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ORIGINAL ARTICLE

Hypothermic continuous machine perfusion improves metabolic preservation and functional recovery in heart grafts

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Conflicts of interest
J. Vercruysse and P. Demuylder are employees of Organ Recovery Systems©, and their technical support is acknowledged with gratitude. These latter authors had no input in the data analysis or report. O. Van Caenegem, C. Beauloye, S. Horman, L. Bertrand, N. Bethuyne, A. Poncelet, P. Gianello, F. Bontemps, and JL Vanovershelde have no financial ties to any commercial entity with an interest in the manuscript's subject, nor have they any other conflicts of interest to disclose.

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Summary

The number of heart transplants is decreasing due to organ shortage, yet the donor pool could be enlarged by improving graft preservation. Hypothermic machine perfusion (MP) has been shown to improve kidney, liver, or lung graft preservation. Sixteen pig hearts were recovered following cardioplegia and randomized to two different groups of 4-hour preservation using either static cold storage (CS) or MP (Modified LifePort© System, Organ Recovery Systems©, Itasca, Il). The grafts then underwent reperfusion on a Langendorff for 60 min. Energetic metabolism was quantified at baseline, postpreservation, and postreperfusion by measuring lactate and high-energy phosphates. The contractility index (CI) was assessed both *in vivo* prior to cardioplegia and during reperfusion. Following reperfusion, the hearts preserved using CS exhibited higher lactate levels (56.63 ± 23.57 vs. 11.25 ± 3.92 μmol/g; $P < 0.001$), increased adenosine monophosphate/adenosine triphosphate (AMP/ATP) ratio (0.4 ± 0.23 vs. 0.04 ± 0.04; $P < 0.001$), and lower phosphocreatine/creatine (PCr/Cr) ratio (33.5 ± 12.6 vs. 55.3 ± 5.8; $P < 0.001$). Coronary flow was similar in both groups during reperfusion (107 ± 9 vs. 125 + /-9 ml/100 g/min heart; P = ns). CI decreased in the CS group, yet being well-preserved in the MP group. Compared with CS, MP resulted in improved preservation of the energy state and more successful functional recovery of heart graft.

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Introduction

Since the introduction of cyclosporine in the 1980's [1], heart transplantation (HTX) has been considered the gold standard therapy for terminal heart failure patients who remain symptomatic despite receiving adequate medical treatment. Although the number of patients listed for transplants has continuously increased, the number of operations remains limited owing to the lack of donors. At the end of 2013, 1268 patients were on the active waiting list in Eurotransplant while only 575 transplants had been performed. Although mortality on the waiting list has slightly decreased over the past 10 years, waiting times are significantly increased. A growing number of patients are put on circulatory support or removed from the list because they were unfit for transplantation [2,3]. In the USA, 27% of patients listed for HTX typically either die or are delisted as too ill 2 years after initially being listed as UNOS Status 2 [4]. The donor pool could be enlarged by taking various approaches, such as extending donor criteria or prolonging the storage period, but with an increased risk of worse outcomes after transplantation. Improvements made to current preservation techniques could impact all of these solutions.

Under normal conditions, the heart requires constant energy supply to sustain its contractile activity. This process involves continuously hydrolyzing and resynthesizing adenosine triphosphate (ATP). More particularly, ATP hydrolysis provides energy for actomyosin interaction and sarcomere shortening, for the Ca^{++} ATPase pump of the sarcoplasmic reticulum, as well as for the maintaining of ion gradients. The total energy requirement is satisfied to the extent of 60–70% by the oxidation of long-chain fatty acids, which is followed by that of glucose (20%) and lactate (10%). Contractile dysfunction is generally the first event to occur after ischemia onset. At this stage, the only metabolic pathway known to generate energy is anaerobic glycolysis, which is primarily responsible for membrane integrity and cell survival [5]. Pyruvate, the end product of glycolysis, is then metabolized into lactate. Anaerobic glycolysis has proven twelve times less efficient than the tricarboxylic acid (TCA) cycle. Moreover, to maintain adequate ATP levels, phosphocreatine (PCr) in the myocardium acts as an energetic buffer by transferring an inorganic phosphate to adenosine diphosphate (ADP).

In previous *in vitro* models, no-flow ischemia severity has been found to correlate with intracellular lactate accumulation, PCr and ATP decreases, and adenosine monophosphate (AMP) concentration increase [6–9]. All the latter changes are typically considered predictors of postreperfusion functional recovery. As an illustrative example, Vanoverschelde has demonstrated that ATP depletion is the major determinant of myocardial ischemic damage [8]. The cold static storage (CS) technique used today is associated with cold ischemia and profound changes in cardiac metabolic state like ATP, PCr, and glycogen breakdown [10]. In preservation models, ATP store conservation strongly correlated with cardiac contractile fuction [9,11].

In the literature, hypothermic machine perfusion (MP) has been shown to improve kidney preservation [12,13]. *Ex vivo* MP has also been tested for heart preservation in rodents and dogs, with encouraging results. Compared with CS, grafts that were preserved by hypothermic MP showed increased ATP stores, reduced oxidative damage, and quicker functional recovery [14]. This work sought to assess the efficacy of MP versus CS in a large animal *in vitro* model of heart preservation in terms of cardiac metabolism alterations during ischemia and following reperfusion, and we sought to determine how they correlated with postreperfusion functional recovery.

Materials and methods

Animal care

The study protocol was approved by the Animal Care and Use Committee of the Catholic University of Louvain's Faculty of Medicine. The study was conducted in accordance with the guidelines outlined in the "Guide for the Care and Use of Laboratory Animals", published by the National Institutes of Health (NIH edition 85-23, revised 1985).

Recovering of the grafts

Anesthesia was induced in 16 landrace pigs (each weighing 50–60 kg) by means of intramuscular injection of tiletamine and zolazepam (Zoletil, Virbac; Carros, France) 6 mg/kg. The intravenous route taken was an ear vein. Following tracheal intubation, inhalation anesthesia was maintained using a composition of enflurane (0–1.5%), nitrous oxide, and oxygen. Electrocardiogram and respiration were monitored using a multiple-channel recorder (Datex-Ohmeda Inc., Beaverton, OR, USA).

The animals were placed in the dorsal position and the heart exposed by way of a median sternotomy. Heparin was administered intravenously (600 IU/kg). After aorta cross-clamping and venting the heart through the superior vena cava and left appendage, the grafts were arrested by flushing 1 l HTK solution (Custodiol©, Dr. F. Köhler Chemie GmbH, Bensheim, Germany) in the ascending aorta via a 16-gauge cannula at a pressure of 65 mmHg. Once the pericardium was opened and the vena cava, pulmonary artery, aorta, and pulmonary veins were dissected, procurement was conducted. Donor blood was harvested in citrate–phosphate–dextrose transfusion bags and washed with an autologous red blood cell salvage device (autoLog©

Autotransfusion System, Medtronic Inc, Minneapolis, MN, USA).

Preservation

The heart grafts were preserved for 4 h. They were randomly assigned to either the static CS ($n = 8$) or MP ($n = 8$) group. In the CS group, the hearts were stored in a plastic bag containing KPS-1© preservation solution (Organ Recovery Systems©, Itasca, Il, USA) and placed on ice to maintain a temperature of 4 °C. The solution included 0.5 mM $CaCL_2$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 25 mM KPO_4, 30 mM mannitol, 10 mM glucose, 80 mM sodium gluconate, 5 mM magnesium gluconate, 5 mM ribose, 50 g hydroxyl ethyl starch, 3 mM glutathione, and 5 mM adenine 5. The solution osmolality was 300 mosm/l. In the MP group, the heart grafts were perfused by means of the HeartPort© System (Modified LifePort© System, Organ Recovery Systems©, Itasca, Il, USA) (Fig. 1). The grafts were suspended, and retrograde aortic perfusion was performed through the brachiocephalic trunk. The perfusion pressure was 13–15 mmHg and the flow 0.2–0.3 ml/g/min. The temperature was maintained constant at 4 °C by an external cooler, following an initial transient increase in perfusate temperature up to 25 °C for 30 min, as previously described by Ozeki *et al.* [15]. The preservation solution applied for the perfusion was 1L asanguineous KPS-1©. The solution was oxygenated to maintain partial oxygen pressure between 150 and 200 mmHg. The temperature and coronary flow were continually recorded during MP.

Figure 1 Portable perfusion pump. The HeartPort© System is a modified version of the LifePort© System (Organ Recovery Systems©, Itasca, Il, USA), designed for kidney graft perfusion and preservation. Heart grafts are suspended inside a sterile cassette and subjected to retrograde perfusion with 1 L asanguineous preservation solution (KPS-1©, Organ Recovery Systems©, Itasca, Il, USA). The perfusate is cooled to 4 °C by a heater exchanger, oxygenated by oxygenator (Minimax Oxygenation System©, Medtronic Inc, Minneapolis, MN, USA), and recirculated by means of a pulsatile roller pump. The system includes a data report unit that allows for temperature, perfusion pressure, and flow to be continuously recorded.

Reperfusion protocol

Following 4 h of preservation, grafts were reperfused for a period of 60 min using the Langendorff technique, at a constant pressure of 60 mmHg, with a Krebs–Henseleit bicarbonate buffer containing 1.5 mM $CaCl_2$ and 5 mM glucose in equilibrium with 95% O2/5% CO_2 gas phase. The Krebs solution was mixed with washed red blood cells of the donor pig so as to achieve a hematocrit level of 26%.

Myocardial edema

The hearts were emptied of blood and weighed at the beginning and end of the preservation period.

Metabolites

In total, three myocardial biopsies were conducted. The baseline biopsy was performed on the donor pig prior to cardioplegia, the second on the graft following 4-h preservation, and the last on the graft following 1-hour reperfusion. The samples were rapidly frozen by means of a Wollenberg clamp cooled with liquid nitrogen, then stored at −80 °C until assayed.

Myocardial tissue lactate, creatine (Cr), and PCr values were measured enzymatically via spectrophotometry in neutralized perchloric acid extracts of the frozen myocardium, as outlined in the Vanoverschelde *et al.* study [8]. All measurements were expressed in micromoles per gram left ventricular dry weight.

Tissue high-energy phosphate content was measured using the same extracts and following separation by high-performance liquid chromatography [8]. AMP, ADP, and ATP were each quantified based on the integration of unknown concentration peaks into known standard concentrations peaks. All measurements were expressed in micromoles per gram left ventricular dry weight.

Ventricular function

A baseline assessment of left ventricular function was performed in the donor pig following heart exposure, prior to cardioplegia, by means of a Millar catheter (MPR-500 Mikro-tip pressure catheter, AD Instrument©, NSW 2153, Australia) connected to an analog–digital converter data acquisition system (Powerlab System©, AD Instrument©, NSW 2153, Australia). During reperfusion on the Langendorff system, a compliant latex balloon was inflated into the left ventricle by way of the left atrium and connected to the same data acquisition system to measure left ventricular

function performance. Left ventricular end-diastolic pressure was maintained at 8–10 mmHg during all the reperfusion period by controlling the balloon volume. Our study included measurement of the contractility index (CI), as this parameter is preload independent [16]. CI was defined as (dP/dTmax)/Pmax. Data were expressed as percentage of recovery 30 min after reperfusion, compared with baseline values.

Statistics

Significance of result differences was assessed using a two-way ANOVA for repeated measurements, with additional post hoc tests (Sidak's and Tukey's multiple comparison methods). Student's *t*-tests were used for testing differences in continuous variables between two groups. All values were expressed as mean ± standard deviation. Values of $P < 0.05$ were considered statistically significant. All calculations were performed using commercially available statistical software (GraphPad Prism-6©, Graphpad software Inc, La Jolla, CA, USA).

Results

Myocardial weight gain after preservation

The hearts that were preserved by MP showed significant weight gain after preservation compared to those that underwent CS preservation (12.5 ± 2.1% vs. 1.88 ± 0.8%, $P = 0.027$), which is indicative of myocardial edema increase.

Myocardial flow during reperfusion

Myocardial flow was observed to be consistently similar in the two groups. Flow measured in the CS group and the MP group were, respectively, 107 ± 30 versus 125 ± 35 ml/min/100 g myocardium 15 min after reperfusion on the Langendorff system, 93 ± 42 versus 127 ± 28 ml/min/100 g myocardium 40 min after reperfusion and 98 ± 36 versus 127 ± 28 ml/min/100 g myocardium 60 min after reperfusion (all P = ns).

Cardiac energy states

The basal lactate levels recorded in both groups were within normal values (Fig. 2). No lactate accumulation was observed in the MP group following preservation and reperfusion. In the CS group, a fourfold increase in lactate concentration was noted after preservation, in comparison with baseline values, along with an approximately 10-fold increase following reperfusion compared with baseline.

At baseline, the AMP/ATP ratio reflected a high myocardial energetic status. In the CS group, preservation induced a sixfold increase in AMP content, while ATP concentration was slightly decreased, resulting in an eightfold increase in the AMP/ATP ratio. Following reperfusion on the Langendorff, the AMP/ATP ratio remained elevated. In contrast, the MP group exhibited no significant variations of this ratio, following either preservation or reperfusion, compared with baseline (Fig. 3a), suggesting a highly preserved energetic status. In the CS group, absolute ATP concentrations decreased by 5.43 ± 4.6 μmol/g dry weight from baseline to postpreservation, then fell further by 2.68 ± 3.53 μmol/g dry weight from postpreservation to postreperfusion. In contrast, the MP group exhibited a decrease of 2.8 ± 4 μmol/g dry weight from baseline to postpreservation, then an increase of 3.18 ± 4.2 μmol/g dry weight from postpreservation to postreperfusion (Fig. 3b).

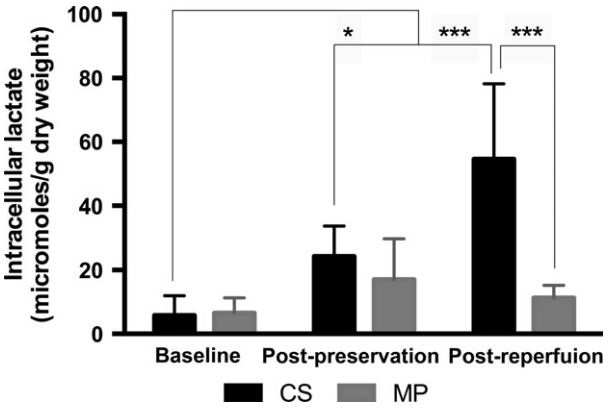

Figure 2 Lactate concentrations measured in myocardial biopsies at baseline, postpreservation, and postreperfusion. Values represent mean ± standard deviation. $N = 8$ in each group; $*P < 0.05$ and $***P < 0.01$.

Energy charge (EC) was defined by Atkinson as (ATP)+0.5(ADP)/(ATP)+(ADP)+(AMP) [17]. This equation reflects the high-energy-charged adenine nucleotide portion, in relation with the total adenylate pool. Normal values typically range from 0.8 to 0.95. EC is a linear measure of the metabolic energy stored in the adenine nucleotide system. In the CS group, EC significantly decreased from baseline to postpreservation and remained inferior to 0.8 following reperfusion (Fig. 4). In the MP group, despite a slight transient decrease in EC observed from baseline to postpreservation, values remained higher than those of the CS group. Following reperfusion, EC returned toward baseline values.

Likewise, the phosphocreatine/creatine (PCr/Cr) ratio at baseline reflected a high percentage of phosphorylated creatine. As expected, in the CS group (Fig. 5), there was a threefold decrease in the PCr/Cr ratio postpreservation. The PCr/Cr ratio increased again following reperfusion, yet remained significantly lower than baseline values in this

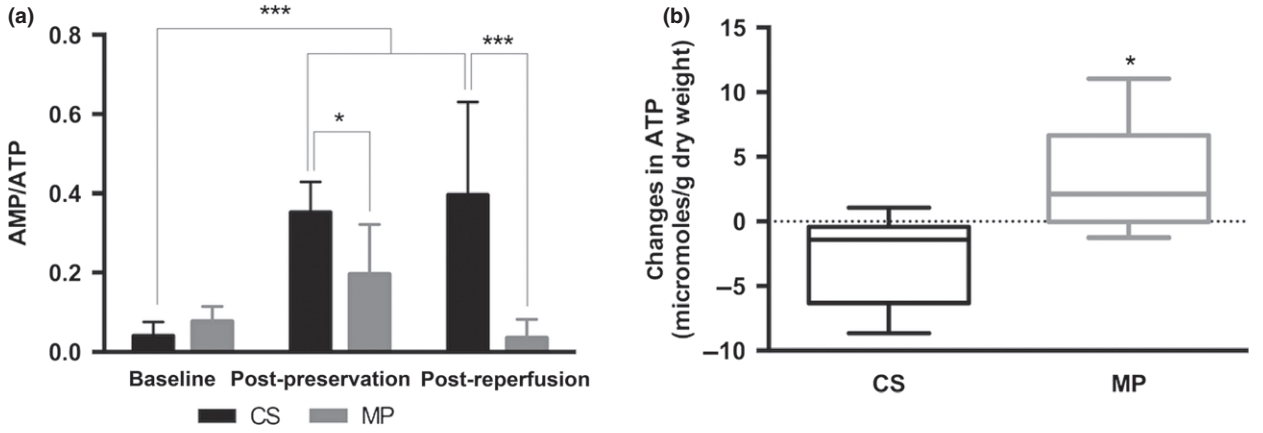

Figure 3 AMP/ATP ratio at baseline, postpreservation, and postreperfusion (a), as well as ATP concentration changes from postpreservation to postreperfusion (b). Values represent mean ± standard deviation. $N = 8$ in each group; $^{*}P < 0.05$ and $^{***}P < 0.01$. In the CS group, the absolute values of nucleotides (μmol/g dry weight) ranged from 0.58 ± 0.35 at baseline to 3.80 ± 0.70 postpreservation ($P < 0.0001$ vs. baseline) and 2.72 ± 0.34 postreperfusion ($P < 0.0001$ vs. baseline) for AMP, and from 16.5 ± 3.7 at baseline to 11.07 ± 2.21 postreperfusion and 8.38 ± 3.08 postpreservation for ATP. In the MP group, values ranged from 0.84 ± 0.31 at baseline to 1.53 ± 0.85 postpreservation ($P < 0.05$ vs. baseline) and 0.37 ± 0.32 postreperfusion (P = ns vs. baseline) for AMP, and from 12.23 ± 3.40 at baseline to 9.42 ± 4.10 postreperfusion and 12.6 ± 2.50 postpreservation for ATP (all P = ns).

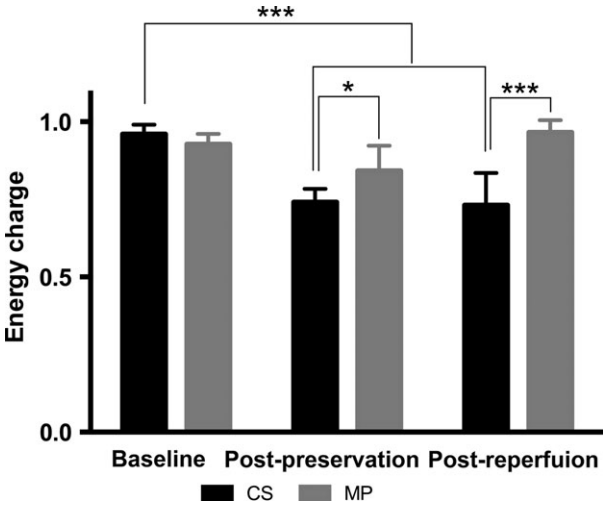

Figure 4 Energy charge at baseline, postpreservation, and postreperfusion. Values represent mean ± standard deviation. $N = 8$ in each group; $^{*}P < 0.05$ and $^{***}P < 0.01$.

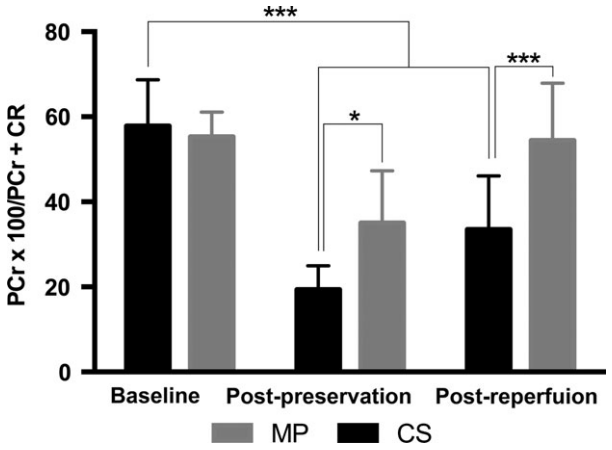

Figure 5 PCr/Cr ratio at baseline, postpreservation, and postreperfusion. Values represent mean+/-standard deviation. $N = 8$ in each group; $^{*}P < 0.05$ and $^{***}P < 0.01$. In the CS group, the absolute values of PCr (μmol/g dry weight) ranged from 13.5 ± 1.9 at baseline to 5. ± 1.4 postpreservation ($P < 0.0001$ vs. baseline) and 8 ± 2.5 postreperfusion ($P < 0.0001$ vs. baseline). In the MP group, the absolute values of PCr (μmol/g dry weight) ranged from 13.2 ± 1 at baseline to 8.9 ± 2.8 postpreservation ($P < 0.01$ vs. baseline) and 12.9 ± 2.6 postreperfusion (P = ns vs. baseline).

group. In contrast, the MP group exhibited a moderate decrease in the PCr/Cr ratio postpreservation compared with baseline. Following reperfusion, this ratio returned toward basal values.

Left ventricular performance

The CI was measured for each donor heart prior to harvesting and 30 min after reperfusion on the Langendorff system. In the hearts preserved using CS, we observed a 25% reduction in CI postreperfusion compared with baseline. In contrast, the MP group exhibited well-preserved CI values (Fig. 6). The absolute values of CI in the CS group ranged from 13.2 ± 2.2 1/s. baseline to 9.7 ± 2.7 1/s. 30 min after reperfusion and from 12.5 ± 2.5 1/s. baseline to 14.4 ± 1.7 1/s. after reperfusion in the MP group ($P < 0.05$).

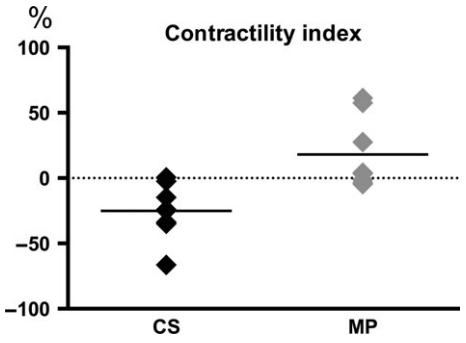

Figure 6 Changes in contractility index measured 30 min after reperfusion. Data are expressed in percentage of the value measured at baseline. Bars represent the mean and plots individual values. $N = 8$ in each group. ($P < 0.05$).

Discussion

This study sought to assess the impact of graft preservation by means of a HeartPort© perfusion machine on changes in energy states and contractile function, following both preservation and *ex vivo* reperfusion.

The salient finding of this study was the observation that this technique caused no energy depletion during both preservation and reperfusion. During organ preservation, changes in both the AMP/ATP and PCr/Cr ratios were considerably less compared with those induced by the commonly used cold preservation method. In addition, no significant drop in ATP was detected, and all these parameters returned toward baseline values following *ex vivo* reperfusion. Of particular interest, the lesser changes observed in PCr contents indicate that the ischemic burden has been considerably reduced in perfused hearts, which probably explains why they also displayed excellent recovery after reperfusion. Our data are consistent with previous findings using different animal models that have reported lower ATP and EC in rat hearts stored using CS compared with those stored using MP [18,19]. Ferrera *et al.* [20,21] demonstrated lower ATP and total adenine content at the end of the preservation period in pig hearts preserved using static storage compared to perfusion without reporting reperfusion data. For the first time, we report energy states after reperfusion, confirming persistent advantage in grafts preserved by MP.

When applying the standard cold preservation method, energy parameters did not recovered completely during reperfusion and reoxygenation. Moreover, a dramatic increase in lactate concentration was reported. In a previous study, Rosenbaum *et al.* [22,23] has already reported lactate accumulation during preservation. It is unlikely that a further increase in lactate concentration postreperfusion would be due to a decrease in metabolite washout, given that coronary blood flow was similar in both groups. We postulate that this could be related to defective oxidative metabolism. We speculate that lactate accumulation and persistent energy deficiency are the consequence of mitochondrial dysfunction, a commonly reported occurrence during reperfusion, but further studies are required to confirm this hypothesis. Machine perfusion is able to remove metabolic end-products that could accumulate during storage, leading to intracellular acidosis or oxygen free radical generation during reperfusion. The H^+ produced by anaerobic metabolism during ischemia exchanges for extracellular Na^+ through stimulation of the Na^+/H^+ exchanger. In the process of reperfusion, the resulting Na^+ increase causes excessive Ca^{++} uptake through Na^+/Ca^{++} exchange, leading to mechanical failure [24]. Ozeki *et al.* [15] demonstrated the existence of a relation between myocardial acidosis and functional recovery following graft preservation.

The persistent energy defect correlated with poorer functional recovery. This observation was made despite the detection of myocardial edema, which was more pronounced in the MP group. Post-MP weight gain has been reported in the literature, ranging from 7% to 44% [18,20,21,25–27]. This factor has been found to be influenced by perfusion (pressure end flow), perfusate (oncotic pressure), and graft (inflammation) characteristics. Nevertheless, myocardial edema may cause diastolic dysfunction [27]. Weight gain higher than 25% has been found to impact on functional recovery [28]. We have only observed a 12% increase in weight following MP, which could be due to our low-pressure and low-flow perfusion characteristics [27,29].

Primary nonfunctioning grafts (PNF) have been reported to occur in 2.3–26% of cases following heart transplantation procedures. PNF is responsible for approximately one-third of early deaths following HTX [2]. The pathophysiology of PNF is multifactorial and not yet fully understood [30,31]. Risk factors related to the recipient (age, pulmonary hypertension, inotrope or mechanical circulatory dependency) have been reported. It has also been suggested that acute ischemia–reperfusion injury with myocardial stunning could contribute to PNF. Finally, the length of ischemic time is consistently recognized as an independent risk factor for PNF [31–33]. Ischemic time longer than 4 hours significantly increases the risk of PNF [34]. Ischemic time is clearly related to post-transplant mortality [2]. By limiting the scope of ischemic graft injury during preservation, MP could reduce PNF incidence and early postoperative mortality... Regarding benefits observed after 4 hours of MP preservation, further studies should determine whether the ischemic time could be safely prolonged using perfusion preservation.

Regarding improved myocardial protection during preservation, there are many potential benefits for the use of

MP, including prolonged ischemic periods or the use of extended criteria donors. In our study, we used hypothermic MP. This technique is cheap and easy to use. In contrast, normothermic MP is technically more complex, expensive and requires the collection of donor blood prior to harvesting to prime the perfusion solution. No data in the literature compare hypothermic MP to normothermic MP. Despite very scarce experimental data, normothermic MP is used currently in human trials. Normothermic MP offers added advantages. The cold ischemic time is shortened. The donor heart is beating in the device, allowing functional and anatomical examination by the use of echocardiography and coronaroangiography. Full aerobic metabolism allows the possibility for organ reconditioning by pharmacologic interventions [35] or even gene therapy. During MP, viral vectors could be perfused to transfer genes targeting inflammatory pathways involved in ischemia–reperfusion injury, acute rejection, or fibroproliferative pathways concerned in primary or late graft failure [36].

This study had several limitations. First, reperfusion was performed *in vitro* on a Langendorff apparatus instead of *in vivo* transplantation. Opting for this apparatus has the advantage of being easy to use, without the numerous confounding factors pertaining to a transplantation model. However, recirculating the blood solution does not eliminate the products that potentially impact graft function, such as inflammatory cytokines. Secondly, to assess postreperfusion functional recovery, we studied contractility parameters. The nonworking model does not allow for a satisfactory assessment of diastolic function, which may be influenced by MP-induced edema. Diastolic dysfunction without any doubt contributes to PNF [28]. Third, we used KPS-1 solution as preservation solution in both groups although KPS-1 has not been tested for cold static preservation of heart grafts. However, MP of donor hearts with KPS-1 has been showed to be superior to CS with Celsior [14,27] and it is very unlikely that KPS-1 decreases the beneficial effect of cold storage. Finally, we used ideal donors and not brain dead donors. Yet, brain death is known to cause greater metabolic changes and cardiac dysfunction related to altered loading conditions due to the loss of the sympathic vasomotor tone [37], mitochondrial and cytosolic calcium overload leading to contracture or necrosis [31] and decreased serum levels of various hormones (triiodothyronine, cortisol, and insulin) [38]. All these conditions may have an impact on postpreservation graft recovery and outcome.

Conclusion

In this *ex vivo* animal model, MP was demonstrated to improve preservation of the heart grafts' energetic state compared with CS. This was proven by superior postreperfusion contractility. The underlying mechanisms could include improved oxidative metabolism or cellular integrity preservation when using the MP method.

Authorship

OVC: designed the research, collected, analyzed, and discussed the data. NB, and AJP: performed surgery. JV, PD, EL, GB, and FB: gave technical support. CB, SH, LB, PG, LMJ, and JLV: participated in data analysis, interpretation, and discussion.

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