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Optimal storage temperature and benefit of hypothermic cardioplegic arrest for long-term preservation of donor hearts: a study in the dog

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Abstract. Currently, for practical clinical purposes, the preservation of donor hearts is limited to about 4 h. Transplantation must be finished within this period to assure complete functional recovery upon reperfusion. From the clinical setting it is well known that hypothermia results in a better myocardial preservation during ischemia. During ischemia, rapid catabolism of high-energy phosphates (e.g., ATP and creatine phosphate) occurs. The purpose of this study was to investigate the influence of temperature during a 24-h preservation period on the rate of catabolism of ATP and on the rate of accumulation of breakdown products (ADP, AMP, adenosine, inosine, hypoxanthine, and xanthine). For this purpose, hearts were excised and stored for 24 h at 0.5°, 12°, or 18 °C. In addition, the effect of initial cardioplegic arrest was compared with simple normothermic excision of the heart followed by 24 h in cold storage. It was found that the higher the storage temperature, the higher the rate of catabolism of high-energy phosphates and, hence, after 24 h, the lower the final ATP level and the higher the level of breakdown products, mainly nucleosides. It was also found that the initial cardioplegic arrest strongly benefits the preservation of high-energy phosphates as a result of the ATP-sparing effect.

Key words: Preservation - High-energy phosphates - Hypothermia - Cardioplegia - Donor hearts.

The preservation of organs - and especially of the heart - is a great challenge to the physiologist, the biochemist, and the surgeon because their immediate functional recovery upon reperfusion is vital for survival of the recipient. Cold cardioplegic arrest and subsequent cooling of the heart are two of the most important components of myocardial protection [16, 24].

In the clinical setting, preservation of the heart is necessary during surgery when a cardiopulmonary bypass is performed, e.g., during valve replacement and aortocoronary bypass grafting. Acute and short-term (1-2 h) myocardial preservation is obtained by cardioplegic arrest and hypothermia. Cardioplegic arrest can be potassium arrest [18], combined potassium and magnesium arrest [18], or arrest induced by sodium reduction to near intracellular concentrations and lowering of the calcium concentration [4]. In short-term preservation, only mild hypothermia is induced for cardiac protection: myocardial temperature varies between 15° and 20 °C in the period following infusion of cardioplegic solution [7].

For cardiac transplantation, preservation is limited to about 4 h to prevent functional deterioration. Slush-ice is used to preserve the heart; hence, hypothermia is more profound and myocardial temperature approximates 0 °C.

For long-term (12-24 h) preservation, different preservation techniques can be applied: single-dose cardioplegia and simple cold storage, multidose cardioplegia, or continuous perfusion. Recently, we showed that optimal functional recovery without any positive inotropic support was possible after cardioplegic arrest, extirpation, continuous hypothermic (2°-4 °C) perfusion with a cardioplegic solution for 24 h, and after subsequent transplantation and blood reperfusion [8]. Other authors found the same result using modified Krebs-Henseleit solutions [13, 30, 31]. However, the optimal storage temperature is still not well established. Some authors

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have found increasing protection with a temperature decrease down to 4 °C [16, 23], but others have concluded that cooling to 10°, 15°, or only 20 °C offers better metabolic and functional recovery of the preserved hearts [5]. Also, for long-distance procurement of donor hearts, higher storage temperatures, e.g., room temperature, would involve less complicated technical devices.

In the present study, we investigated the influence of storage temperature on myocardial preservation. The evaluation of myocardial preservation was based upon the relationship between function and high-energy phosphate tissue level [3, 10]. The breakdown of energy-rich phosphates is most probably the triggering mechanism of ischemic damage, and a critical level of these phosphates will determine functional recovery during reperfusion. Therefore, high-energy phosphates were determined in myocardial tissue stored for 24 h at different temperatures.

Methods

Eight dogs were anesthetized by subcutaneous administration of 0.4 ml/kg Hypnorm, Duphar (10 mg fluanizone/0.2 mg fentanyl pro ml) followed by intravenous injection of 10-15 mg/kg sodium pentobarbital (Nembutal, Abbott). Following tracheal intubation, artificial respiration was maintained with air and oxygen (60/40) by means of a Mark 7 (Bird) respirator. The respiration frequency was 15 cycles/min.

In four dogs (series A), a median sternotomy was performed and the heart excised after cross-clamping of the aorta without the use of any cardioplegia. The heart, which was still beating, was immediately immersed in a cold Ringer solution. Following bradycardia, the heart stopped beating within 1-2 min after extirpation. The heart was immersed in an ice-cold (2°-4 °C) Ringer solution and placed in the cold room (4 °C) in a box filled with slush-ice. After equilibration, the temperature of the myocardium reached 0.5 °C. The heart was stored in this condition for 24 h. Serial transmural left-ventricular needle biopsy specimens (Trucut needle, Travenol Laboratories) were taken before cardiac arrest (control) and at regular time intervals during the preservation period.

In another four dogs (series B), a median sternotomy was performed, the pleural cavities entered, and the pericardium transected. After cutting the inferior vena cava and closure of the superior vena cava, Liquemine (heparin, Roche, 300 units/kg) was administered. The ascending aorta was cross-clamped and infusion of Bretschneider cardioplegic solution[1] [4] was begun (200 ml/min for 8-10 min). In this way, the heart was arrested in diastole within 1 min after the start of infusion. A temperature probe was inserted into the lower part of the interventricular septum, and the temperature was recorded continuously from the beginning of cardioplegia. After infusion, the heart was excised and immediately immersed in an ice-cold (2°-4 °C) Ringer solution. In the cold room, 15 to 20 min after the onset of cardioplegia, the left ventricle was cut into three slices: the apex, the middle portion, and the base. These three parts were randomly stored at 0.5°, 12°, or 18 °C in fresh Ringer solution for the subsequent 24 h. The temperatures 12° and 18 °C were maintained with a thermostatic cooling-heating system (WTH 500) placed in the cold room. The 0.5 °C was obtained by putting the myocardial section into a glass beaker filled with Ringer solution. This beaker, in turn, was immersed in slush-ice water. Serial transmural myocardial needle biopsies were taken prior to aortic cross-clamping (control biopsy) and at regular time intervals after cardiac arrest during the subsequent preservation period.

The biopsy specimens were immediately cooled in liquid nitrogen, lyophilized, and kept frozen (−80 °C) until analysis. The lyophilized tissue was weighed and homogenized in ice-cold 0.6 N $HClO_4$ (1 ml/1.5 mg dry weight), first with a Polytron homogenizer (Pleuger) for 2 times 10 s and, second, manually with a simple glass homogenizer. One milliliter of this solution was centrifuged for 20 s (Eppendorf centrifuge) and to 0.6 ml of the supernatant, 0.4 ml of 1 N ice-cold $KHCO_3$ was added for neutralization.

Adenosine triphosphate (ATP) and creatine phosphate (CrP) were determined by bioluminescence techniques (LKB 1250; ATP CLS-luminescence kit, Boehringer). The nucleotides [adenosine di- (ADP) and monophosphate (AMP)], the nucleosides [adenosine (ADO) and inosine (INO)], and the purine bases [hypoxanthine (HX) and xanthine (X)] were determined in one single 30-min run [32] by means of high-performance liquid chromatography (HPLC). The instrument used was a Varian HPLC model 5500 equipped with an UV detector (Varian model UV-5, 254 nm). A linear concentration gradient from 100% of 0.15 *M* ammonium dihydrogen phosphate (pH 6.0) to 80% $NH_4H_2PO_4$ and 20% of a 50/50 mixture acetonitrile/methanol was induced over a reversed phase column (LiChrosorb RP-18, 4 µm, Merck). All chromatograms were monitored on a microcomputer data system (Varian, DS 601). The peak identification was based on the retention times, which were checked daily with a synthetic mixture of standards. Tissue levels were expressed as micromolars per gram dry weight (µmol/g DW).

Results

Temperature of the myocardium during cardioplegia and preservation period

Myocardial temperature was registered in both experimental series before aortic cross-clamping, during cardioplegia, and during the subsequent preservation period. In series A, temperature started to decrease only after excision and after immersion in the ice-cold (2-4 °C) Ringer solution. It has to be stressed that in the initial period after excision, the hearts were still beating. In series B, temperature decreased from the onset until the end (8-10 min) of cardioplegia. The hearts stopped beating within 1 min and were excised at the end of cardioplegia. Thereafter, myocardial temperature further declined as a result of immersion in the ice-cold Ringer solution. The temperature curves obtained in both experimental series are shown in Fig. 1. In the first moments, a sharp decrease in temperature was observed in both series despite an initial delay of

[1] Composition Bretschneider cardioplegic solution (mM): NaCl = 15; KCl = 9; $MgCl_2$ = 9; histidine = 180; histidine × HCl = 15; tryptophane = 2; mannitol = 30

5-10 min in series B as a result of the cardioplegic infusion. In series A, the temperature after 4 min immersion was (mean ± SD) 18.2 ± 1.98 °C; after 30 min it was 8.3 ± 1.33 °C, after 60 min 3.4 ± 0.90 °C, and after 4 h 0.6 ± 0.14 °C. In series B, in the 0.5 °C preservation subgroup, myocardial temperature was 13.9 ± 1.56 °C 4 min after the start of infusion of cardioplegic solution, 4.9 ± 0.49 °C after 30 min, 2.7 ± 0.33 °C after 60 min, and 0.6 ± 0.11 °C after 4 h. In the 12 °C and 18 °C subgroups, myocardial temperature equilibrated completely within 1 h after the start of cardioplegia. As can be delineated from the temperature shifts between 15 and 60 min in series A and between 8 and 30 min in series B, simple storage of the heart in a completely closed ice-box but kept at room temperature would cause a considerable delay in reaching the final equilibration temperature of slush-ice water. Storage in the cold room (4 °C air temperature) and refreshment of the immersion solution caused, again, a faster decrease in the myocardial temperature between 30 and 60 min in series A and between 15 and 30 min in series B in the 0.5 °C subgroup. Myocardial temperature in this subgroup was only significantly different from the temperatures observed in series A at 8, 30, and 180 min ($P<0.01$).

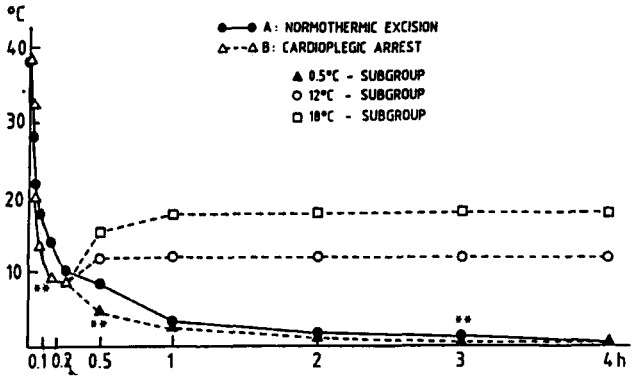

Fig. 1. Myocardial temperature during cardioplegic arrest, excision, and subsequent 24 h storage at 0.5°, 12°, or 18 °C. Statistically significant differences between simple normothermic excision, followed by immersion in ice-cold Ringer, and cardioplegic arrest, followed by immersion in ice-cold Ringer, are indicated: ** $P<0.01$

Normothermic ischemic arrest followed by cold storage: effects on high energy phosphate content (series A)

Normothermic excision of the heart and subsequent cold storage for 24 h caused a rapid and progressive breakdown of high-energy phosphates (see Fig. 2). A significant depletion of the myocardial ATP content occurred already during the first hour of cold storage. At that time, the ATP level dropped to 64% of the control value ($P<0.05$). CrP levels decreased even faster during this time interval and reached 15% of the control value ($P<0.001$). After 6 h of cold storage, there was only 6% of the normal myocardial ATP content and 3% of the normal CrP content ($P<0.001$). After 24 h of cold storage, the same low levels of ATP and CrP were found.

Determination of the optimal temperature of cold storage (series B)

After excision of the heart and cutting the left ventricle into slices, these three parts of the four investigated hearts were stored at different temperatures (0.5°, 12°, 18 °C) for the following 24 h. Transmural biopsy specimens were taken at distinct time intervals during the experiments. The first two biopsies were taken before dividing the heart into three parts: the first biopsy was taken as a control from the beating heart before arrest (indicated as "−5"), the second immediately after cardioplegic arrest ("0"). The following biopsies were taken at the same

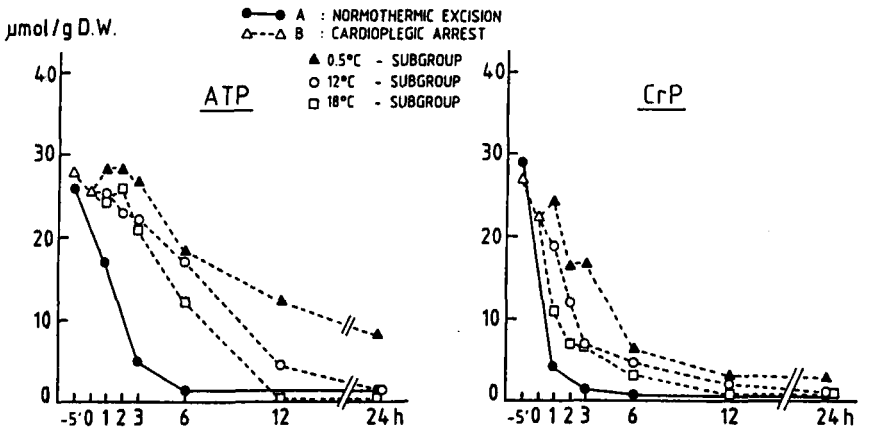

Fig. 2. ATP and CrP levels measured in the myocardial tissue stored for 24 h at different temperatures after cardioplegic arrest or after simple normothermic excision and stored at 0.5 °C

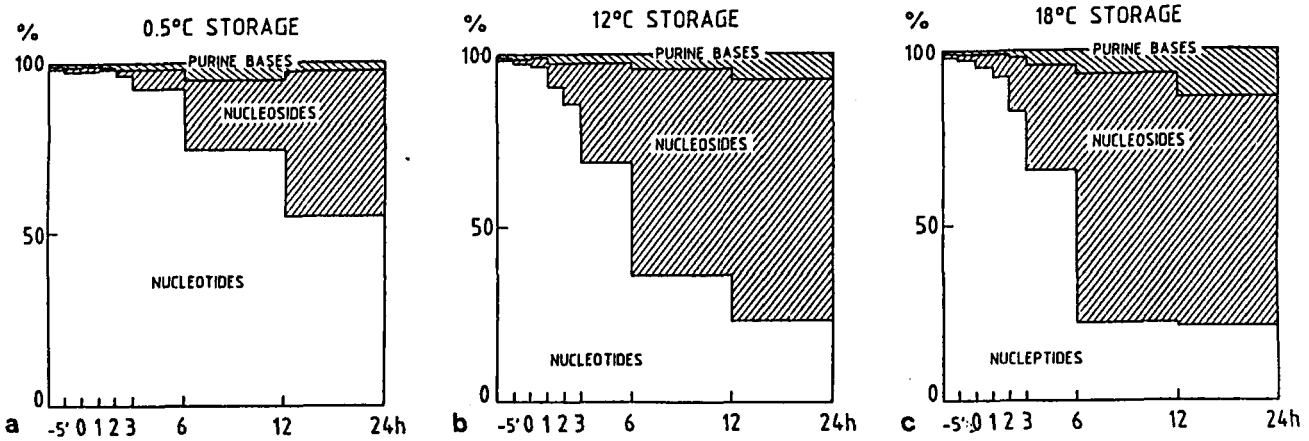

Fig. 3a-c. Total amount of nucleotides, nucleosides, and purine bases measured at different times during the 24-h preservation period: **a** 0.5 °C-storage temperature; **b** 12 °C-storage temperature, and **c** 18 °C-storage temperature

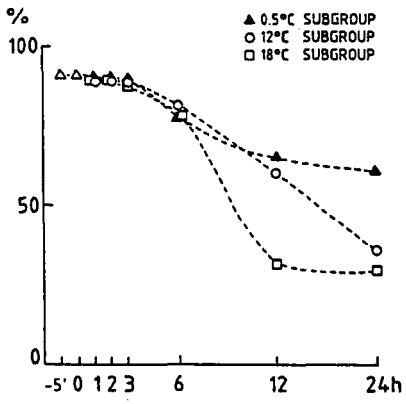

Fig. 4. Energy charge (Bretschneider-cardioplegic arrest) measured as (ATP+1/2ADP)/(ATP+ADP+AMP) obtained in myocardial tissue stored for 24 h at different temperatures

moment in the three left ventricular parts 1, 2, 3, 6, 12, and 24 h after the onset of infusion of cardioplegic solution.

The ATP content remained unchanged in the 0.5 °C subgroup during the first 3 h of cold storage and declined progressively from that time on during the whole period of storage (Fig. 2). At 6 h of storage, ATP was 65% and at 12 h 44% of the control value. This difference with the control value was significant ($P<0.05$ and $P<0.01$, respectively). At 24 h, the ATP content was still about 30% of its control value (8.58 μmol/g dry weight ±4.57 SD versus 28.27 μmol/g dry weight ±2.25 SD, respectively, $P<0.01$). In the 12° and 18 °C subgroups, the ATP level declined continuously during the whole period of storage. The rate of ATP breakdown was faster when the temperature during storage was higher (see Fig. 2). The ATP was already significantly lower than the control value after 3 h of storage (22.4 μmol/g dry weight ±3.11 SD at 12 °C and 21.34 μmol/g dry weight ±4.10 SD at 18 °C, $P<0.05$). Less than 4% of the initial amount of ATP was left after 24 h of storage at 12° or 18 °C.

Changes in CrP level occurred even faster. CrP had already decreased significantly in the 0.5 °C subgroup after 2 h of cold storage (16.79 μmol/g dry weight ±7.74 SD versus 27.60 μmol/g dry weight ±2.89 SD, respectively, $P<0.05$). After 6 h 24%, after 12 h 14%, and after 24 h only 8% of the original CrP level was left ($P<0.01$). In the 12° and 18 °C subgroups, the CrP level had, after 1 h storage, already decreased significantly as compared to the control value ($P<0.01$). After 6 h of storage, respectively, 20% and 14% and after 12 h 8% and 4% of the control CrP levels were left. In both subgroups, CrP was only 4% of the control value after 24 h of storage.

During storage ATP was catabolized. In the 0.5 °C storage group the breakdown products, ADP and AMP, accumulated to the highest levels observed. As a result the total level of nucleotides (ATP+ADP+AMP) decreased only slightly during the 24-h preservation period (Fig. 3, left panel). In this subgroup only a slight accumulation of nucleosides (ADO+INO) was observed. Changes in the level of major purine bases (HX+X) were hardly measurable.

In the 12 °C storage group there was a more pronounced breakdown of ATP, while no accumulation of ADP or AMP was observed. This resulted in a more pronounced decline in total nucleotide level and in a greater accumulation of nucleosides (Fig. 3, middle panel). In the 18 °C storage group the ATP breakdown occurred the fastest, with a more pronounced accumulation of ADO and INO and even of HX and X (Fig. 3, right panel).

Probably the strongest argument for the statement that the degree of breakdown of high-energy phosphates correlates with the temperature of storage is shown in Fig. 4. The energy charge, calculated as (ATP+1/2 ADP)/(ATP+ADP+AMP), decreased in the three subgroups to the same extent for up to 6 h of storage. After 6 h, the energy charge declined faster at 18 °C, and after 12 h the same rapid decline was observed in the 12 °C subgroup. Beyond 12 h of preservation, the energy charge remained only relatively constant in the 0.5 °C subgroup; after 24 h, the energy charge still amounted to 63%. At 24 h, the energy charge was low in both the 12 °C and 18 °C groups.

Discussion

Hypothermia

Toward the end of the nineteenth century, Van't Hoff (1884) and Arrhenius (1889) studied the thermal coefficient for chemical processes. Van't Hoff found that the velocity of various organic reactions diminished by a factor of two for every 10 °C reduction in temperature and he introduced the concept of Q_{10} to describe these relationships. Arrhenius, however, found that although biochemical reactions exhibited exponential decreases in velocity in relation to decreases in absolute temperature, this relationship does not always yield a Q_{10} of 2. Empirically, Arrhenius developed equations which state that there is a linear relationship between the logarithm of the rate of chemical process and the reciprocal of absolute temperature (which, for a given reaction, has come to be known as its Arrhenius plot), and that the activation energy of these processes can be estimated indirectly through the measurement of their velocities at different temperatures [1]. Generally, chemical reactions that depend upon a high energy of activation have higher Q_{10} values (above 2 or 3) than reactions with low activation energies. Different Q_{10} characteristics between biochemical and biophysical reactions lead to a heterogeneous cooling response between different cellular processes and between organs. Cellular energy processes such as phosphorylation may decline rapidly, whereas diffusion of ions, metabolites, and water may be reduced to a lesser extent.

The heterogeneity of cellular responses to hypothermia is further compounded by the phenomenon of lipoprotein phase transitions. According to the Singer-Nicholson fluid-mosaic model of membrane structure, plasma membranes and intracellular membranes are composed of a classic bilayer in which protein elements are embedded. In normothermia, the proteins exist predominantly in a fluid state, permitting a high degree of freedom for conformational changes of membrane-bound protein and for enzyme complexes to be formed. As cooling progresses, membrane lipids undergo a phase transition from their dynamic fluid state to a more highly ordered gel state characterized by closer packing of phospholipid molecules, an increase in bilayer thickness, and a reduction in mobility of membrane proteins and enzyme complexes [2, 12]. One important consequence of membrane phase transitions is the reduction of permeability for molecular transport [2]. There is a steep increase in the activation energy of membrane-bound reactions in hypothermia. Such reactions may, therefore, show a dramatically decreased rate of activity, expressed as a break in the linearity of the Arrhenius plot. Lipid phase transitions and their associated metabolic consequences may largely determine the lower limits for organ function during hypothermia [28]. Even at 4 °C, metabolic processes are very slowly operative [14].

A clear relationship between high-energy phosphate preservation and storage temperature can be delineated (Fig. 3): the total level of nucleotides (ATP+ADP+AMP) is best preserved at the lowest temperatures; the breakdown of these nucleotides into nucleosides (adenosine and inosine) and the further catabolism of these nucleosides into the purine bases hypoxanthine and xanthine progresses at an increased rate, the higher the preservation temperature and the longer the preservation time become. This may suggest gradually increasing enzymatic (myokinase, 5'nucleotidase, adenosine deaminase) activity at progressively higher storage temperatures.

Cardioplegia

Although myocardial ischemia itself induces a rapid and dramatic reduction in contractility, residual mechanical activity may persist for some time. In addition, ventricular fibrillation will occur rapidly, and both mechanisms will drain the heart of vital and limited supplies of cellular energy that otherwise might be used for the preservation of cellular integrity.

The most effective method for prolonging the ischemic tolerance of the heart is to switch off the main consumer of energy - the contractile system - by artificial arrest of the heart. In the experiments described above, the heart was arrested by a reduction of the sodium level to the intracellular concentrations with a simultaneously decreased level of

calcium (Bretschneider cardioplegia). Using cardioplegia, we were able to conserve the myocardial high-energy phosphate level completely for at least 3 h. As compared to normothermic ischemic arrest of the heart, the myocardial tissue ATP level remained at a normal level much longer using cardioplegia. In the case of simple ischemic arrest, ATP content had already decreased significantly within 1 h (see Fig. 2). Because all hearts, including those excised after cardioplegic arrest, were stored cold at the same temperature, the ATP-sparing effect must have been due to the immediate diastolic arrest of the heart. Indeed, magnesium, which is contained in the Bretschneider cardioplegic solution (9 m*M*) has been shown to be a highly effective component of protective infusates [6, 20, 21, 27, 29]. Magnesium and potassium [as contained in the well-known N.I.H. (National Institutes of Health) or St.Thomas' Hospital cardioplegic solutions] are the most abundant intracellular cations. Magnesium forms a complex with ATP to act as a substrate for enzymatic reactions underlying muscle contraction and relaxation and as a cofactor for energy-transferring reactions, oxidation, synthesis, and transport. In ischemia, with the decrease in ATP, there is an increase in ionized magnesium and a loss of magnesium and potassium to the extracellular space. Hearse et al. [17] demonstrated, in a detailed dose-response study (0-50 m*M*), that the increased concentration of cytosolic ionized calcium that occurs with reperfusion can be alleviated by magnesium at a dose of 16 mmol/l. Hearse et al. [17, 18] and Hearse [15] are of the opinion that magnesium is protective at the extracellular site as a result of: (1) the reduction of the trans-sarcolemmal magnesium gradient, (2) the reduction of the potassium efflux and calcium influx during ischemia, and (3) the reduction of the incidence of dysrhythmias. As reported recently, however, a 50% reduction in the magnesium concentration, i.e., to 4 m*M*, in the cardioplegic solution seems to have better protective properties for high-energy phosphates than high-dose cardioplegia [11, 26].

The reduction in the sodium and mainly in the calcium levels in a cardioplegic solution (e.g., in the calcium-free Bretschneider cardioplegic solution) renders a potential danger to the myocardium. It has long been known that calcium-free perfusion of the heart induces the development of a "calcium paradox" when the heart is reperfused with a calcium-containing solution [25]. However, it was shown that hypothermia protects the heart from the deleterious effects of a calcium paradox [19]. A protective action of low sodium against the calcium paradox was also demonstrated and can be explained by a delay in the calcium removal from an essential membrane site during the calcium-free perfusion [22, 33]. Upon addition of 50 μmol/l of calcium ions to a calcium-free solution, the risk of a calcium paradox is significantly reduced [9]. As recently reported, no calcium paradox occurred after orthotopic transplantation of dog hearts preserved for 24 h using continuous hypothermic perfusion with Bretschneider cardioplegic solution [8].

Conclusions

In conclusion, the influence of storage temperature (0.5°, 12°, and 18 °C) on high-energy phosphate preservation was studied using the low-sodium, calcium-free Bretschneider cardioplegia. The best preservation of high-energy phosphates was found at the lowest temperature; hence, the optimal temperature for long-term preservation is 0.5 °C. However, mere storage of the heart at this low temperature is no guarantee of adequate preservation of high-energy phosphates. As compared to simple normothermic excision, cardioplegic arrest strongly favors the constancy of the nucleotide pool as a result of the ATP-sparing effect.

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