Nonischemic myocardial acidosis adversely affects microvascular and myocardial function and triggers apoptosis during cardioplegia

Kamal R. Khabbaz, MD, Jun Feng, MD, PhD, Munir Boodhwani, MD, Richard T. Clements, PhD, Cesario Bianchi, MD, PhD, and Frank W. Sellke, MD

Objectives: We investigated whether the degree of nonischemic myocardial acidosis during a period of cardioplegic arrest differentially affects the recovery of microvascular/left ventricular function, the profile of Bcl2-family protein expression, and the occurrence of apoptosis.

Methods: Isolated hearts from donor rabbits were perfused with oxygenated diluted blood on a modified Langendorff apparatus. The hearts were arrested for 60 minutes with cold (15°C ± 0.5°C) diluted-blood cardioplegic solution (hematocrit: 18%–25%) administered continuously under nonischemic conditions (flow rate; 10 mL/min). The myocardial pH was adjusted and measured continuously with a glass electrode system. Myocardial pH was maintained at 7.2, 6.5, or 6.2, respectively (n = 6 per group) during 60 minutes of arrest. Hearts were then reperfused for 120 minutes with oxygenated diluted blood.

Results: Recovery of left ventricular and microvascular endothelial function was better with a myocardial pH of 7.2 than with a pH of 6.5 or 6.2 (P < .05). There were no significant differences in total Bcl2, phospho-Bcl2-serine 70, phospho-Bad-serine 112, and phospho-Bad-serine 136 levels among groups. Myocardial pH of 7.2 also induced less caspase 3 activation and apoptotic cells (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling) than a pH of 6.5 or 6.2 (P < .05). Regression analysis demonstrated that a significant relationship existed between the recovery of endothelial microvascular (r² = 0.38, P = .004) or left ventricular (r² = 0.37, P = .007) function and myocardial pH.

Conclusion: Severe acidosis during cardioplegic arrest, independent of ischemia, adversely affects recovery of microvascular and left ventricular function and increases indices of apoptosis. This effect on apoptosis may influence long-term outcome after cardiac surgery.

Intraoperative measurements of myocardial tissue pH during cardiac surgery have shown that regional myocardial acidosis is frequently encountered during the course of a cardiac surgical procedure.1 Myocardial acidosis can reach profound levels during the period of aortic crossclamping and early reperfusion,1,3 and the severity of this myocardial acidosis has been shown to be an independent determinant of early adverse outcomes after surgery.1,3 Furthermore, poorer long-term outcomes and reduced survival have been shown to be related to the severity of intraoperative acidosis in a large cohort of patients followed up for a mean of 10 years.2,3 There is evidence from in vitro studies to suggest that acidosis can induce apoptosis in human and porcine cardiac myocytes.4 Therefore, it is possible that the myocardial tissue acidosis during the period of aortic crossclamping can trigger apoptosis, in addition to acutely affecting myocardial functional recovery. This
Abbreviations and Acronyms

ADP = adenosine 5’-diphosphate
LV = left ventricular
LVDP = left ventricular developed pressure
LVSP = left ventricular end-systolic pressure
SDS-PAGE = sodium dodecylsulfate-polyacrylamide gel electrophoresis
SNP = sodium nitroprusside

ongoing cell death triggered by acidosis during cardiac surgery may explain the poor long-term outcomes seen in patients who had higher degrees of acidosis during aortic crossclamping. Consequently, the implications of adequate myocardial protection and avoidance of acidosis during cardiac surgery go beyond the acute recovery of myocardial function in the immediate postoperative period. The purpose of this study was to examine the effects of myocardial acidosis on recovery of myocardial and microvascular function, as well as on the profile of Bcl2-family proteins and the degree of apoptosis in the setting of cardioplegic arrest and reperfusion.

Materials and Methods

All of the experiments were approved by the Beth Israel Deaconess Medical Center Animal Care and Use Committee and the Harvard Medical Area Standing Committee on Animals (Institutional Animal Care and Use Committee) and conformed to the National Institutes of Health guidelines regulating the care and use of laboratory animals (NIH publication No. 5377-3, 1996).

Isolated Blood-perfused Heart Preparation

An isolated blood-perfused Langendorff preparation was used inasmuch as this preparation is considered to be superior to a crystalloid-perfused Langendorff system. The preparation has been described in detail by Stringham and associates. In each experiment, a blood-donor and a heart-donor rabbit were used.

Blood donor. New Zealand White rabbits (3.8–4.5 kg, Millbrook Farm, Mass) were anesthetized with ketamine (35 mg/kg) and xylazine (2.5 mg/kg, intramuscularly) and anticoagulated with heparin (2000 U/kg, intravenously). After cannulation of the ear vein, the animals were intubated with a cuffed endotracheal tube, mechanically ventilated, and maintained under deep general anesthesia with a gas mixture of oxygen at 1.5 to 2 L/min and isoflurane at 0.75% to 3.0% concentration. After anesthetic induction, the pericardium was opened through a median sternotomy. After the pericardial cradle was created, a needle was inserted into the aorta and arterial blood was obtained. Blood was also harvested from the chest cavity of the heart donor after heart excision and added to the prime. Heparinized blood was diluted with modified Krebs–Henseleit buffer. The perfusate was equilibrated with 95% oxygen and 5% carbon dioxide, adjusted to a pH of 7.35 to 7.4 at 37°C. Dilution of donor blood with Krebs–Henseleit buffer was allowed to maintain perfusate hematocrit between 18% and 25%.

Membrane Oxygenator Management

A Lilipuke D901 (Dideco, Mirandola, Italy) pediatric membrane oxygenator was used in all experiments. A single miniroller pump was used to perfuse solution from the reservoir through the oxygenator and into the aortic perfusion column. A mixture of 95% oxygen and 5% carbon dioxide was insufflated into the oxygenator at a rate of 1 to 2 L/min. Perfusate PO2 was maintained above 500 mm Hg and PCO2 at 35 to 45 mm Hg by adjustment of the rate of gas flow. Perfusate pH was further adjusted to 7.35 to 7.45.

Heart donor. Rabbits were anesthetized and anticoagulated as described above. The heart was rapidly exposed. Before the heart was excised, 60 mL of arterial blood was withdrawn from the aorta to prepare the blood cardioplegic solution. The heart was then excised and the aorta cannulated. The heart was mounted and perfused with diluted-blood perfusate at 70 mm Hg in an organ chamber on the perfusion system. Left ventricular (LV) performance was measured isovolumetrically with a compliant latex balloon connected to a pressure transducer inserted in the LV across the mitral valve. LV performance was assessed by measurement of LV systolic pressure (LVSP, mm Hg) and LV end-diastolic pressure (mm Hg; LVSP – LV end-diastolic pressure = LV developed pressure [LVDP]).

Myocardial pH Measurements

Myocardial pH was measured by the Khuri myocardial pH monitoring (KmPH) system (Terumo Cardiovascular System Corp, Ann Arbor, Mich), which was used in 3 treatment groups as described previously. This system consists of a small digitalized monitor, pH probes, temperature probes, reference electrode, and KmPH calibration cuvette. After the heart was mounted and blood-perfused in the Langendorff system, the first calibrated pH probe with unstriped wire was inserted perpendicularly. The second calibrated pH probe with unstriped wire and the reference (ground) electrode were placed in the venous-returning blood cardioplegic solution. The venous-returning blood and myocardial pH and temperature were continuously recorded during 30 minutes of baseline period, 60 minutes of cardioplegic arrest, and 10 minutes of early reperfusion.

Experimental Protocols

In the cardioplegia groups, after 30 minutes of equilibration, hearts were initially infused with 20 mL of cold blood cardioplegic solution (10°C-15°C) and then reinfused (10 mL/min) continuously during 60 minutes of cardioplegic arrest. The diluted-blood cardioplegic solution consisted of equal volumes of 60 mL crystalloid cardioplegic solution (BIDMC Surgical Associates, Boston, Mass) and 60 mL of oxygenated blood removed from the heart-donor rabbit. Rabbits were randomly divided into 3 experimental groups (myocardial pH 7.2, 6.5, and 6.2 groups, respectively) by altering the pH of diluted-blood cardioplegic solution. BIDMC Surgical Associates solution contained the following (in mmol/L): KCl, 30; MgSO4, 15; glucose, 5; THAM (tris [hydroxymethyl] aminothane), 10; and NaCl, 118. The myocard-
dial pH was adjusted with 0.1N HCl or 0.3N NaOH as needed to maintain the desired pH range. The pH was measured continuously with a Khuri glass electrode system. Myocardial pH was maintained at 7.2, 6.5, or 6.2, respectively, during 60 minutes of cardioplegic arrest (n = 6 per group). The blood cardioplegic solution (120 mL) was maintained to 15°C by a Brinkmann RM6 refrigerator-circulating bath (Brinkmann Instruments, Inc, Lauda, Germany) and recirculated by a Masterflex roller pump (Cole Parmer Instrument Co, Chicago, Ill). In the control group, hearts (n = 5) were continuously perfused with diluted oxygenated blood for 150 minutes without cardioplegic arrest.

After a total of 60 minutes of cardioplegic arrest, the heart was reperfused for 120 minutes with oxygenated diluted blood (37°C). The hearts were then excised and LV tissue cut into 3 pieces for microvessel study, apoptosis analysis, and molecular comparisons.

**In Vitro Coronary Microvessel Studies**

Coronary arterioles (100–180 μm internal diameters) were dissected from the LV free wall tissue of the isolated rabbit hearts after cardioplegia and sham control. Microvessel studies were performed by in vitro organ bath videomicroscopy as previously described.6,7 After equilibration, the coronary microvessels were constricted with the thromboxane A2 analog U46619 (1 μmol/L) by 30% to 50% of the baseline diameter. Once the steady-state tone was reached, endothelium-independent/dependent relaxation to sodium nitroprusside (SNP)/adenosine 5’-diphosphate (ADP) was examined, respectively.

**Sodium Dodecylsulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting**

SDS-PAGE and immunoblot were performed as previously described.6,7 Total protein (40 μg) was fractionated on 8% to 16% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Bedford, Mass). Membranes were incubated with rabbit polyclonal anti-Bcl2 (BD Biosciences, San Jose, Calif), anti-phospho-Bcl2-serine 70 (p-Bcl2-70) (Santa Cruz Biotechnology, Santa Cruz, Calif), mouse monoclonal anti-phospho-Bad-serine 136 (p-Bad-136), anti-phospho-Bad-serine 112 (p-Bad-112), and rabbit monoclonal anti-phospho-Bad-serine 136 (p-Bad-136) (Cell Signaling Tech, Inc, Beverly, Mass) for 1 hour at room temperature. The membranes were incubated with the appropriated horseradish peroxidase–conjugated secondary antibody for 1 hour, washed 3 times in Tris-buffered saline solution, and detected by chemiluminescent detection (Pierce, Rockford, Ill). Bands were measured by densitometric analysis of autoradiograph films.

**Caspase 3 Activity**

Caspase 3 activity in the LV tissue was measured with a caspase 3–like activity assay kit (BioVision, Mountain View, Calif) according to the manufacturer’s directions.7 LV tissue (50 mg) was homogenized in lysis buffer followed by centrifugation (16,000g, 4°C, 10 minutes). The supernatants were incubated with reaction buffer and DEVD-7-amino-4-trifluoromethyl coumarin substrate at 37°C for 60 minutes. Caspase 3–like activity was detected in a luminescence spectrometer (LS50B, EG&G; Perkin-Elmer, Shelton, Conn). The fold-increase in caspase 3 activity was determined by comparing these results with the level of the uninduced controls.

**Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling (TUNEL) Staining**

Heart ventricular tissues were fixed in formalin for 24 hours, embedded in paraffin, and sectioned. The apoptotic cells were identified by TUNEL using an apoptosis detection kit according to the manufacturer’s protocol (Chemicon Inc, Temecula, Calif).7 Ten photographs (magnification, ×200) of each tissue section were taken. The TUNEL-positive cells were viewed and manually counted by 2 observers, who were blinded to the experimental conditions. The number of cardiomyocytes with clear nuclear staining, indicating apoptosis, was expressed as the number of TUNEL-positive cells per total nuclei, respectively.

**Drugs**

U46619, SNP, and ADP were obtained from Sigma Chemical Company, St Louis, Missouri. All of the drugs were dissolved in ultrapure distilled water and the solutions were prepared on the day of the study.

**Data Analysis**

The data are presented as mean ± SEM. Repeated-measures analysis of variance, with the Tukey correction for multiple comparisons, was used to compare the variables between groups. A paired t test was used to compare preischemic to postischemic hemodynamic variables within groups. Simple linear regression analysis was used to evaluate the relationship between LV functional recovery (or microvessel function) and myocardial pH during cardioplegic arrest.

**Results**

**Myocardial pH Measurements and LV Function**

Data from the 3 separate experimental groups (pH 7.2, 6.5, and 6.2) are shown in Figure 1 to illustrate the representa-
tive pH profile. These data show that myocardial pH could be controlled within a reasonable range by altering the pH of the cardioplegic solution. Myocardial pH values at baseline were not significantly different among the 3 groups. The average values of mean myocardial pH during cardioplegic arrest were 6.2 ± 0.03 in the pH 6.2 group, 6.5 ± 0.02 in the 6.5 group, and 7.2 ± 0.06 in the 7.2 pH group. The myocardial pH was rapidly normalized after early reperfusion.

The effects of different myocardial pH values during cardioplegic arrest on functional recovery after reperfusion are illustrated in Figure 2, A. There were no significant differences in baseline LV performance before cardioplegic arrest among the groups. After 60 minutes of cold blood cardioplegic arrest and 120 minutes of reperfusion, there were significant decreases in the recovery of LVDP in all 3 treatment groups. The recovery of LVDP was significantly greater in pH 7.2 group than that in pH 6.2 group (P < .05). The recoveries of LVDP tended to be greater with pH 7.2 than that in the pH 6.5 group, but this difference failed to reach statistical significance. The recovery of LVDP also appeared to be increased in the pH 6.5 group compared with recovery in the pH 6.2 groups, but no significant differences were observed between the 2 groups. There was a significant positive correlation between the recovery of LVDP and the mean myocardial pH during cardioplegic arrest ($r^2 = 0.37, P = .007$) (Figure 2, B).

**Vessel Characteristics**

Coronary microvessels ranged from 100 to 180 μm in internal diameter, averaging 150 ± 5 μm in the control, 135 ± 6 μm in the pH 7.2, 140 ± 7 μm in the pH 6.5, and 143 ± 5 μm in the pH 6.2 groups, respectively. The percentage of contraction after the application of the thromboxane A2 analog U46619 was similar among the groups (40% ± 4% in control, 35% ± 3% in pH 7.2, 39% ± 5% in pH 6.5, and 37% ± 4% in pH 6.2 groups, respectively).

**Endothelium-dependent and -independent Relaxation**

The endothelium-dependent relaxation of microvessels to ADP was significantly impaired in the pH 6.5 and pH 6.2 groups as compared with that in the pH 7.2 group (P < .05; Figure 3, A). Recovery of endothelium-dependent relaxation in response to ADP ($10^{-4}$ mol/L) was also significantly correlated with the mean myocardial pH during cardioplegic arrest ($r^2 = 0.38, P = .004$, Figure 3, B). The endothelium-independent relaxation of coronary microvessels to SNP was also significantly reduced in the pH 6.2 group, suggesting mild impairment in vascular smooth-muscle responsiveness (Figure 3, C).

**Bcl2-Family Protein Expression and Phosphorylation**

Expression of total Bcl2, phospho-Bcl2-serine 70, and phospho-Bad-serine 112, phospho-Bad-serine 136 proteins were analyzed by Western blot (Figure 4, A and C). The expression of total Bcl2 was not different among the groups. Decreases in myocardial pH were associated with reduced phospho-Bcl2-serine 70; however, there were no statistically significant differences between groups (1-way analysis of variance; P = .16) (Figure 4, B). There was no significant difference in the amount of phospho-Bad-serine 112, or phospho-Bad-serine 136 (Figure 4, D).

**Caspase 3 Activity and TUNEL Staining**

Activated caspase 3 in the pH 7.2 group (2.8 ± 0.2-fold vs control) was significantly lower than that in the pH 6.5
group (4.2 ± 0.3-fold vs control; \( P < .05 \)) and pH 6.2 groups (5.5 ± 1.0-fold vs control; \( P < .05 \)), respectively (Figure 5). The caspase 3 activity tended to be higher in the pH 6.2 group compared with that in the pH 6.5 group, but this difference was not statistically significant. Figure 6, A to D, shows the TUNEL-positive myocyte nuclei photographed at a magnification of \( \times 200 \). Figure 6, E, shows the percentage of TUNEL-positive nuclei in LV sections in the 4 groups. The percentage of TUNEL-positive myocyte nuclei was significantly increased in pH 6.5 (1.6% ± 0.2%) and pH 6.2 (2.5% ± 0.7%) groups compared with pH 7.2 group (0.41% ± 0.05%; \( P < .05 \), respectively). The percentage of TUNEL-positive cells in pH 6.5 group was also significantly lower than that of the pH 6.2 group (\( P < .05 \)).

**Discussion**

This study demonstrates that myocardial acidosis during cardioplegic arrest, independent of ischemia, has deleterious effects on recovery of myocardial function and endothelium-dependent and -independent microvascular relaxation, and is a trigger for apoptosis.

The finding that the recovery of LVDP was significantly worse in the more acidotic group (pH 6.2) is in agreement with other investigators’ work evaluating the effect of acidosis on contractility in numerous studies.\(^1\),\(^2\),\(^4\) Thatte and colleagues\(^4\) have demonstrated that recovery of myocardial contractility is affected by the degree of myocardial acidosis during a period of cardioplegic arrest.

The effect of myocardial acidosis on recovery of microvessel relaxation and endothelial function is also evident from this study. Endothelium-dependent and endothelium-independent relaxation were adversely affected in the hearts subjected to lower pH during aortic crossclamping. However, the effect of acidosis during cardioplegic arrest was more pronounced on the endothelium than on microvessel smooth muscle, as reflected by the stronger correlation between myocardial pH and response to ADP. This adverse effect of acidosis on microvascular function may in part explain the poor functional recovery seen in the more acidic group and could contribute to diminished long-term recovery.

Using isolated myocytes or endothelial cells, previous studies have demonstrated that not only ischemia/acidosis induced apoptosis, but hypoxia/acidosis also caused a significant increase in myocardial and endothelial cell apoptosis.\(^9\)\(^-\)\(^12\) Acidosis-induced apoptosis may require activation of caspase 12 and expression of Bcl- 1-family BH3-only protein, but is independent of p53.\(^9\)\(^-\)\(^12\) Our group\(^6\),\(^7\) has previously demonstrated that the expression of molecular indices of apoptosis is influenced by the type of cardioplegic solution delivered in a similar model.\(^6\)\(^,\)^7 We\(^6\),\(^7\) have found that continuous or intermittent cardioplegic ischemia does not
alter the expression of Bcl2-family proteins, but induces the phosphorylation of Bad and activation of caspase 3. Intermittent blood cardioplegia is superior to the other cardioplegic solutions in increasing phosphorylated Bad and in inhibiting the activation of caspase 3, which are associated with improved LV function and endothelium-dependent relaxation of coronary microvessels. These results suggest that changes in phosphorylation or translocation of the Bcl2-family proteins, rather than its total protein amount, may be the primary indicators of apoptosis induction during cardioplegic ischemia. Recently, we13 have also confirmed these findings in humans, demonstrating that there is an increase in expression of apoptotic markers during a period of cardiopulmonary bypass and cardioplegic arrest, despite optimal myocardial protection. Cardioplegic ischemia and cardiopulmonary bypass induced phosphorylation of Bad on serine 112 and Bcl2 on serine 70 in human atrial tissue.

In the present study, decreases in myocardial pH to 6.5 or 6.2 did not change the amount of p-Bcl2 70, p-Bad 112 or p-Bad 136. These discrepancies between the previous studies12,13 and the present one may be due to the different animal models and different experimental protocols used. First, in our previous studies, intermittent cardioplegia was compared to continuous blood cardioplegia (10 mL/min for 60 minutes). This may afford improved myocardial protection. Second, 30 minutes of reperfusion was used in our previous study, compared with 120 minutes in the present study. Furthermore, in human atrial tissue, a very brief period (10 minutes) of reperfusion after ischemic arrest triggered Bcl2 and Bad phosphorylation.13 Indeed, Bcl2-family proteins and many other survival protein kinases are activated or phosphorylated during the very early minutes of myocardial reperfusion or preconditioning ischemia/reperfusion instead of late-phase reperfusion.14 The absence of significant differences in the modification of apoptotic proteins among the 3 groups at the end of 120 minutes of reperfusion may be explained by the longer duration of the reperfusion period. It is possible that more pronounced differences in Bcl2-family proteins may have been evident had expression been examined earlier in the reperfusion period.

Numerous clinical scenarios have been documented whereby myocardial pH reaches levels as low as those achieved in this experimental model. Kumbhani and colleagues3 have presented compelling data from a large cohort

Figure 4. Immunoblots and graphs showing the protein levels and fold changes in total Bcl2 (A), p-Bcl2-70 = phospho-Bcl2-serine 70 (B), p-Bad-112 = phospho-Bad-serine 112 (C), and p-Bad-136 = phospho-Bad-serine 136 (D) from control, pH 7.2, pH 6.5, and pH 6.2 groups, respectively.

Figure 5. Graph showing the amount of caspase 3 activity in different groups. *P < .05 versus control; †P < .05 versus pH 7.2; #P < .01 versus pH 7.2.
confounded by the degree of ischemia to which the myocardium is subjected. In this study, we were able to modulate myocardial pH without affecting the degree of ischemic injury. The effect of acidosis on apoptosis as measured by caspase 3 activity and by TUNEL staining indicates that in hearts in which the pH reached a level of 6.2, apoptotic activity was significantly increased. This degree of acidosis is not infrequently encountered during cardiac surgery. Therefore, acidosis alone, independent of ischemia, may be an important trigger for apoptosis. This does not imply that ischemia may not be another trigger of apoptosis.

The statistically significant correlations between myocardial pH and the recovery of LV and microvessel function further strengthen our conclusions and suggest a dose-dependent effect. The avoidance of profound levels of myocardial acidosis is beneficial to LV and microvascular function and may improve short- and long-term outcomes after cardiac surgery.

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References


