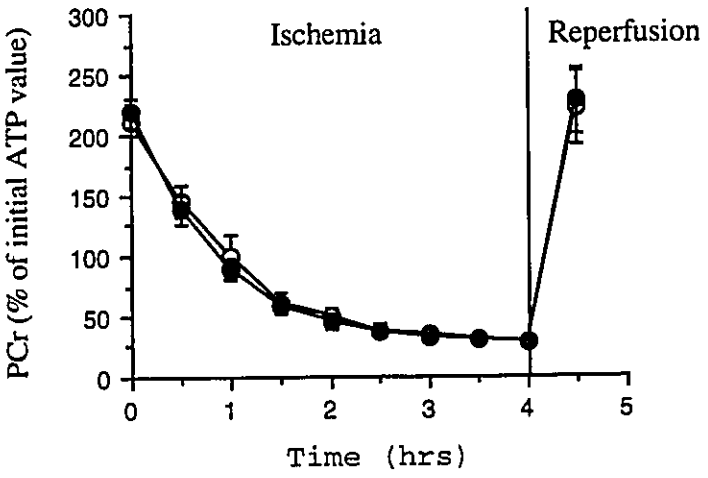


Figure 26. Time course of phosphocreatine (PCr) levels during 4 hours of ischemic preservation and reperfusion. Pig hearts were stored in St Thomas' solution #2 containing either 0 (open circles) or 16 mmol/L Mg^{++} (solid circles). Data are presented as mean ± S.E.

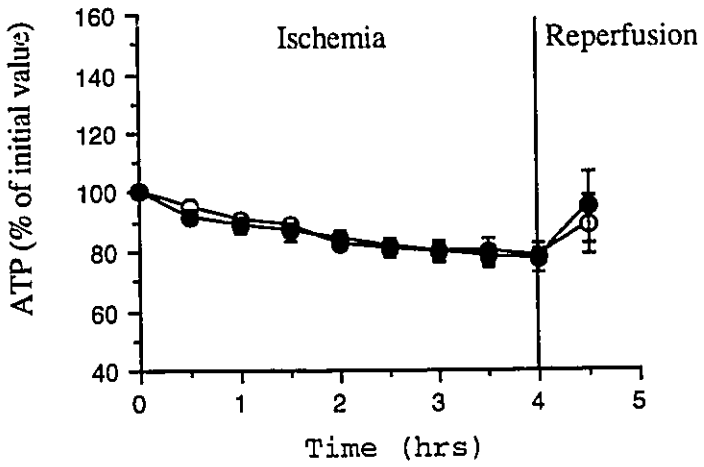


Figure 27. Time course of adenosine triphosphate (ATP) levels observed during ischemic preservation and reperfusion in the hearts arrested with St Thomas' solution #2 containing 0 (open circles) or 16 mmol/L Mg^{++} (solid circles). Data are presented as mean ± S.E.

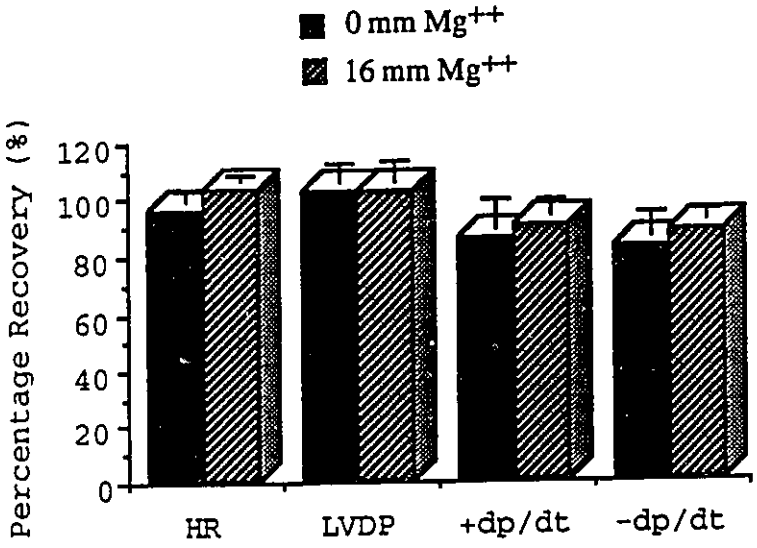


Figure 28. Mechanical performance during reperfusion following 4 hours of ischemic preservation in hearts stored in 0 or 16 mml/L Mg^{++} St Thomas' solution #2. HR: heart rate; LVDP: left ventricular developed pressure; ±dp/dt: derivatives of pressure changes.

3.2. **The Effect of 16 mmol/L Mg^{++} in a Re-arrest Perfusion Solution on Postischemic Myocardial Recovery.**

Hearts reperfused with secondary cardioplegic solution (S-C-S) containing 16 mmol/L $MgCl_2$ did not show better recovery of high phosphate energy levels, HR, LVDP, +dp/dt and -dp/dt than hearts reperfused with S-C-S containing 0 mmol/L $MgCl_2$ (Figures 29, 30 and 31), indicating that magnesium did not provide additional protection during reperfusion. There were no significant differences in recovery of metabolism or contractile function between the hearts subjected to S-C-S or 16 mmol/L KCl K-H perfusion prior to K-H solution (Figures 29, 30 and 31), suggesting that the high concentration of potassium in the early period of reperfusion is mainly responsible for beneficial effect of re-arrest perfusion.

As presented in Table 11, all hearts perfused with K-H alone developed reperfusion-induced ventricular fibrillation upon reperfusion, which usually required several countershocks for reversion to normal sinus rhythm. None of the hearts subjected to S-C-S or K-H solution containing 16 mmol/L KCl showed ventricular fibrillation.

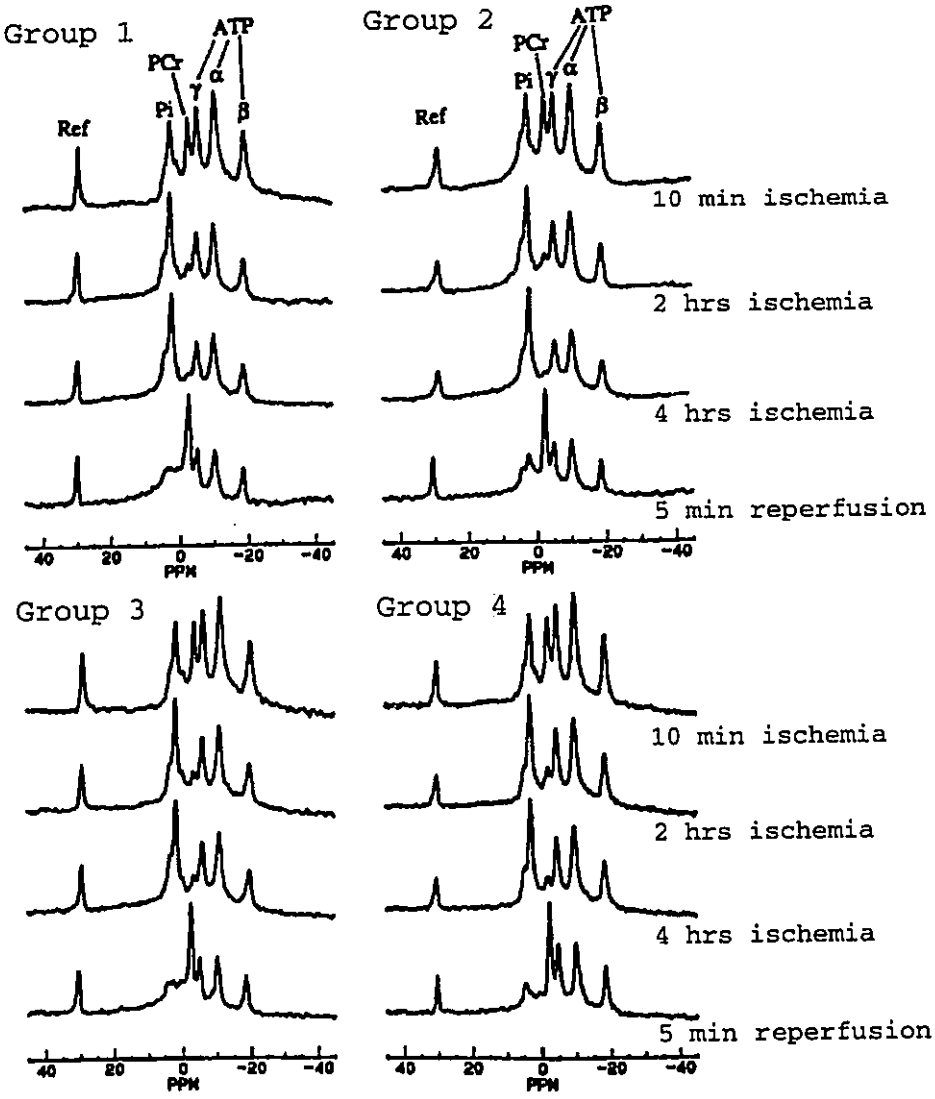


Figure 29. Representative ^{31}P NMR spectra obtained throughout preservation and reperfusion. Group 1: hearts reperfused with K-H solution alone; Group 2: hearts reperfused with S-C-S containing 16 mmol/L Mg^{++} prior to K-H solution; Group 3: hearts reperfused with S-C-S containing 0 mmol/L Mg^{++} prior to K-H solution; Group 4: hearts reperfused with K-H solution containing 16 mmol/L K^{+}.

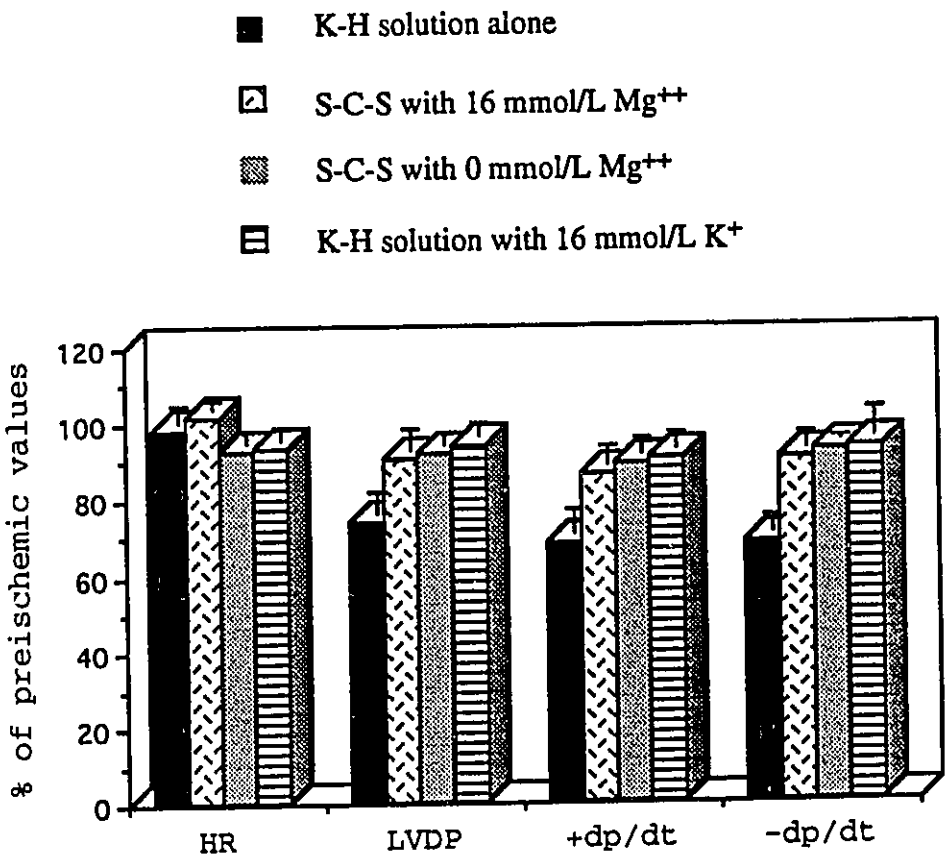


Figure 30. Postischemic recovery of heart rate (HR), left ventricular developed pressure (LVDP), maximum rate of pressure rise (+dp/dt) and decrease (-dp/dt) in the hearts of the four groups described above. The hearts reperfused with K-H solution alone showed significantly poorer recovery in LVDP and ±dp/dt than the hearts reperfused either with S-C-S (0 or 16 mmol/L Mg^{++}) or with 16 mmol/L K^+ K-H ($p<0.05$).

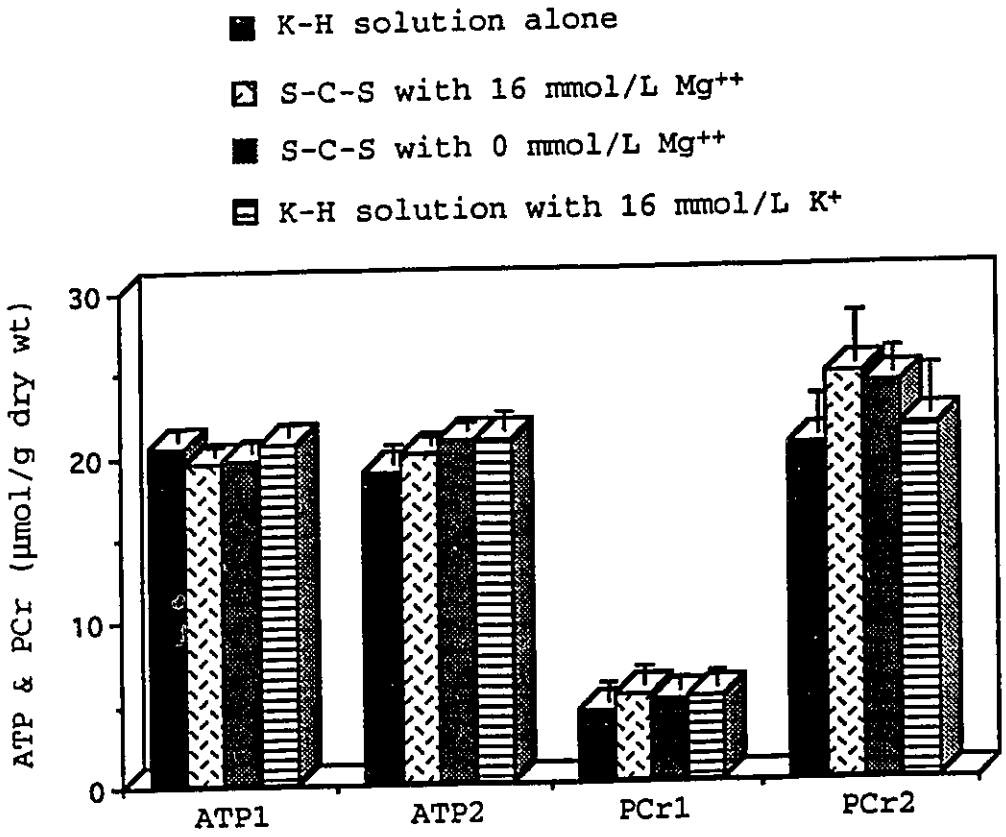


Figure 31. Adenosine triphosphate (ATP) and phosphocreatine (PCr) levels in the four groups of hearts described above. The values obtained at the end of ischemia are marked as ATP1 or PCr1, and those obtained at the end of the reperfusion are indicated as ATP2 or PCr2. There were no significant differences in the levels of ATP or PCr among the four groups.

TABLE 11. Comparison of the incidence of ventricular fibrillation

Group	Incidence of VF
1 (K-H solution alone)	6/6
2 (S-C-S with 16 mmol/L Mg^{++})	0/6
3 (S-C-S with 0 mmol/L Mg^{++})	0/6
4 (K-H with 16 mmol/L Mg^{++})	0/6

VF, ventricular fibrillation; K-H, Krebs-Henseleit solution; S-C-S, secondary cardioplegic solution.

4. The Efficacy of an Intracellular-Type Cardioplegic Solution

4.1. A Comparison of Metabolic and Functional Preservation by UW Solution and St Thomas' Solution #2 at 4°C and 12°C.

Figure 32 shows typical spectra obtained from hearts stored at either 4°C or 12°C using either St Thomas' solution #2 or UW solution. At 4°C, the PCr peak decreased and then disappeared during preservation. Upon reperfusion, the PCr peak was restored in both groups. The peak of ATP decreased slightly during preservation. At 12°C, hearts stored with St Thomas' solution #2 showed the similar changes in ATP and PCr peaks as those preserved at 4°C. However, in the hearts stored with UW solution, the ATP and PCr peaks were not restored upon reperfusion. Only a large Pi peak was observable.

Figure 33 shows the changes in ATP content as a function of time measured by ^{31}P NMR spectroscopy for the four groups of hearts. The top panel shows the mean curves of ATP content in the hearts stored at 4°C. There was no significant difference between the two curves. The bottom panel illustrates the mean curves of ATP content in the hearts stored at 12°C. The hearts stored in UW solution at 12°C showed a rapid decrease in ATP content ($P<0.01$).

Comparison of the PCr content measured by ^{31}P NMR spectroscopy among the hearts preserved with St Thomas' solution #2 and UW solution at 4°C and 12°C is shown in Figure 34. There was no significant difference between the hearts stored at 4°C with either solution (top panel). Significant differences were noted between the hearts stored at 12°C (bottom panel) particularly during the reperfusion period.

The effects of this cardioplegic solution on the functional recovery as indicated by the ventricular function curves constructed during reperfusion period are shown in Figure 35. At 4°C (top panel), hearts stored with St Thomas' solution #2 showed a slightly higher rate pressure product than did hearts preserved with UW solution. At 12°C (bottom

panel), the differences were greatly exaggerated and reached significant level, with four hearts becoming "stone heart". The latter is refered to a heart that is rigid and has no contractile function with disappearance of ATP and PCr upon reperfusion. These findings demonstrate that UW solution is not superior to St Thomas' solution #2 at 4°C, and is inferior to St Thomas' solution #2 at 12°C.

Figure 36 shows the myocardial concentration of energy metabolites measured with HPLC at the end of reperfusion. Metabolite levels are significantly lower in the hearts stored with UW solution at 12°C than in other hearts.

Figure 37 shows the dry/wet weight ratio measured at the end of reperfusion in the hearts stored in UW solution and St Thomas' solution #2 at both temperatures. There were no significant differences between the groups.

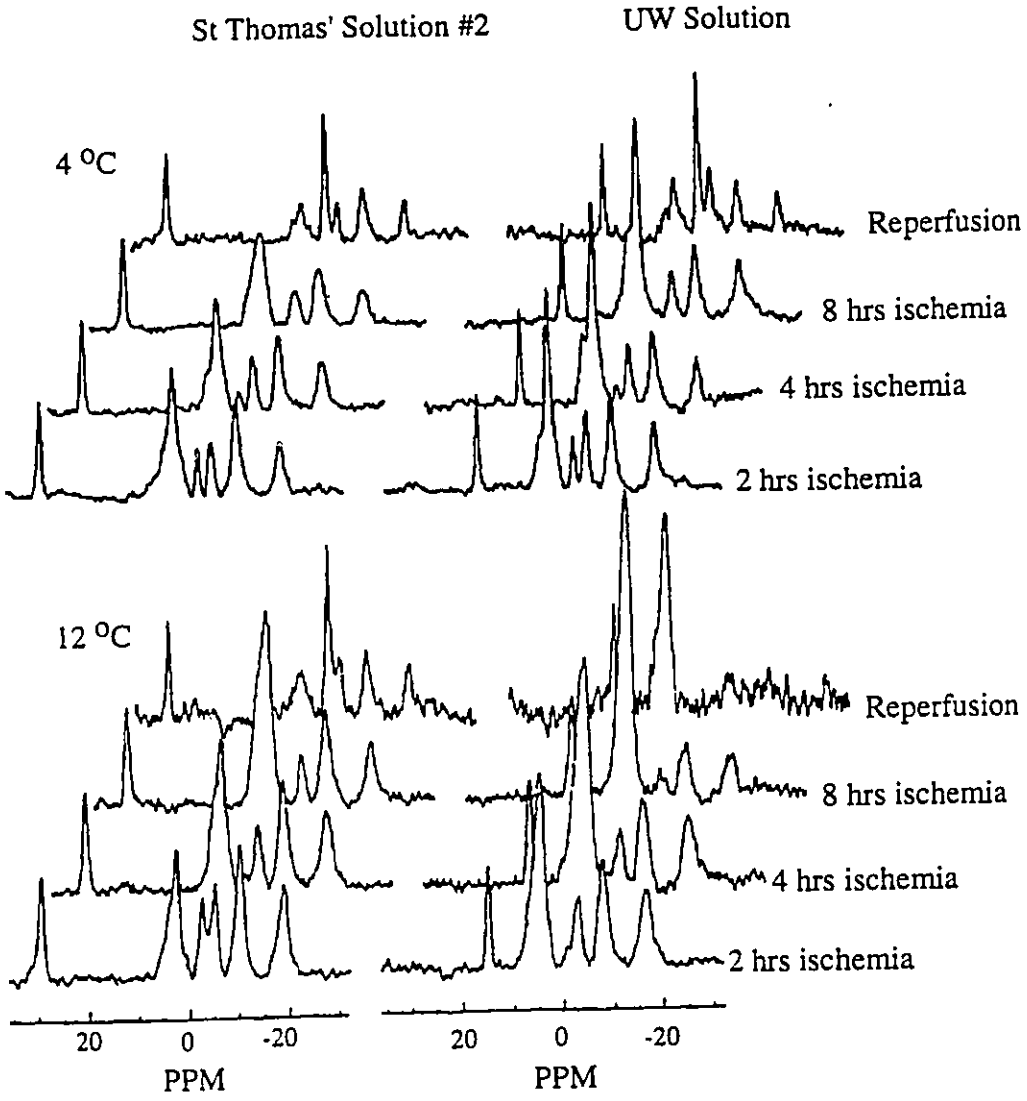


Figure 32. Typical time course of the ^{31}P NMR spectra of hearts preserved at 4°C (top panels) and 12°C (bottom panels). The spectra in the left panels were obtained from hearts stored in St Thomas' solution #2. Those on the right were obtained from hearts stored in UW solution. Note a rapid decrease in PCr and a gradual decrease in ATP during preservation. ATP and PCr disappeared upon reperfusion in the heart stored with UW solution at 12°C. In the other hearts, the Pi peaks decreased significantly and PCr peaks were restored upon reperfusion.

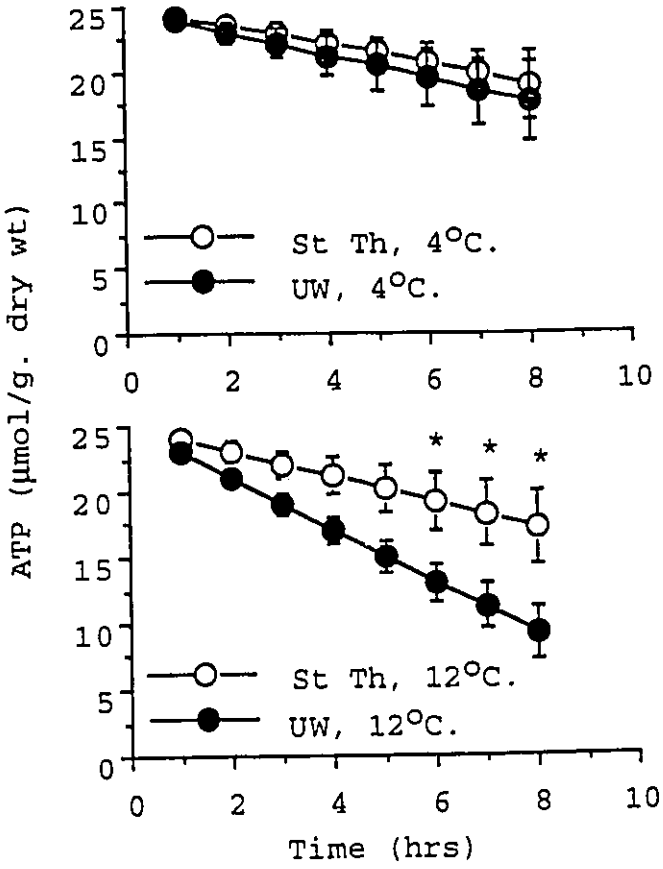


Figure 33. Time course of changes in ATP level. Bars represent standard errors. St Th: St Thomas' solution #2; UW: University of Wisconsin solution (*, $p<0.05$).

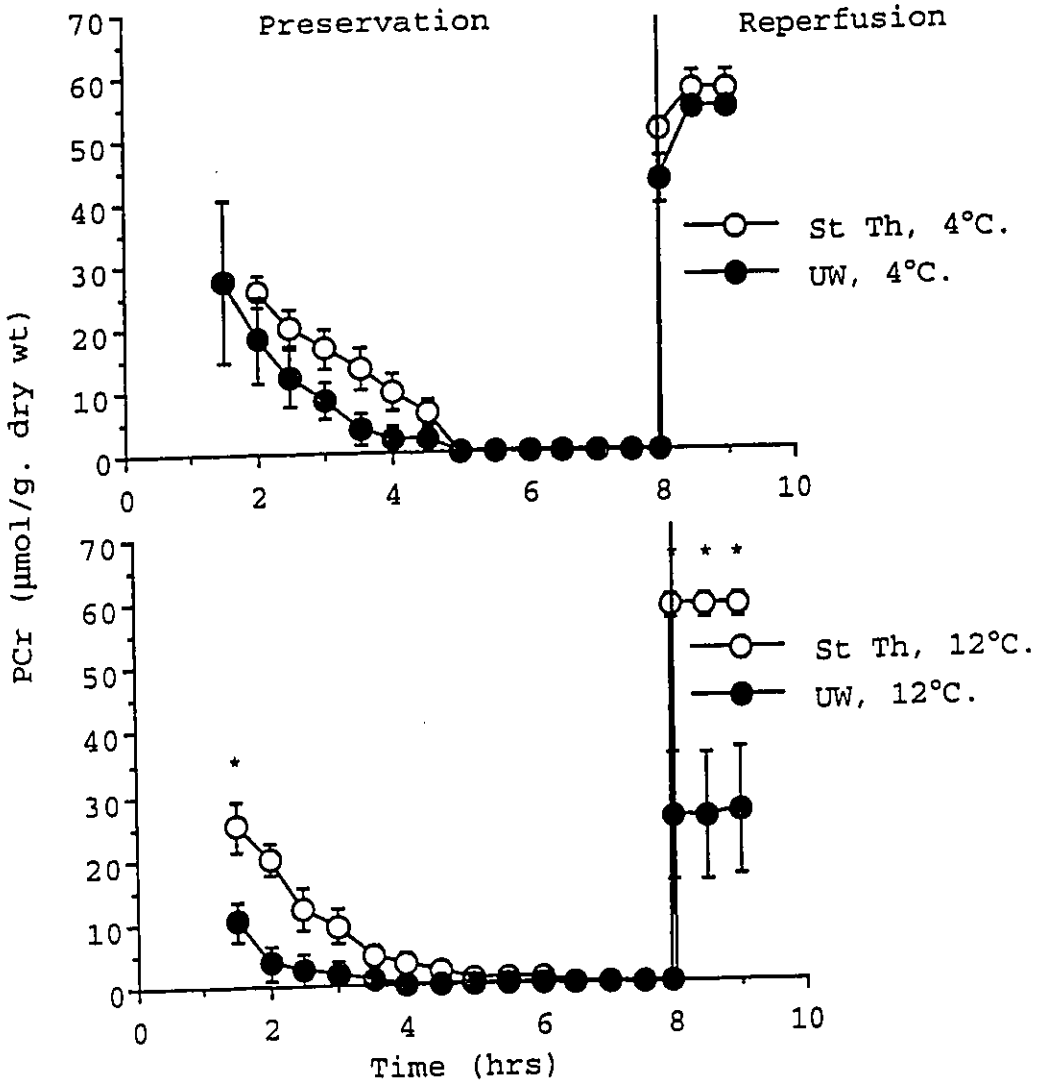


Figure 34. Time course of changes in PCr levels at 4°C (top panel) and at 12°C (bottom panel). The PCr level decreased rapidly during preservation. The PCr level was significantly lower in the hearts stored with UW solution at 12°C than in other hearts (*, $p<0.05$). St Th: St Thomas' solution #2; UW: University of Wisconsin solution.

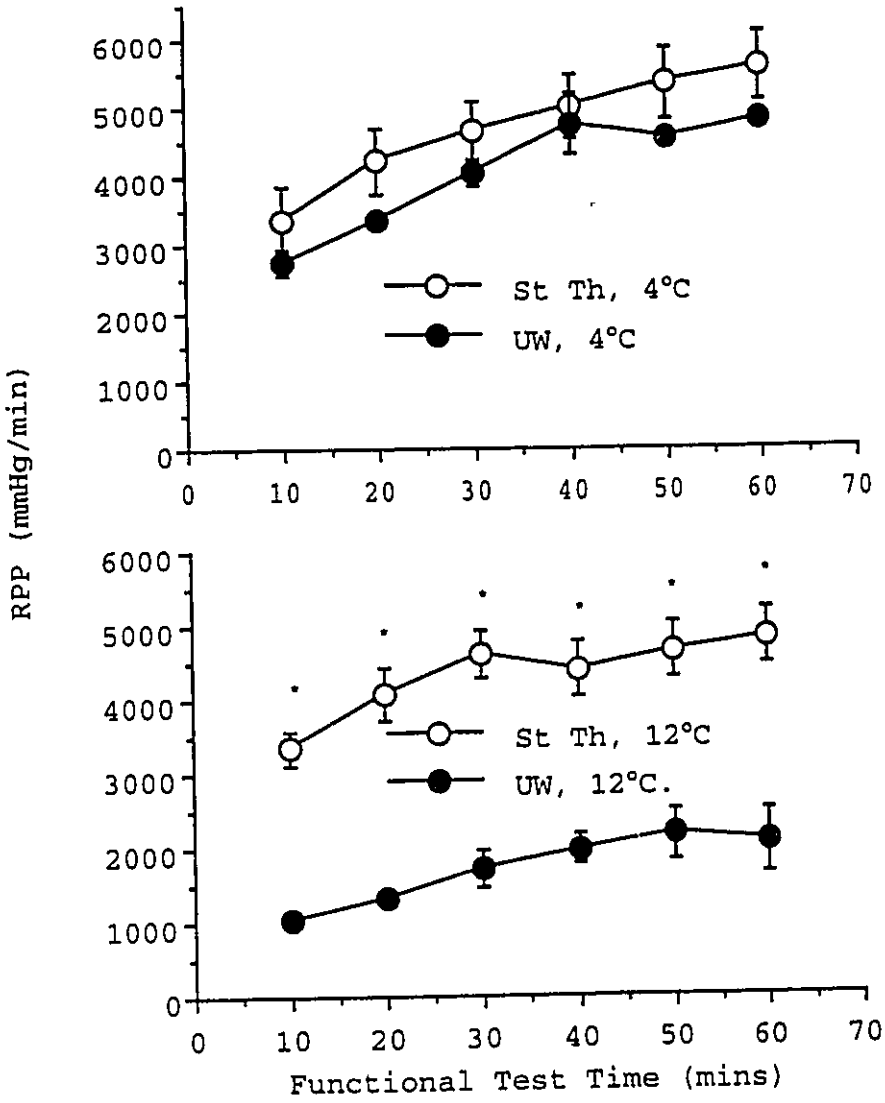


Figure 35. Time course of the rate pressure product (RPP) during reperfusion from hearts stored in either St Th or UW solution at 4°C (top panel) or at 12°C (bottom panel). Functional recovery was extremely poor in the hearts stored with UW solution at 12°C (*, $p<0.05$). St Th: St Thomas' solution #2; UW: University of Wisconsin solution.

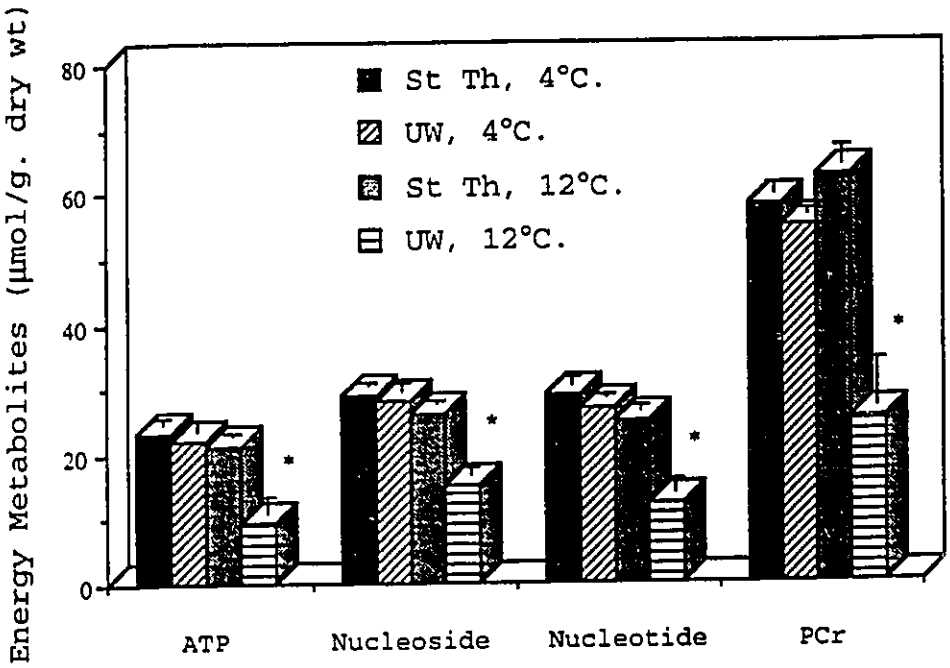


Figure 36. Energy metabolites in the four groups of hearts described above at the end of reperfusion (*, $p<0.05$). St Th: St Thomas' solution #2; UW: University of Wisconsin solution.

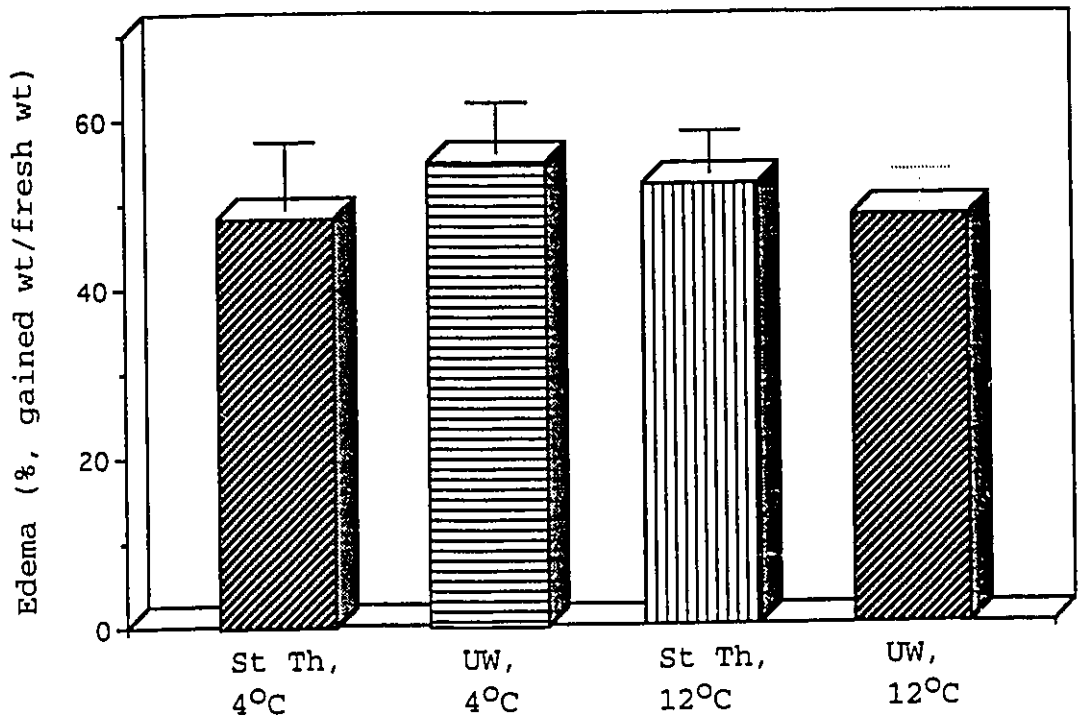


Figure 37. Edema measured in hearts at the end of reperfusion. St Th: St Thomas' solution #2; UW: University of Wisconsin solution.

4.2. **Advantage and Disadvantage of Adding Ca^{++} to UW Solution.**

The free calcium concentration measured directly with calcium electrode was 0.08 ± 0.002 mM (n=7) when 0.5 mmoles of $CaCl_2$ was added to 1 L of UW solution.

Figure 38 shows the left ventricular developed pressure (LVDP) observed during reperfusion following 8 hours of ischemic preservation of hearts in either unmodified UW solution or Ca^{++}-containing UW solution. Compared to hearts stored with unmodified UW solution, hearts arrested with the Ca^{++}-containing UW solution showed a significantly improved ($p<0.001$) recovery of contractile function upon the reperfusion, and none of the "stone heart" phenomenon that was observed upon reperfusion in four of the eight hearts stored in unmodified UW solution (Table 12). In addition, at the onset of reperfusion, the ^{31}P NMR spectra of hearts preserved with the Ca^{++}-containing UW solution did not show the loss of high energy phosphates that was observed in hearts stored in unmodified UW solution (Fig. 39).

Figure 40 depicts the effect of the Ca^{++}-containing UW solution on PCr levels during 30 minutes of ischemia. ^{31}P NMR spectra show that the rate of PCr decline was more rapid ($p<0.001$) in hearts arrested with the Ca^{++}-containing UW solution than in hearts arrested with unmodified UW solution. The comparison of recovery of contractile function is given in Figure 41. There is no significant difference in the recovery of contractile function between hearts arrested with either Ca^{++}-containing UW solution or unmodified UW solution following 30 minutes of ischemia.

Figure 42 shows representative traces of diastolic pressure measurement from hearts arrested with either Ca^{++}-containing UW solution (top panel) or Ca^{++}-free UW solution (bottom panel). The heart arrested with Ca^{++}-containing UW solution showed a transient increase of diastolic pressure.

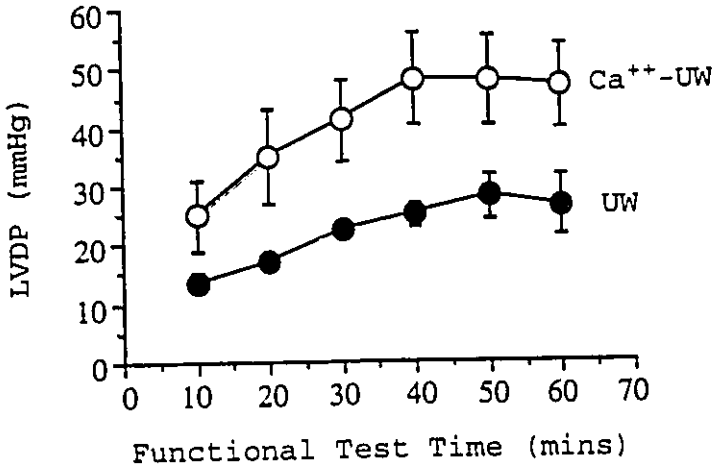


Figure 38. Time course of the left ventricular developed pressure (LVDP) following 8 hours of ischemic preservation. The curves represent means, and vertical bars denote standard errors of the means. Ca^{++}-UW or UW represent, respectively, UW solution containing 0.5 mmol/L $CaCl_2$ or unmodified UW solution. The difference between the two mean curves is statistically significant ($p<0.01$).

TABLE 12. Incidence of "stone heart" upon reperfusion

Group	Stone heart
Ca^{++}-UW	0/7
UW	4/8

The "stone heart" is refered to a heart that is rigid and has no contractile function upon reperfusion. UW and Ca^{++}-UW represent unmodified or Ca^{++}-containing University of Wisconsin solution, respectively.

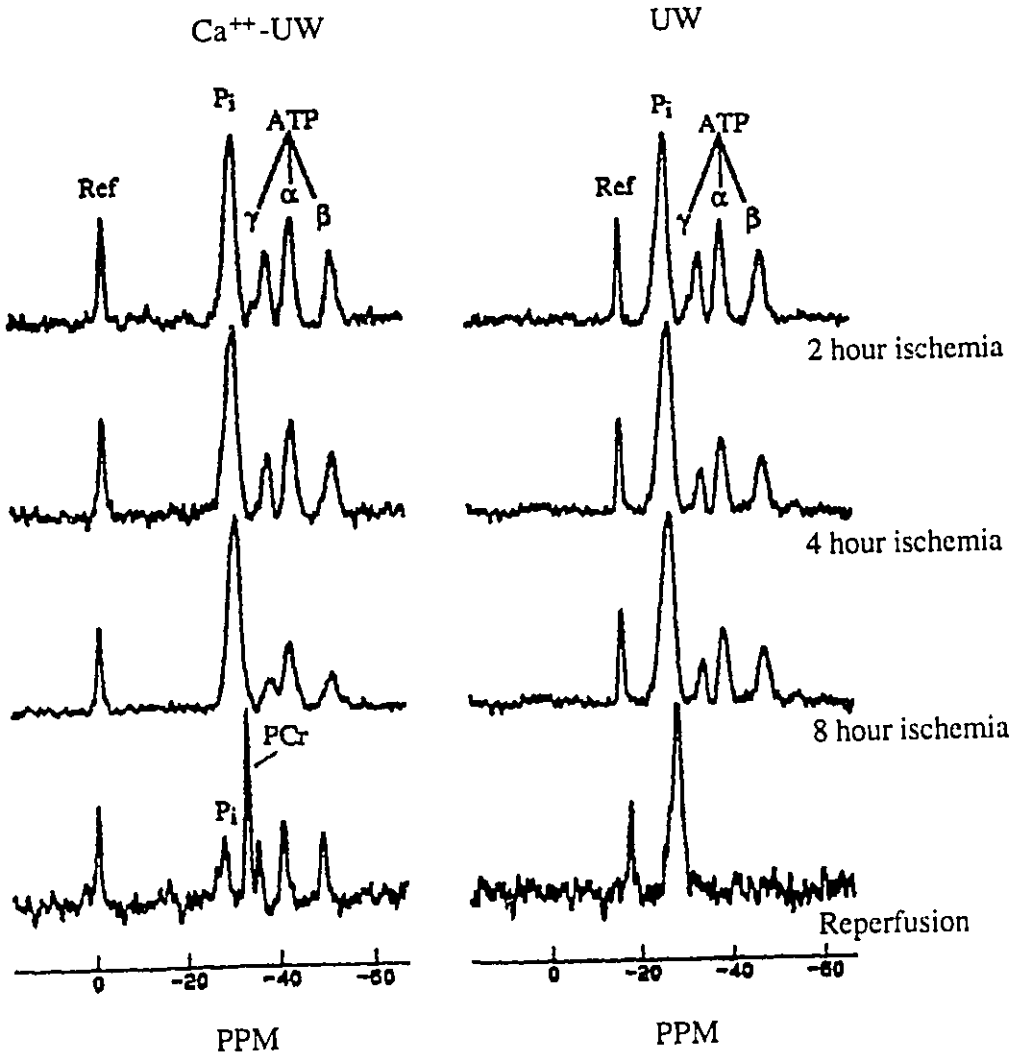


Figure 39. Representative ^{31}P NMR spectra during preservation and reperfusion of hearts stored in Ca^{++}-containing UW solution (left panel) or with the unmodified UW solution (right panel). The peaks of ATP and PCr disappeared on reperfusion in hearts preserved with unmodified UW solution. Hearts stored in UW solution with addition of 0.5 mmol/L $CaCl_2$ showed a decrease in the Pi peak and restoration of the PCr peak on reperfusion.

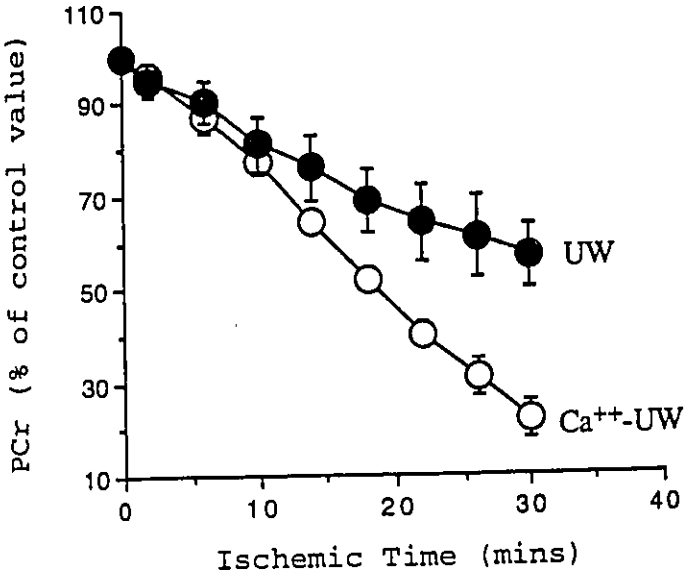


Figure 40. Comparison of the phosphocreatine (PCr) levels during 30 minutes of ischemia in hearts arrested with UW solution and those stopped with Ca^{++}-containing UW solution (Ca^{++}-UW). The decrease in PCr is more rapid in the hearts arrested with Ca^{++}-containing UW solution ($p<0.01$).

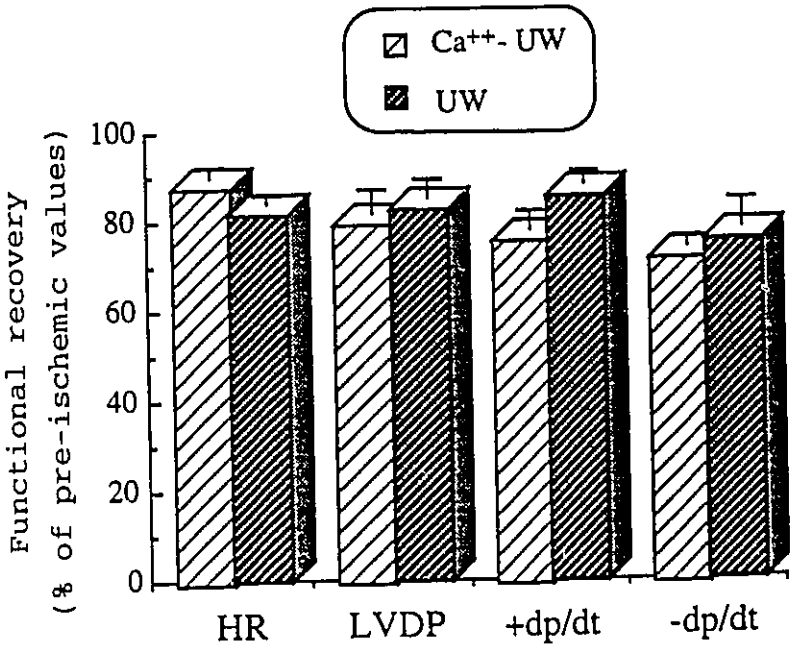


Figure 41. Postischemic recovery of heart rate (HR), left ventricular developed pressure (LVDP), maximum rate of pressure rise (+dp/dt) and decrease (-dp/dt) after 30 minutes of ischemia in hearts arrested with either Ca^{++}-containing UW solution (Ca^{++}-UW) or unmodified UW solution. No significant differences were noted in these parameters between the two groups of the hearts. All data are expressed as mean ± standard error of the mean.

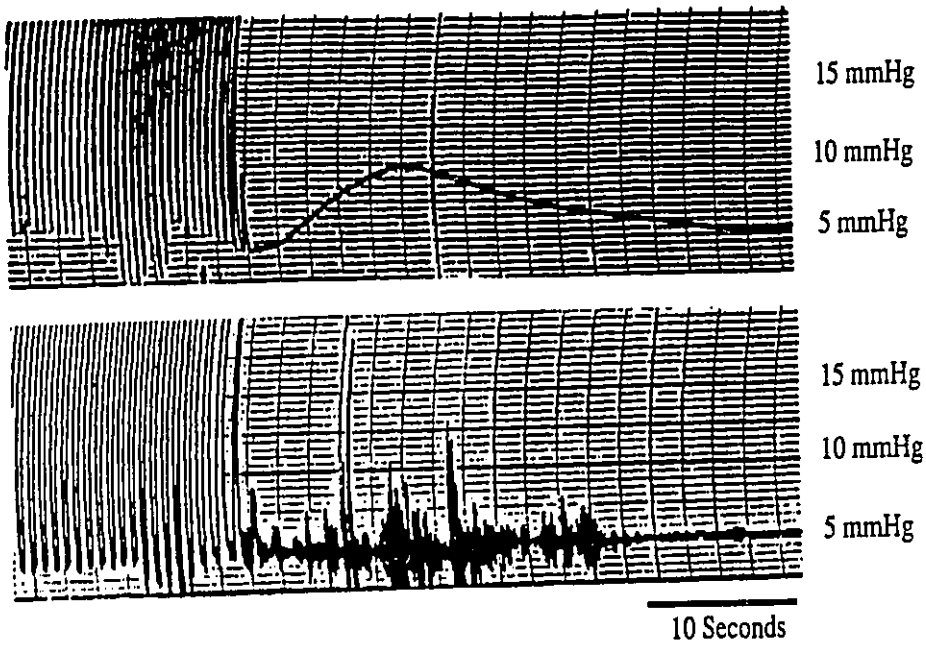


Figure 42. Representative traces of diastolic pressure recorded during the infusion of the cardioplegic solution. The top panel was obtained from a heart arrested with Ca^{++}-containing UW solution and bottom one from a heart arrested with unmodified UW solution. The heart arrested with Ca^{++}-containing UW solution showed a transient increase in diastolic pressure whereas the heart arrested with unmodified UW solution did not.

DISCUSSION

1. Major Findings

This study shows that re-arrest perfusion using S-C-S following long-term hypothermic preservation improved myocardial functional recovery whereas it did not alter high energy phosphate concentrations. Increasing the buffer capacity of cardioplegic solution did not significantly affect energy metabolites and contractile function in long-term preservation (8 hours) while it helped to maintain intracellular pH at slightly higher level during such preservation. Moreover, 16 mmol/L Mg^{++} in cardioplegic solution and S-C-S did not offer additional protection to ischemic myocardium. Comparison of UW and St Thomas' solution #2 showed that pig hearts preserved at 4°C with either solution showed comparable changes in energy metabolites and mechanical performance, whereas at 12°C, hearts stored in UW solution showed more rapid decrease in ATP and PCr during preservation and poorer functional recovery during reperfusion than those stored in St Thomas' solution #2. "Stone heart" phenomenon was observed in some of the hearts stored in UW solution. Furthermore, addition of Ca^{++} to UW solution improved its ability to preserve the heart albeit with a transient and modest increase in diastolic pressure.

2. Discussion of the Findings

It is important to recognize that the recovery of the cardiac contractile function is a very complex process in which multiple factors may interact; it includes intracellular components (restoration of energy production, shuttles, organelle function and sensitivity to regulatory processes), as well as extracellular components (such as coronary flow, substrate and oxygen supply, interstitial collagen coupling for efficient force transmission, etc). In view of the complexity of the recovery process, the assignment of the presence or absence of adequate recovery to any one or combination of factors is uncertain at best.

As mentioned above, ^{31}P NMR spectroscopy is a valuable means of obtaining information about myocardial metabolism, for it is able to provide simultaneously and non-invasively estimates of ATP, PCr and inorganic phosphate as well as to monitor intracellular pH. However, quantification of the metabolites with ^{31}P NMR is not absolute and is "averaged" overall whole heart and "average" within cell.

2.1. **The Benefit of Re-arrest Perfusion on Myocardial Recovery Following Long-Term Ischemic Preservation**

As mentioned previously, myocardial ischemic injury is characterized by a rapid loss in contractile function, depletion of high energy phosphates, particularly phosphocreatine, with elevation of inorganic phosphate and resulting intracellular acidosis and accumulation of various metabolic products[142]. However, reperfusion injury is mainly associated with abrupt and massive Ca^{++} influx and generation of free radicals which result in severe damage to the sarcolemma and organelles[3-4]. Therefore, reperfusion injury is distinct from ischemic injury. But it should be noted that reperfusion injury and its severity is clearly dependent on the factors associated with the preceding ischemic event[3-4]. Since it is a somewhat distinct entity, it is expected that reperfusion injury may be alleviated by modifying reperfusion conditions. In the present study, we proposed that re-arrest perfusion could reduce reperfusion injury and improve postischemic recovery. To this end, we studied the effects of a solution designed specially for use during early reperfusion on postischemic myocardial recovery, formulated according to the principles described earlier; namely, that (1) lowering energy demands by hyperkalemia; (2) reducing calcium influx by hypocalcemia; (3) increasing recovery of intracellular pH with additional buffering capacity. We found that hearts subjected to S-C-S prior to perfusion with K-H solution showed a significantly better recovery of mechanical performance (Fig. 8-10) than those initially

reperfused with K-H solution alone. No ventricular fibrillation occurred upon reperfusion in the hearts receiving S-C-S, whereas hearts reperfused with K-H solution alone showed ventricular fibrillation in every instance. The results indicate that functional recovery after long-term preservation can be improved by modifying the reperfusion solution.

It has been well known that reperfusion injury is closely related to a rise in intracellular calcium level[4]. Excessive calcium gain during reperfusion can result in irreversible myocardial injury by activating cellular and membrane-bound phospholipases and proteases and causing precipitation of calcium crystals in mitochondria, etc[57,61,176,198]. Additionally, calcium is the major determinant of myocardial energy utilization through its activation of myofibrillar adenosine triphosphatase and subsequent energy-dependent sequestration by Ca^{++}-ATPase in the sarcoplasmic reticulum and cell membrane[68]. Therefore, elevation of intracellular calcium during reperfusion would hasten the breakdown of residual energy stores and "waste" precious energy generated by recovery of the producing processes. Consequently, low calcium reperfusion may be beneficial by reducing the calcium gradient and thereby its influx, decreasing energy consumption during this early phase. Thus, more of the adenosine triphosphate resynthesized during the early phase of reperfusion would be available for reparative processes. The salutary effects of low calcium reperfusion has been reported by Shine and his colleagues[142]. They found that rabbit hearts reperfused with 0.7 mmol/L Ca^{++} solution showed a significantly improved recovery in the levels of ATP and PCr when compared to hearts reperfused with 2.5 mmol/L Ca^{++} buffer. They concluded that low calcium reperfusion is important for the recovery of myocardial metabolic function. Therefore, the beneficial effects of re-arrest perfusion may be related to a lower concentration of calcium.

Since myocardial acidosis impairs cellular metabolism, and since ionic regulation and most cellular processes are pH-dependent, intracellular acidosis could conceivably impede postischemic recovery[95]. Additional buffer capacity was incorporated to S-C-S to counteract intracellular acidosis, to allow enzymatic systems to function more optimally. This modification may also have some contribution to the beneficial effect of re-arrest perfusion.

As described above, cellular homeostasis is lost during prolonged ischemic preservation and the process of resumption of homeostasis requires a continuous supply of energy[93-97]. Additionally, ischemically injured myocardium has a limited capacity for oxygen uptake due to its impaired mitochondrial function[34,205]. This provides the basis for using a high concentration of potassium in S-C-S. Because of the cardioplegic effect of hyperkalemia, the re-synthesized high energy phosphates can be used exclusively to restore activity of the sarcolemmal Na^+-K^+ pump and Ca^{++} pump, thereby accelerating the recovery of the ionic gradients across the cell membrane. The re-establishment of a normal transmembrane Na^+ gradient reduces cell swelling that occurs during ischemia and helps restore normal cell homeostasis.

The three components (hypocalcemia, hyperkalemia and buffer) in S-C-S has provided the aforementioned beneficial effects to the ischemic myocardium. To determine the major component responsible for beneficial effect of S-C-S, we compared the recovery of hearts reperfused with S-C-S or with K-H containing 16 mmol/L K^+. However, both groups of hearts showed a similar degree of contractile functional recovery and comparable changes in high energy phosphates during ischemia and reperfusion (Fig. 29-31). These findings suggest that the cardioplegic effect (16 mmol/L K^+) is likely to be the principal mechanism underlying the benefit of S-C-S and other components may not be essential; further suggesting that resumption of mechanical activity during early

reperfusion severely disturbs myocardial reparative processes.

Ventricular fibrillation (VF) often occurs during reperfusion, even after a short period of ischemia[206]. VF can result in some deleterious effects on myocardial function and metabolism, particularly in the ischemically damaged myocardium[207]. Early studies demonstrated that ventricular fibrillation induced as a cardioplegic intervention during open-heart surgery resulted subsequently in reduced recovery of mechanical performance and of energy metabolites[208]. Using the isolated buffer perfused rat heart preparation, Heuer and his colleagues[207] found that ventricular fibrillation led to an excessive release of creatine kinase (CK) with a moderate increase in coronary flow and myocardial oxygen consumption; in contrast, the CK release was very small in hearts which were permanently stimulated with rhythmic impulses of 10Hz, where the increase in coronary flow and oxygen consumption were significantly greater. Moreover, during reperfusion of globally ischemic hearts, only minor amount of CK were released amounting only to a very small percentage of release from fibrillating hearts. These results led to the conclusion that VF could lead to profound myocardial injury that is not completely due to ischemic injury although ischemia plays a role. It has been proposed that VF itself has direct effects on the permeability of the myocardial membrane (leading to the release of the enzyme), that results from impairment of the integrity of the cell membranes induced by the asynchronous contractile activity of many small parts of the myocardium within the whole heart[207]. Whether this is a truly mechanical or chemical process is not known.

More recently, attention has shifted to the ionic consequences of VF. Using ^{19}F NMR spectroscopy and the Ca^{++} indicator 5-F-BAPTA, Koretsune et al[209] demonstrated a five-fold increase of intracellular Ca^{++} concentration induced by VF in isolated perfused ferret hearts. After VF was

terminated, the hearts showed reduced mechanical performance. The authors postulated that VF causes intracellular Ca^{++} overload, which in turn is responsible for the deleterious effects of VF. This idea has been confirmed by the work of Ingwall[210]. In addition, it has been shown that ventricular fibrillation can cause an unwanted change in the distribution of coronary flow with a significant reduction in flow to the subendocardium, in spite of the observation that the decrease in overall coronary flow may not be so dramatic[208], and the resulting ischemic injury being mostly confined to the subendocardium. The present study has shown that following 8 hours of ischemic preservation, hearts subjected to re-arrest perfusion for 15 minutes prior to reperfusion with K-H solution began to beat with a regular rhythm (Table 10). In contrast, hearts reperfused with K-H solution alone fibrillated upon reperfusion and electrical defibrillation was necessary. This result demonstrates that reperfusion-induced ventricular fibrillation can be abolished by manipulating the myocytes' ionic environment with a re-arrest perfusion technique. The underlying mechanism has not been fully established. It seems clearly related to the re-establishment of intracellular sodium and calcium levels presumably by improvement of the function of the sodium and calcium pumps.

Although a secondary cardioplegic solution abolishes ventricular fibrillation and improves postischemic functional recovery, direct chemical assay on biopsy specimens did not show any significant differences in ATP or PCr levels between the two groups of hearts (Fig. 11), suggesting that postischemic contractile functional recovery may not be limited by the levels of high energy metabolites within the prevailing range and is predominantly determined by other factors under our experimental conditions. In other words, we suggest that the mechanism underlying the salutary effect of re-arrest perfusion is not likely to act through direct improvement of myocardial energetic metabolism. It has been

shown that there is no clear correlation between myocardial ATP levels and recovery of contractile function[211]; and low levels of energy metabolites do not necessarily limit functional recovery during reperfusion[211]. Furthermore, some investigators found that functional recovery during reperfusion can be improved by modifying the conditions of reperfusion without affecting the levels of high energy metabolites[212-224]. Accordingly, the fundamental cause of postischemic dysfunction in our experiments is not likely to be insufficient availability of energy. Using isolated ferret hearts, Kusuoka et al[212] observed that postischemic myocardium exhibits decreased responsiveness to calcium, as manifested by a decrease in the maximal calcium-activated force. They concluded that postischemic dysfunction is mainly due to the reduced sensitivity of the contractile apparatus to calcium. Additionally, dysfunction of the sarcoplasmic reticulum (SR) is another likely cause of poor functional recovery during reperfusion[120]. It is known that the ability of the SR to sequester and release calcium from the ischemic myocardium is decreased[215]. Less calcium uptake by the SR will lead to an increase in the resting level of the intracellular calcium. The latter can cause some damage to myocytes as described above. On the other hand, reduced calcium sequestration by the SR means that less calcium will be available to be released from the SR for contraction, leading to a reduction in the calcium transient and thereby to poor contractile function[216]. Damage to the extracellular collagen matrix has also been considered as one of possible additional mechanisms responsible for cardiac dysfunction following a short period of ischemia[217-219]. It is not clear whether damage of the collagen matrix plays a role in the poor functional recovery observed in our experiments since the hearts were subjected to 8 hours of ischemia.

On the other hand, the levels of ATP and PCr measured with ^{31}P NMR are only a measurement of the apparent overall concentrations of these metabolites in a quasi-steady state

and is not indicative of the actual energy fluxes (rate of resynthesis and consumption) which is more valuable to assess metabolic homeostasis and function of mitochondria. It is possible that the myocardial capacity to generate ATP was significantly lower in the hearts reperfused with K-H solution alone compared to those subjected to S-C-S. Because poor contractile function presumably consumes less energy, any real distinction between the energy metabolic conditions of the two groups would not be observed. Therefore, it is not clearly evident from our study whether re-arrest perfusion has any impact on myocardial energetic metabolism. A second limitation of both direct chemical and non-invasive (i.e. NMR) determinations of high energy phosphates is due to the overall "averaging" of these methods. The intracellular distribution of these compounds is not uniform and utilization of compartmented substrates requires the operation of efficient transport shuttles. On a higher order, the averaging also abolishes the possibly heterogeneous distribution of energy phosphates between cells and regions of the myocardium. Thus, the results of experiments using these methodologies must be interpreted with caution, recognizing that they provide only first order approximations of the real cellular events.

2.2. **The Effect of Buffer Capacity in Cardioplegic Solution on Long-Term Cardiac Preservation**

Intracellular acidosis plays an important role in ischemic injury[1,220]. We reasoned that an increase in buffer capacity in the extracellular space could be a potential strategy to attenuate the fall of intracellular pH (pHi) and thereby to reduce ischemic injury during preservation. We found that the rate of decline of pHi was in fact significantly slower in the hearts stored in high buffer cardioplegic solution than those stored in St Thomas' solution #2. The pHi at the end of 8 hours of preservation of hearts in high buffer cardioplegic solution and St Thomas'

solution #2 was 6.61 ± 0.03 and 6.34 ± 0.07 ($p<0.05$), respectively. Our results demonstrate that the extra buffer added by the cardioplegic solution to the extracellular space reduced the rate at which intracellular acidosis developed, when compared to the low buffering capacity of St Thomas' solution #2 (Fig. 12). We also observed that hearts in both groups showed nearly identical changes in energy metabolites at comparable times during preservation (Fig. 13-16) and comparable recovery of their contractile function during reperfusion (Fig. 17-19). This clearly suggests that this intervention may not have a significant impact on myocardial cellular homeostasis (ionic gradients and function of SR and mitochondria, etc). A corollary to this finding is that, evidently, reducing the cellular acidosis to this extent does not prevent the ischemia-induced functional and metabolic damage. This cannot be extrapolated to complete abolition of cellular acidosis.

Possible explanations for this finding may be suggested. Firstly, the increase of buffer capacity created by adding 150 mmol/L MOPS to the cardioplegic solution is physiologically limited in spite of the finding of statistical significance. The extracellular space in which this buffer is confined is only about 20% of the total tissue volume[221]. Therefore, if the entire extracellular space is occupied by the cardioplegic solution after the introduction of the solution, the high buffer cardioplegic solution (containing 150 mmol/L MOPS) can only provide 30 mmoles of buffer per liter of myocardium. It can be estimated that 30 mmoles MOPS in the cardioplegic solution at a pH 7.5 can only neutralize 11.6 mmoles of acid at 12°C to maintain pH above 6.8. The rate of proton generation in ischemically preserved myocardium is relatively high; Deslauriers et al.[222] found that lactate was produced by human myocytes at a rate of 104 nmoles/min/g tissue at 12°C. Using these two factors, the buffer delivered to the extracellular compartment by the high buffer cardioplegic solution would neutralize the lactic acid

flux in the myocardium during ischemic preservation only for about 110 minutes without replenishment if the aim is to keep pH_i above 6.8. This shows that high buffer cardioplegic solution would increase the buffer capacity of the whole heart only to a limited extent for cardiac preservation exceeding about two hours. This indicates further the possibility that periodic replenishment of the contents of the extracellular space with additional cardioplegic infusions may provide substantial additional benefits.

Secondly, the protons are generated intracellularly and ideally need to be buffered by intracellular buffer systems. The extracellular buffer only helps outward diffusion of proton because the sarcolemma is not permeable to MOPS. Furthermore, the decrease in pHi is only one of several interacting factors responsible for myocardial injury. Although decrease of intracellular pH was attenuated by the high buffer cardioplegic solution, the salutary effect of a small increase in pHi on other deleterious ischemic changes may be very limited. Moreover, this increase in pHi might not be able to significantly alleviate the calcium and sodium overload and other events particularly during reperfusion. In addition, the fate of the reperfused myocardium is not solely determined by events occurring during ischemia, but also by the conditions of reperfusion. Thus, despite the fact that added buffer capacity helped maintain intracellular pH at relatively higher levels, it is possible that reperfusion-associated events may mask any of its beneficial effects. This may be one of the possible explanations for the failure of the high buffer cardioplegic solution and emphasizes the possible significance of reperfusion conditions.

In conclusion, the extra buffer capacity (150 mM) added to the cardioplegic solution increased only slightly the total buffer capacity of the whole heart, particularly for long-term cardiac preservation. This does not result in an improvement of postischemic recovery following 8 hours of preservation at 12 °C. This does not, however, preclude the

possibility that it may be beneficial when it is combined with other modifications for long term cardiac preservation, such as low flow intermittent perfusion of cardioplegic solution.

2.3. **High Concentrations of Magnesium for Heart Protection**

After reviewing the controversies about the effect of high concentrations of magnesium on cardiac preservation, we compared the effects of 0 and 16 mmol/L Mg^{++} in cardioplegic and reperfusion solutions (in the presence of 16 mmol/L K^{+}) on energy metabolites during ischemic preservation and the metabolic and functional recovery during reperfusion. We found that hearts stored with solutions containing either 0 or 16 mmol/L Mg^{++} showed similar changes in high energy phosphates during preservation (Fig. 25-27) and functional recovery during reperfusion (Fig 20-23, 28); likewise, hearts reperfused with solutions containing either 0 or 16 mmol/L Mg^{++} also exhibited comparable changes in energy metabolites and mechanical performance (Fig. 29-31). Our results strongly suggest that a high concentration of Mg^{++} in presence of 16 mmol/L K^{+} is not essential for preventing ischemic and reperfusion injury.

Magnesium is an abundant intracellular cation and an important constituent of cells[12]. It has been shown to be involved in many cellular processes under both physiological and pathological conditions[12]. Alteration of the magnesium concentration in intra- and extracellular spaces has an important impact on cellular function. It has been well known that cyclic adenosine monophosphate, the "second messenger", has an important regulatory function on cardiac cellular activity through phosphorylation of a variety of regulatory proteins, including Ca^{++} channels, troponin-C, phospholamban and C-protein[8]. The activation of G-protein (guanine nucleotide protein) is necessary for activating adenylate cyclase and is regulated by intracellular Mg^{++} [8]. Therefore, it has been suggested that intracellular Mg^{++} is important in

the cascade of functions regulating c-AMP and thereby myocardial function.

In addition, intracellular Mg^{++} has important effects on both potassium and sodium channels. Using patches of membrane containing background inwardly rectifying K^+ channels (I_{k1}), Matsuda[223] found that when the solution bathing the cytoplasmic side of the membrane contained Mg^{++}, these channels rectified inwardly. When Mg^{++} was removed, the channels no longer rectified and carried current in both directions. This suggests that intracellular Mg^{++} may prevent outward movement of K^+. Similar mechanisms may be responsible for rectification of other K^+ channels, such as the ATP-sensitive K^+ channel. These channels are activated when ATP is depleted (such as in ischemia). However, a concomitant rise of intracellular Mg^{++} would promote inward rectification of the channels. As a result, the loss of intracellular K^+ would be alleviated. Additionally, intracellular Ca^{++} levels are also modulated by both intra- and extracellular Mg^{++} [9,12]. It has been widely accepted that extracellular Mg^{++} is natural Ca^{++} channel antagonist[9,12]. Increase in extracellular Mg^{++} results in a decrease in the inward Ca^{++} current[9,224-225]. Intracellularly, Mg^{++} modulates the activity of Ca^{++}-ATPase in sarcoplasmic reticulum (SR) and regulates intracellular Ca^{++} level[9,12]. Since Ca^{++} movement across the SR membrane is a primary mechanism of regulating the levels of intracellular Ca^{++}, it would be expected that intracellular Mg^{++} also has a major effect on intracellular Ca^{++}. Furthermore, intracellular Mg^{++} is an important co-factor for Na^+/K^+ ATPase activity[8] as well as for cellular energy metabolism[192].

In considering a role of magnesium for heart preservation, two major mechanisms are thought to be involved in the "beneficial" effect of increased extracellular Mg^{++}: prevention of the loss of intracellular Mg^{++} and attenuation of the overload of intracellular calcium[185].

It is well known that intracellular Mg^{++} exists in two forms. Some is ionized and the rest (about 90%) is bound to, principally, ATP, other adenine nucleotides and RNA[9]. As expected, intracellular free ionized Mg^{++} would increase during myocardial ischemia as ATP decreases[188]. Under these conditions, one could expect to observe a loss of intracellular Mg^{++}. Conceivably, the use of a high concentration Mg^{++} extracellularly in cardioplegic and reperfusion solutions may be expected to reduce this loss, particularly during reperfusion. However, it has been shown that Mg^{++} equilibrates very slowly across the cell membrane, due to its low membrane permeability[12,192]. According to the evidence available presently, a low capacity Na^{+}/Mg^{++} exchange is the only Mg^{++} transport mechanism in some types of cells[12], and evidence for Na^{+}/Mg^{++} exchange in the myocardium has been not clearly defined[12,192]. Using ion-selective microelectrodes and fluorescent indicators, respectively, Fry[195] and Murphy[188] found that intracellular Mg^{++} is unchanged during 20 minutes of Mg^{++}-free perfusion. Using the Mg^{++}-sensitive NMR indicator, F-APTRA, Murphy and co-workers[188] showed that the basal intracellular Mg^{++} concentration in perfused rat hearts was 0.85 ± 0.1 mmol/L and 15 minutes of ischemia resulted in an increase to 2.1 ± 0.4 mmol/L. The elevated intracellular Mg^{++} concentration did not return to the normal level during 20 minutes of reperfusion. Likewise, Kirkels and his colleagues[187] reported that the intracellular Mg^{++} concentration increased tenfold to 6 mmol/L, from a basal level of 0.6 mmol/L during 15 minutes of ischemia. More importantly, they also found that reperfusion after 30 minutes of ischemia did not result in loss of total cell Mg^{++} [187]. These studies demonstrate that myocardial ischemia leads to elevation of the intracellular free ionized magnesium concentration, but reperfusion did not result in a loss of the accumulated excess of intracellular Mg^{++}.

In addition, if the Mg^{++} gradient across the membrane is in electrochemical equilibrium with a 1 mmol/L

extracellular Mg^{++} concentration and a membrane potential of -80 mV, the intracellular Mg^{++} concentration would be expected to be about 188 mmol/L as calculated by the Nernst equation[12]. This suggests that the electrochemical potential strongly favors the inward movement of extracellular Mg^{++}. Therefore, the loss of intracellular Mg^{++} during ischemia and reperfusion may not be significant. More important is that myocardial recovery during reperfusion is not limited by the level of intracellular Mg^{++}, as suggested by some investigators[187].

It was thought that ischemic and reperfusion injury resulted in damage to the membrane structure, allowing a significant passive efflux of intracellular Mg^{++} [226]. However, if membrane damage is so severe, one would expect to observe a transmembrane movement of sodium and calcium ions as well, which would result in more profound myocardial injury than caused by the loss of intracellular Mg^{++} alone. Under this condition, using high concentration of Mg^{++} in cardioplegic solution is futile.

In summary, loss of intracellular Mg^{++} during ischemia and reperfusion may not be significant. Conceivably, the prevention of this loss by increased Mg^{++} extracellularly could not be important.

Attenuation of calcium overload by blocking the calcium channels is another mechanism proposed for the possible "beneficial" role of increasing Mg^{++} concentration in perfusates[226]. Because an increase in the extracellular Mg^{++} concentration does not alter the level of intracellular Mg^{++} significantly[188,195], any effect of an increase in extracellular Mg^{++} concentration is likely related to sarcolemmal, rather than intracellular, sites. Using rat and rabbit hearts, Hall[179] found that an elevated Mg^{++} concentration only resulted in a decrease in the inward calcium current and the calcium transient but had no effect of resting basal calcium levels. Reduction of the calcium transient by Mg^{++} will lessen the amount of calcium available for coupling electrical and contractile events[179], leading to

a negative inotropic effect. Therefore, increasing the Mg^{++} concentration in the perfusate containing normal [K^+] prior to ischemia, would of course enhance myocardial energy stores and may improve postischemic recovery[11]. Conceivably, this is merely a cardioplegic effect of increased Mg^{++} concentration. Bersohn and his co-workers[11] found in the rat heart that increasing the Mg^{++} concentration in K-H solution before ischemia resulted in improved postischemic functional recovery. On the other hand, elevation of Mg^{++} concentration during the early period of reperfusion would reduce energy consumption by mechanical work and direct energy phosphates toward recovery of myocardial homeostasis[189-190]. Using ^{31}P NMR spectroscopy, Borchgrevink[189] found that after 9 minutes of normothermic ischemia, rat hearts subjected to 15 mmol/L Mg^{++} in K-H solution, during the early period of reperfusion, showed significantly higher levels of ATP and PCr and improved recovery of contractile function when compared to hearts reperfused with normal K-H solution. These authors concluded that an increased Mg^{++} concentration in the reperfusion solution reduced ischemic injury and improved postischemic recovery. However, in the presence of a high K^+ concentration, such as in cardioplegic and re-arrest perfusion solutions, the effect of magnesium on the calcium channels may become negligible. Membrane depolarization induced by a high K^+ concentration abolishes the action potential and sodium channels are in an inactivated state[16,131]. As a result, most of the calcium channels are likely to be inactivated[131,224]. Apparently, the beneficial effect of Mg^{++} can be achieved more efficiently by high concentration of potassium.

An early representative study on Mg^{++} in cardioplegic solutions was performed by Hearse and co-workers[10] in 1977. Using isolated ejecting rat hearts, they reported that Mg^{++}-free St Thomas' solution #2 resulted in less than 10% recovery of aortic flow following 30 minutes of ischemia at 37°C, whereas using a solution containing 15 mmol/L Mg^{++},

aortic flow recovered to an average of 96%. The extent of recovery declined gradually as the Mg^{++} concentration was increased further. It is now clear that this functional depression at Mg^{++} concentrations above 15 mmol/L was, at least partially, due to lowering the Na^{+} concentration, which was deliberately adjusted to compensate for the osmolarity change induced by increasing the Mg^{++} concentration, because the normal Na^{+} gradient across the membrane plays a significant role in the maintenance of intracellular calcium levels[55]. However, at present we cannot explain why the functional recovery of the hearts arrested with Mg^{++}-free cardioplegic solution was so much poorer than those arrested with 15 mmol/L Mg^{++} cardioplegic solution in their experiments (10% vs 96% recovery), apart from the general suggestion that this may be related to the experimental conditions, species and preparations. However, most recent studies have shown that recovery of cardiac output in hearts arrested with Mg^{++}-free cardioplegic solution is about 60-70%. Geffin[180] and Reynolds[227], respectively, found that 0 and 16 mmol/L Mg^{++} cardioplegic solution resulted in similar recoveries of cardiac output after 2 hours of preservation at 8°C with intermittent infusion of cardioplegic solution every 15 minutes. Using the same animal model, Takemoto[182] reported that hearts arrested with 0, 4, 8, 12, and 16 mmol/L Mg^{++} St Thomas' solution #2 with constant Na^{+} concentration of 140 mmol/L showed similar recovery of aortic flow after 30 minutes ischemia at 37°C, which is in agreement with the findings of the present study. In general, our results suggest that increased concentration of Mg^{++} in cardioplegic and reperfusion solutions in the presence of a high concentration of K^{+} does not provide substantial additional benefits for long term heart preservation.

Nevertheless, our findings should not be interpreted to suggest that Mg^{++} plays no role in cardioplegic and reperfusion solutions. It is possible that some beneficial effects of Mg^{++} may have occurred but could not be documented

in our study because of the design and conditions of our experiment. These may include its effects on the coronary vessels and membrane fluidity[228-230].

2.4. Intracellular-Type Cardioplegic Solution

In evaluating an intracellular-type of cardioplegic solution, we compared the changes in energy metabolites and mechanical performance of hearts stored in UW solution and St Thomas' solution #2. We found that hearts stored with either UW solution or St Thomas' solution #2 at 4°C showed similar changes in energy metabolites during 8 hours of preservation and mechanical performance during reperfusion, suggesting that an intracellular-type cardioplegic solution (UW solution) does not provide significant benefit on heart preservation. However, hearts preserved with UW solution at 12°C exhibited a more rapid decrease in ATP and PCr during preservation and significantly poorer recovery of contractile function than those preserved with St Thomas' solution #2 at the same temperature. These findings indicate that an intracellular-type cardioplegic solution (UW solution) is comparable to an extracellular-type cardioplegic solution for cardiac preservation only at lower temperature (4°C); but it is less effective than an extracellular-type of cardioplegic solution at 12°C. This temperature-dependence of the effects has an important clinical impact because rigid control of heart temperature during open heart surgery is difficult practically. Also, if UW solution is used for heart transplantation, temperature control is very important.

Because of its intrinsic low osmolarity, an intracellular-type cardioplegic solution usually contains high concentrations of added osmotically active impermeants. UW solution contains about 130 mmol/L of such substances (100 mmol/L Lactobionate, 30 mmol/L Raffinose) to which the cell is normally impermeable. These substances are used in the hope of preventing cellular swelling[231]. Because edema is an important consequence of ischemia and reperfusion, limiting

of water accumulation is important for successful heart storage. However, for long-term heart preservation after a single infusion of cardioplegic solution, the accumulation of water in the intracellular and interstitial spaces is unlikely to be significant in spite of the uneven distribution of water that occurs during preservation, because the total vascular space is very limited[221]. Therefore, the impermeants in UW solution may not play a significant role in cardiac preservation initiated by a single infusion of cardioplegic solution, but they may be valuable when the heart is subjected to continuous hypothermic perfusion during preservation.

It has long been recognized that oxygen-derived free radicals play an important role in tissue damage during reperfusion[232]. Xanthine oxidase is one of the major mediators in the production of free radicals. The incorporation of allopurinol into St Thomas' solution #2 has been shown to enhance postischemic recovery in rat hearts[233] However, human and pig hearts appear to have very little or no xanthine oxidase[234]. The potential usefulness of allopurinol in UW solution is therefore debatable in human applications. The role of reduced glutathione, as an antioxidant, still remains unclear[235], however, it may have more general utility as a radical quencher. Thus, many of the empirically-decided constituents of UW solution may not be essential for heart preservation.

A low concentration of sodium ions is one of the major characteristics of intracellular-type cardioplegic solutions and this may reduce sodium influx during preservation. However, it significantly reduces the sodium gradient which is important for the maintenance of other ionic gradients. In addition, low concentrations of sodium and lack of calcium in the extracellular space (such as with the use of UW solution) can have deleterious effects on membrane permeability[106,236] which may counteract part of the beneficial effect for heart preservation. These factors may provide a partial

explanation for the observation that UW solution was not found to be better than St Thomas' solution #2 for heart preservation at 4°C.

In conjunction with low concentration of sodium, most intracellular-type cardioplegic solutions also contain no calcium. Although this attenuates calcium overload, the use of an intracellular-type cardioplegic solution may predispose the heart to the calcium paradox. However, the use of calcium-free solutions does not necessarily result in a calcium paradox, because this phenomenon depends on other factors as well. For instance, the calcium paradox may be inhibited by hypothermia[30]. The severity of the calcium paradox is also dependent on the duration of the period during which the heart is exposed to a calcium-free solution[31]. If this period is extended, the protective effect of cooling is decreased[30]. In the present study, we have found that, some hearts (4/8) stored with UW solution at 12°C exhibited rigor, showed no recovery of contractile function upon reperfusion, and no ATP or PCr peaks were observed with ^{31}P NMR. These findings suggest that the myocardium completely lost these energy metabolites possibly due to damage of the cell membrane, a manifestation of the calcium paradox. However, hearts stored at 4°C with UW did not show such evidence of the calcium paradox.

The concept of the calcium paradox was introduced by Zimmerman and co-worker[237] in 1966, referring to the sudden loss of electromechanical activity, release of cellular constituents and occurrence of microscopic hypercontracture bands during Ca^{++} replenishment following a period of Ca^{++} depletion or Ca^{++}-free perfusion[237]. Although several theories have been proposed, the exact mechanism whereby this occurs has not been fully elucidated[31]. Recently, evidence from different groups suggests that disruption of the cell membrane plays a critical role in this phenomenon[238-240]. Briefly, the adhesive material connecting cells at the intercalated disc junctions is the calcium dependent

calhedrin protein[238]. Removal of Ca^{++} from the perfusate results in loosening of the intercalated disc junctions. When Ca^{++} is replenished, it enters the cells through this weakened junction, leading to hypercontracture. The contracture in turn tears the intercalated disc junctions and cell membranes. According to this hypothesis, an overload in intracellular sodium may not be a prerequisite for the calcium paradox[31, 241], and this is supported by Van Echteld[242] using ^{23}Na NMR spectroscopy to monitor intra- and extracellular Na^+ in isolated rat hearts during 30 minutes of Ca^{++} depletion, who found that there was no significant increase in intracellular sodium. Therefore, UW solution can predispose hearts to the calcium paradox.

If the calcium paradox plays a role in reperfusion injury, then the addition of 0.5 mmol/L calcium to the UW solution should improve cardiac preservation by attenuating its occurrence. This is consistent with our observation that hearts arrested with the Ca^{++}-containing UW solution showed no loss of high energy phosphates and no occurrence of "stone heart" upon reperfusion while some of the hearts arrested with unmodified UW solution did so.

We found that the addition of a small amount of calcium to UW solution apparently prevented the manifestation of the calcium paradox, but it imposed another stress on the heart, namely an increase in diastolic pressure, which presumably is due to an increase of intracellular calcium via Na^+/Ca^{++} exchange. It is well known that the Na^+/Ca^{++} exchange system is important in regulating intracellular Ca^{++} levels[243-45]. Under physiological conditions, calcium which enters the cell during each action potential, is extruded mainly by the Na^+/Ca^{++} exchange system, energized from the Na^+ electrochemical gradient across the cell membrane. Thus, the maintenance of a low level of intracellular Ca^{++} is to some extent dependent on the Na^+ gradient across the cell membrane, or on extracellular Na^+ [13,246]. A large decrease, or removal, of Na^+ extracellularly can reverse the Na^+/Ca^{++}

exchange and bring about an efflux of Na^+ coupled with a gain of Ca^{++}, which is the case when Ca^{++} is added to UW solution. The exact relationship between sodium and calcium in intra- and extracellular spaces has be decrisbed above (Introduction, 2.2.1). Because UW solution contains about 140 mmol/L K^+, the membrane potential (E) is near zero. When this solution is used to arrest the heart, the intracellular Ca^{++} concentration is theoretically zero, because $[Ca^{++}]_o$ = 0 mmol/L, suggesting that Na^+/Ca^{++} exchange strongly favors Ca^{++} efflux, or at least that $[Ca^{++}]_i$ cannot be increased by the Na^+/Ca^{++} exchange system. This may be why hearts arrested with UW solution did not show an increase in diastolic pressure. When 0.5 mmol/L $CaCl_2$ is added to UW solution, leading to 0.08 mmol/L free Ca^{++} in the vascular space, $[Ca^{++}]_i$ will increase significantly by about two orders of magnitude, to pCa=5 from a normal value of 7.1 as estimated from the equation ,

$$[Ca^{++}]_i = [Ca^{++}]_o\{\frac{[Na^+]_i}{[Na^+]_o}\}^3$$

$$= [0.08]\{\frac{10}{20}\}^3$$

$$= 0.01 \text{ mmol/L (pCa=5)}$$

This intracellular Ca^{++} level can, theoretically, lead to contracture with 70 % maximum tension[247].

Because partial recovery of the Na^+ gradient occurs, a proportion of increased intracellular Ca^{++} can be extruded by Na^+/Ca^{++} exchange and the intracellular Ca^{++} overload is also buffered by the sarcoplasmic reticulum and the mitochondria[247-248], the actual concentration of intracellular Ca^{++} may not reach the level estimated above. Therefore, hearts arrested with the Ca^{++}-containing UW solution showed only a transient increase in diastolic pressure of 5 mmHg and lasting only about 2 minutes. It is not clear how significant this increase in diastolic pressure (intracellular Ca^{++}) is because of its small magnitude and transient duration. It is possible that this Ca^{++} increase may result in some deleterious effects on the function of SR and mitochondria in

the myocardium to which supply of oxygen and substrates are terminated. Thus, an increase in intracellular Ca^{++} exacerbates high energy phosphate breakdown as shown by the more rapid decrease in PCr (Figure 40). In general, the results suggest that an intracellular-type cardioplegic solution is comparable (at 4°C) or inferior (at 12°C) to an extracellular-type of cardioplegic solution for cardiac preservation. The calcium paradox is partially responsible for the failure of UW solution in cardiac preservation at 12°C. Addition of 0.5 mmol/L $CaCl_2$ to UW solution improves its cardiac preservation potential, but induces a transient and modest decrease in diastolic compliance, which may be attributable to an increase in intracellular calcium, and accelerates high energy phosphate breakdown. Therefore, the sodium concentration in an intracellular-type cardioplegic solution may need to be increased.

2.5. Long-Term Cardiac Preservation and Modifications of Cardioplegia and Reperfusion

As mentioned before, the primary factor limiting the number of heart transplantations performed is the lack of adequate donor hearts. One of the main factors contributing to this is the relatively short period for which a donor heart can be preserved. At present, it is not yet possible to store donor hearts longer than five hours before implantation, which was achieved in the late 1970's. Clinical studies show that hearts subjected to preservation longer than five hours may show early failure after transplantation. The present study was attempted to determine whether cardiac preservation time may be significantly increased without the risk of heart damage by "optimizing" cardioplegia and reperfusion management. We investigated some selected points in cardioplegia and reperfusion.

We found that re-inducing cardiac arrest during the early period of reperfusion, by using a specially designed secondary cardioplegic solution, significantly improved

postischemic recovery of the heart's contractile function after 8 hours of hypothermic preservation. This manipulation did not affect the levels of myocardial ATP, PCr and P_i. Furthermore, this beneficial effect of secondary cardioplegic solution was also achieved by increasing the potassium concentration in K-H solution during reperfusion. This suggests that inhibition of myocardial electromechanical activity during early reperfusion is critical to improve postischemic recovery, but the underlying mechanism needs further investigation. Moreover, the results demonstrate that the fate of preserved myocardium is closely dependent on the conditions of reperfusion after even 8 hours of hypothermic preservation.

In addition, this study showed that neither an increase of the buffer capacity (to 150 mmol/L) nor increase of Mg^{++} concentration (to 16 mmol/L) in a cardioplegic solution, not the use of an intracellular-type cardioplegic solution provided substantial benefit to the myocardium that had been subjected to 8 hours of ischemic preservation, when compared to the commonly-used cardioplegic solution (St Thomas' solution #2).

Beside the conclusions of each individual study described above, this work may suggest that under the condition of single infusion, modifications of cardioplegic solution itself may have very limited potential to ameliorate ischemic injury and improve postischemic recovery of the heart that is subjected to long-term preservation; this may be due to the small volume of the extracellular space. On the other hand, this may also suggest that multiple infusions of cardioplegic solution (intermittent cardioplegia) may be an alternative or necessary refinement to prolong the safe preservation time. With respect to the composition of the cardioplegic solution, we think that neither an extra- nor intracellular-type cardioplegic solution may be optimal. Extracellular-type cardioplegic solution contains "high" concentrations of sodium (120-140 mmol/L) and calcium (1.2-2

mmol/L), which provides large pools of sodium and calcium in extracellular space and could aggravate sodium and calcium overload in the ischemic myocardium. In contrast, intracellular-type cardioplegic solution may subject the heart to the calcium paradox because it contains very little or no calcium. Adding calcium to an intracellular-type cardioplegic solution could be expected to cause an increase of intracellular calcium through Na^+/Ca^{++} exchange because the solution usually contains very little sodium. Therefore, we propose that the concentrations of sodium and calcium in an cardioplegic solution should be in the intermediate range between those of intra- and extracellular-type cardioplegic solutions.

This study was performed in isolated buffer perfused rat and pig hearts, which were not in normal physiological conditions. It has been noted that perfusion of isolated hearts with a crystalloid solution subjects the myocardium to "ischemia," suggesting that the experimental model in this study was not ideal. Variations in the recovery of metabolism and contractile function following 4 or 8 hours of ischemic preservation may be partially related to the instability of the preparation. It must be noted, however, that the "foundations" of cardioplegic practice, namely those of Hearse et al, were similarly non-physiologic preparations.

It has been demonstrated that interventions of cardioplegia or reperfusion may change either rate or/and extent of recovery during reperfusion. However, it is almost impossible to distinguish between an accelerated rate and an improved extent of recovery with isolated heart models because contractile function and the levels of some metabolites may take several days or weeks to recover fully, even after a short period of ischemia. Therefore, there are limitations associated with most experimental models used currently for the study of myocardial ischemia and preservation. Although S-C-S was shown to improve postischemic functional recovery, we are still not sure

whether this intervention increases the extent of recovery (irrespective of the duration of reperfusion) or whether it merely accelerates the rate of recovery without affecting its ultimate extent.

As noted by other investigators, we found that the extent of functional recovery during reperfusion did not correlate with the recovery of metabolic parameters. Metabolic parameters are quickly normalized during early reperfusion, whereas the depression of ventricular function is more long-lasting, suggesting that myocardial energy metabolism is not only factor to determine functional recovery. In other words, functional recovery following cardiac preservation may not be predicted by metabolic parameters alone. Since cardiac function ultimately determines the survival of the patient, contractile functional recovery is the most sensitive and important parameter to characterize the recovery of hearts and to assess heart protection. In addition, the present study focused only on myocardial contractile element (myocyte). It has now become clear that other tissue components of the heart may be equally vulnerable to ischemic insult and might be determinant of the recovery and survival of heart as functioning whole organ. There is considerable evidence that reperfusion injury is associated with functional and structural alterations of the coronary vessels, which range from increased capillary permeability and impaired vasodilator response to capillary obstruction and vascular rupture. Thus, in future studies, it will be important to consider the role of other tissue components.

GENERAL CONCLUSIONS

The present study was carried out to determine whether selected modifications of current techniques could be expected to improve cardiac preservation and to provide a physiological rationale for the modifications. It was hoped that this study would provide the beginning of a rational, comprehensive approach to the improvement of cardiac preservation. Isolated pig hearts and rat hearts were subjected to various protocols in which myocardial high energy metabolites, intracellular pH and contractile function were followed using ^{31}P NMR spectroscopy and functional measurements. On the basis of our findings, we conclude that:

1. Re-arrest perfusion during the early period of reperfusion improves heart recovery following ischemic preservation. This does not appear to be due to improved energetic metabolism.

2. Increased buffer capacity in a cardioplegic solution helps maintain intracellular pH during preservation, but the effect is very limited, presumably due to the small volume of the extracellular space and relatively high rate of proton generation. Furthermore, it does not offer a significant improvement to energy storage or generation and recovery of contractile function following ischemia. However, it might be beneficial to combine high buffer with other modifications in a cardioplegic solution.

3. A high concentration of Mg^{++} in the presence of increased K^+ concentration (>15 mmol/L) does not offer any additional benefit for cardiac preservation, because the salutary effects of Mg^{++} can be achieved more efficiently by K^+. Thus, it may be superfluous to incorporate a high concentration of Mg^{++} to cardioplegic and re-arrest perfusion solutions.

4. An intracellular-type cardioplegic solution may not be appropriate for cardiac preservation because of rapid decrease of high energy phosphates and the possibility of the development of the calcium paradox. Adding calcium to an intracellular-type cardioplegic solution prevents the occurrence of the calcium paradox, but it induces a transient increase in ventricular diastolic pressure and a rapid decrease of energy metabolite concentration. We have no information on any changes in metabolite fluxes under these conditions. The observed effects (rapid decrease of PCr and transient increase of diastolic pressure) may likely be due to an increase in intracellular calcium. Increasing the sodium concentration appears to be very logical and/or necessary for a means of modifying an intracellular-type cardioplegic solution.

The present study has significant clinical implications because the conditions of cardioplegia and reperfusion are under the complete control of the cardiac surgeon and proper manipulation may be expected to prolong the safe preservation time and concurrently improve postischemic myocardial recovery. The data presented here may also be useful for routine open heart surgery because a short period of S-C-S can prevent ventricular fibrillation. However, this study was performed in isolated rat and pig hearts and extrapolation to clinical settings must be done with caution. Nevertheless, we believe that the principles established here deserve further study with a view to clinical applicability.

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