Efficacy of myocardial initial reperfusion with 2,3 butanedione monoxime after cardioplegic arrest is time-dependent

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Abstract

Objective: In a previous study, initial reperfusion of isolated hearts after cardioplegic arrest with 2,3 butanedione monoxime (BDM) for 5 min was markedly superior to warm hyperkalemic reperfusion in improving the initial oxygen balance and reducing reperfusion arrhythmias. However, left ventricular contractility was only marginally enhanced. The goal of the present study was to test, whether the efficacy of BDM reperfusion can be enhanced by prolonging the application period. Methods: 32 Langendorff perfused guinea pig hearts were subjected to 50 min of cardioplegic arrest in St. Thomas Hospital II solution at 37°C for 50 min. Control hearts (n = 8) were immediately reperfused with normal Krebs solution for 30 min. In BDM-5, BDM-20, and BDM-40 hearts (n = 8, each), a 5, 20, or 40 min period of initial BDM reperfusion preceded perfusion with normal Krebs. Results: BDM markedly improved the O balance during initial reperfusion by reducing O demand by over 50% (p < 0.01) in all treatment groups while coronary flow was maintained. Reperfusion contracture, estimated by the end-diastolic balloon pressure was inhibited by more than 50% in BDM-20 and BDM-40 hearts. Recovery of left ventricular developed pressure, dP/dt max and dP/dt max was significantly enhanced throughout the reperfusion period only in the BDM-20 group (p < 0.05). Myocardial ultrastructure was best preserved in BDM-20 hearts. Conclusions: 20 min of initial BDM reperfusion were clearly superior to immediate Krebs reperfusion or a shorter (5 min) or longer (40 min) BDM treatment period in attenuating reperfusion damage. Thus, contraction uncoupling during initial reperfusion by BDM or similarly acting drugs may prove a viable technique to reduce myocardial reperfusion damage in patients undergoing open heart surgery.

Keywords: Heart; Cardioplegia; Reperfusion injury; 2,3-butanedione monoxime; BDM; Guinea pig

1. Introduction

Ischemic cardioplegic arrest and reperfusion as performed for cardiac transplantation and during most cardiopulmonary bypass protocols can result in decreased myocardial contractility (stunning) [1–3]. Strategies aimed at ameliorating posts ischemic myocardial injury include the improvement of cardioplegic solutions as well as modifications of the reperfusion protocol, such as warm cardioplegic blood reperfusion, or the use of agents directed at improving substrate metabolism, scavenging free O radicals, or inhibiting leukocyte adhesion to endothelium.

A novel strategy embraces the use of 2,3 butanedione monoxime (BDM) as a protective additive during ischemia or cardioplegic arrest [4–6] as well as during reperfusion [7–9]. BDM is an effective negative inotropic and vasodilating agent, believed to decrease the sensitivity of contractile proteins to Ca²⁺, or to directly inhibit actin-myosin crossbridge cycling [10–15]. BDM also considerably improves the myocardial O₂ balance, by reducing myocardial O₂ demand and simultaneously increasing O₂ delivery [9,16,17].

In a previous study we demonstrated that initial reperfusion with BDM for 5 min was more effective than initial hyperkalemic reperfusion in improving left ventricular (LV)
function in isolated Langendorff perfused guinea pig hearts [9]. BDM reperfusion also allowed for a faster return to regular electric activity, i.e. to sinus rhythm than hyperkalemic reperfusion. However, the improvement of LV function by BDM over hyperkalemic reperfusion in our experimental model was only marginal. A study by Schlüter et al. indicates that 5 min of BDM application during initial reperfusion may be too short to elicit the maximum beneficial effect of BDM [8]. In an isolated heart model of the oxygen paradox, BDM suppressed LDH and CK release during re-oxygenation after 60 min of hypoxic perfusion, but had to be applied for up to 40 min to avoid a rebound of tissue damage after drug washout [8]. However, the biochemical and structural alterations of oxygen paradox induced tissue damage may differ considerably from the injury induced by cardioplegic arrest and reperfusion.

We, therefore, tested the hypothesis that after cardioplegic arrest initial reperfusion with BDM for 20 or 40 min is superior to only 5 min initial BDM reperfusion in reducing reperfusion damage, and such improving the recovery of myocardial function and maintaining cellular integrity. If that were the case, contraction uncoupling by BDM or equivalent drugs during early reperfusion might prove superior to the established protocols of warm cardioplegic reperfusion in reducing reperfusion damage of the heart after cardioplegic arrest.

2. Materials and methods

Following Animal Studies Committee approval, guinea pigs (250–350 g, Charles River—Savo, Kisslegg, Germany) were injected intraperitoneally with 20 mg of ketamine and 1000 units of heparin, and were decapitated when unresponsive to noxious stimuli. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1985). Our methods have been published in detail [3,9,18]. After thoracotomy, the aorta was cannulated distal to the aortic valve and the venae cavae and pulmonary artery were cut. Each heart was immediately perfused through the aortic canula and excised. All hearts were perfused at a controlled perfusion pressure of 55 mmHg, measured at the aortic root. The perfusate, a modified Krebs–Ringer solution, was filtered in-line (5 μm pore size; Minisart, Sartorius, Göttingen, Germany) and contained (in mM): Na⁺, 137; K⁺, 4.5; Mg²⁺, 1.2; Ca²⁺, 2.5; Cl⁻, 134; HCO₃⁻, 15.5; H₂PO₄⁻, 1.2; glucose, 11.5; pyruvate, 2; mannitol, 16; ethylene-diaminetetraacetic acid (EDTA), 0.05; and insulin, 5 U/l. Perfusionate and bath temperatures were maintained at 37°C using a thermostatically controlled water circulator (RM 3, MGW Lauda, Lauda-Königshafen, Germany). Both solutions were equilibrated with a gas mixture of 97% O₂ and 3% CO₂, which resulted in a pH of 7.4. Left ventricular pressure (LVP) was measured isovolumetrically with a transducer (Viggo-Spectramed DT-XX, Ohmeda, Erlangen, Germany) connected to a flexible, saline-filled latex balloon (Hugo Sachs Elektronik, March-Hugstetten, Germany) inserted into the left ventricle through a cut in the left atrium via the mitral valve. Balloon volume was adjusted to maintain an end-diastolic LVP of approximately 5 mmHg and left ventricular developed pressure (LVDP) was calculated as LVP systolic — LVP end-diastolic. Positive and negative dP/dt max were obtained electronically with an analogue differentiator (Recomed 216 080 02, Hellige, Freiburg, Germany). Heart rate was determined electronically using the positive peaks of the dP/dt curve as input signal (Recomed 236 045 06, Hellige).

Coronary flow was measured on-line with an electromagnetic flow probe (Recomed 236 046 01, Hellige). Coronary sinus effluent was collected by a cannula placed in the right ventricle via the pulmonary artery after ligating the venae cavae. Coronary inflow and outflow O₂ tensions were measured periodically with a self-calibrating analyzer system (ABL-300, Radiometer, Copenhagen, Denmark). Derived parameters, O₂ delivery, O₂ consumption and the cardiac efficacy in utilizing consumed oxygen were calculated according to standard formulae [9].

All directly measured electronic signals were displayed on a six-channel recorder (Recomed 330-P, Hellige), and stored on a personal computer via a 16 channel AD-converter (ME 26, Meilhaus Electronic, Puchheim, Germany) for offline analysis.

2.1. Protocol and statistical analysis

Following completion of the preparation and a 30 min period of stabilization, baseline values of all measured variables were obtained and cardioplegic arrest was induced by 20 ml of St. Thomas Hospital II solution (55 mmHg, 37°C). The hearts remained unperfused and submerged in the cardioplegic solution at 37°C for 50 min. On reperfusion hearts were divided into four groups: Control hearts (C, n = 8) were immediately reperfused with normal Krebs solution. In the study groups reperfusion was initiated with 20 mM BDM in Krebs solution for either 5 min (BDM-5, n = 8), 20 min (BDM-20, n = 8), or 40 min (BDM-40, n = 8) before the perfusate was switched to normal Krebs. The LV-balloon remained inflated during cardioplegic arrest and initial reperfusion to record the effect of contracture, but balloon volume was adjusted to yield again 5 mmHg end-diastolic pressure after 10 min of perfusion with normal Krebs. At this time all hearts were also bolus infused with lidocaine (0.05 ml of 1%) to convert any hearts not already in sinus rhythm. Coronary flow was measured at baseline, at the peak of reactive hyperemic flow, i.e. at 1 min of reperfusion, at the end of BDM perfusion, and at 5, 10, 15, and 30 min of reperfusion with normal Krebs. Postischemic recovery of all other variables was determined after 30 min of reperfusion. At
the end of the experiments hearts were fixated for electron microscopy by perfusion with Karnovsky solution and a tissue sample from the LV anterior free wall was excised for further processing.

All data are expressed as means ± standard error of the mean. After testing for normal distribution of data, statistical differences among groups were obtained by student t-test, and for within group comparisons by paired t-test. p-Values were corrected for multiple comparisons according to Bonferroni-Holm as appropriate. Differences were considered significant at \( p < 0.05 \).

3. Results

3.1. Heart rate

Initial heart rate was not different among groups. No significant changes in heart rate occurred after 30 min reperfusion or among the experimental groups.

3.2. LV systolic and diastolic function

Left ventricular developed pressure (LVDP) was significantly depressed in all groups following cardioplegic arrest and reperfusion. However, in hearts initially reperfused with BDM, LVDP recovered faster and to a better

Fig. 2. Left ventricular end-diastolic balloon pressure at an unchanged volume is given for preischemic baseline (BL), the end of 50 min STH cardioplegia (End-Isch) and after 5 or 10 min of reperfusion. means ± SEM, * \( p < 0.01 \) vs. C.

Fig. 3. Coronary flow is given in percent of preischemic baseline at peak reactive hyperemic reflow (RH) and after 5, 10, 15, and 30 min reperfusion with normal Krebs solution. All data are means ± SEM.
Table 1

Parameters of oxygen metabolism

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>BDM</th>
<th>30 min rep.</th>
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<tbody>
<tr>
<td>O₂ extr. [%]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>78±3</td>
<td>-</td>
<td>73±3</td>
</tr>
<tr>
<td>BDM-5</td>
<td>74±3</td>
<td>44±4*</td>
<td>75±1</td>
</tr>
<tr>
<td>BDM-20</td>
<td>69±3</td>
<td>33±5*</td>
<td>74±2</td>
</tr>
<tr>
<td>BDM-40</td>
<td>79±3</td>
<td>49±5*</td>
<td>80±2</td>
</tr>
<tr>
<td>MVO₂ [μl/min]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>132±11</td>
<td>-</td>
<td>83±6*</td>
</tr>
<tr>
<td>BDM-5</td>
<td>119±12</td>
<td>69±17*</td>
<td>98±15</td>
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<td>BDM-20</td>
<td>130±11</td>
<td>47±9*</td>
<td>107±7</td>
</tr>
<tr>
<td>BDM-40</td>
<td>126±11</td>
<td>63±8*</td>
<td>96±8</td>
</tr>
<tr>
<td>O₂ efficiency [mmHg/μl]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>178±13</td>
<td>-</td>
<td>126±14*</td>
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<tr>
<td>BDM-5</td>
<td>208±22</td>
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<td>119±23*</td>
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<tr>
<td>BDM-20</td>
<td>196±19</td>
<td>-</td>
<td>149±15*</td>
</tr>
<tr>
<td>BDM-40</td>
<td>181±16</td>
<td>-</td>
<td>123±18*</td>
</tr>
</tbody>
</table>

Parameters of oxygen metabolism are oxygen extraction (O₂ extr.), oxygen consumption (MVO₂), and the efficiency of oxygen utilization (O₂ efficiency). All data are means ± SEM; *p < 0.05 vs. baseline.

percentage at the endpoint of 30 min reperfusion as compared to the control group. The best recovery of LVDP was obtained with 20 min of BDM application (66 ± 5% of baseline), which proved superior to both, the 5 min (53 ± 5%) and the 40 min (54 ± 4%) BDM perfusion protocol (Fig. 1). Baseline values: 105 ± 4, 99 ± 5, 106 ± 3, and 104 ± 4 mmHg in C, BDM-5, BDM-20, and BDM-40 hearts, respectively.

In the isolated Langendorff perfused heart, dP/dt max is a direct indicator of myocardial contractility. 50 min of cardioplegia at 37°C considerably depressed dP/dt max during reperfusion in all groups. Depression of dP/dt max was most pronounced in control hearts, intermediate in BDM-5 and BDM-40 hearts and least in the BDM-20 group. This order remained unchanged until 30 min of reperfusion, when recovery of dP/dt max amounted to 50 ± 3% in C, 60 ± 7% in BDM-5, 62 ± 5% in BDM-40, but 70 ± 6% in BDM-20 hearts (Fig. 1). Baseline values: 1753 ± 61, 1773 ± 92, 1882 ± 122, and 1840 ± 131 mmHg/s in C, BDM-5, BDM-20, and BDM-40 hearts, respectively.

LV relaxation during early diastole was assessed by −dP/dt max, which was significantly reduced after 50 min of cardioplegic arrest at 37°C in all groups. Already after 5 min of reperfusion, relaxation was best maintained in BDM-20 hearts as compared to all other groups. In the course of reperfusion −dP/dt max recovered least in the control group, intermediate in BDM-5 and BDM-40 hearts, and best in hearts treated with 20 min of BDM.

Fig. 4. Electron microscopy graph of a control heart after 50 min cardioplegic arrest in STH at 37°C and 30 min reperfusion. Sarcomeres are in a state of severe contracture, Z-lines are irregularly shaped and broadened. The mitochondria are drastically swollen, the matrix is widely cleared and only few intact cristae are visible. Scale bar: 0.6 μm.
perfusion (Fig. 1). Baseline values: $-1459 \pm 40$, $-1373 
\pm 74$, $-1533 \pm 74$, and $-1444 \pm 86$ mmHg/s in C, BDM-5, BDM-20, and BDM-40 hearts, respectively.

During cardioplegic arrest and initial reperfusion LV diastolic pressure at constant balloon volume was monitored as an indicator of myocardial contracture. In all hearts balloon pressure increased similarly during cardioplegic arrest (Fig. 2). With the onset of reperfusion diastolic balloon pressure rapidly increased even further and reached its peak after about 5 min. The presence of BDM in the reperfusate significantly attenuated this increase in all study groups. However, a sustained effect even after washout of the drug was only obtained after 20 or 40 min of BDM perfusion (Fig. 2).

3.3. Coronary flow and $O_2$ consumption

In all groups, coronary flow increased by 25 to 30% over preischemic baseline during reactive hyperemic reflow within the first min of reperfusion. Within 5 min of reperfusion (control group) or within 5 min of BDM washout (treatment groups), coronary flow decreased in all groups to about 80 to 90% of baseline and remained at this level until the end of the reperfusion period (Fig. 3).

The vasodilatory action of BDM during initial reperfusion is reflected by the markedly decreased oxygen extraction at the end of the 5, 20 and 40 min of initial BDM perfusion (for all oxygen data: see Table 1). After BDM washout, oxygen extraction returned to preischemic baseline values and was not different among groups at the end of reperfusion. Oxygen demand was depressed during BDM perfusion by about 50%. Following washout of BDM, oxygen consumption increased, but remained significantly reduced in comparison to preischemic baseline only in control and BDM-40 hearts. Cardioplegic arrest and reperfusion decreased the efficiency of oxygen utilization similarly in all groups.

3.4. Electron microscopy

Control hearts, fixated for electron microscopy after 50 min cardioplegia and 30 min reperfusion displayed severe hypercontracture as indicated by the shortening of sarcomeres and the wide and irregular Z-lines. Mitochondria were drastically swollen, the matrix widely cleared and only few intact cristae visible (Fig. 4). BDM-5 and BDM-40 hearts showed slight improvement of ultrastructural damage in comparison to control hearts, with moderate contracture and less mitochondrial edema. In BDM-20 hearts, contrac-

Fig. 5. Electron microscopy graph of a heart from the BDM-20 group, i.e. after 50 min of cardioplegic arrest in STH at 37°C, and 20 min initial reperfusion with BDM followed by 30 min perfusion with normal Krebs. Only discreet signs of reperfusion damage are visible. The sarcomeres are contracted normally, without indication of hypercontracture. Z-lines are narrow and regularly shaped. Slight mitochondrial edema can be detected but cristae are widely preserved. Scale bar: 0.6 $\mu$m.
tured during early reperfusion. As increased in the control hearts resulting in the typical and the onset of reperfusion and washout of the cardioplegic solution, or by a combination of both mechanisms. With the protective properties of the St. Thomas Hospital II balloon pressure increased only moderately during cardio-

lar injury 6.

and may attenuate the contracture induced structural cellu-

lar damage after cardioplegic arrest in isolated perfused guinea pig hearts are: (1) 20 mM BDM added to the perfusate during reperfusion considerably reduced reperfu-

sion contracture, improved recovery of postischemic my-
ocardial function and attenuated ultrastructural damage; and (2) the optimal beneficial effect was achieved with 20 min as compared to only 5 min or the longer period of 40 min BDM perfusion.

The putative mechanism of the BDM effect in our study can be derived from the pathophysiology of reperfusion damage in the heart and the pharmacology of BDM. During ischemic cardioplegic arrest, cardiac myocytes are depleted of energy and Ca\(^{2+}\) accumulates in the cytosol. The increased intracellular Ca\(^{2+}\) concentration activates the contractile apparatus to induce contracture, but this effect is limited by the finite amount of Ca\(^{2+}\) present in the tissue and the lack of energy necessary for actin-myosin interaction. With the onset of reperfusion, extracellular Ca\(^{2+}\) is restored allowing for further Ca\(^{2+}\) influx while elimination of Ca\(^{2+}\) from the cytosol is inadequate. Simultaneously, rapid resumption of ATP synthesis provides sufficient energy for Ca\(^{2+}\) mediated actin-myosin interac-

tion, resulting in hypercontracture which may structurally damage cardiac myocytes by disrupting myofibrils, the cytoskeleton, and the sarcolemm.

BDM uncouples muscle contraction from excitation and the cytosolic Ca\(^{2+}\) concentration by decreasing the Ca\(^{2+}\) sensitivity of contractile proteins [10–13], or by directly affecting actin-myosin crossbridge kinetics [14,15]. In concentra-

tions above 5 to 10 mM (20 mM in the present study) BDM also alters intracellular Ca\(^{2+}\) kinetics and transsarcolemmal Ca\(^{2+}\) transport [12,14,19–21], lowers heart rate and increases AV conduction time [11,16]. Thus BDM reduces the intracellular Ca\(^{2+}\) load as well as the Ca\(^{2+}\) induced hypercontracture during early reperfusion and may attenuate the contracture induced structural cellular injury [6].

In the present study contracture was estimated by recording diastolic balloon pressure at a constant volume during cardioplegia and early reperfusion. In all groups balloon pressure increased only moderately during cardio-

plegia, indicating that contracture was sufficiently inhib-

ited either by the lack of energy during ischemic arrest, by the protective properties of the St. Thomas Hospital II solution, or by a combination of both mechanisms. With the onset of reperfusion and washout of the cardioplegic solution, however, balloon pressure rapidly and markedly increased in the control hearts resulting in the typical and damaging hypercontracture during early reperfusion. As expected, the presence of BDM in the reperfusate attenuated this increase in balloon pressure. However, 5 min of BDM perfusion were insufficient to protect the heart from hypercontracture also after washout of the drug, while this goal was achieved after 20 and 40 min of BDM perfusion (Fig. 2).

In addition, BDM improves the myocardial O\(_2\) balance during initial reperfusion by reducing O\(_2\) demand (attenuated contracture, negative inotropy) and dilating the coronary vasculature [16,22] [as indicated by decreased oxygen extraction at unchanged flow in the present study]. Because these effects, which markedly enhanced the my-
ocardial O\(_2\) balance, were abolished with the onset of BDM washout, the assumed protection through both, the improved O\(_2\) delivery to demand ratio and the increased perfusion rate acted only while BDM was present and may thus also depend on the duration of BDM perfusion.

Our hypothesis that BDM improved functional recovery of the hearts by attenuating hypercontracture induced cellular damage during initial reperfusion after cardioplegic arrest is supported by the electron microscopy studies: control hearts show severe ultrastructural damage including markedly shortened sarcomers with widened and fuzzy Z-lines, indicating contracture. The mitochondria are swollen, the mitochondrial matrix is severely cleared, and most cristae are fragmented. Amorphous densities, which would indicate irreversible ischemic injury in cardiac mito-

chondria [23], were, however, not observed. These alter-

ations were only slightly attenuated in BDM-5 and BDM-40 hearts, while sarcomeric and mitochondrial damage was markedly reduced only in hearts from the BDM-20 group, which also rendered the best functional recovery.

The reason for the partial loss of BDM mediated car-
diac protection by increasing the duration of the initial BDM reperfusion from 20 to 40 min is not quite clear. However, longtime perfusion of non beating hearts has been demonstrated to increase tissue edema and to deteriorate cardiac function [24]. Also, BDM may exert a directly damaging effect on cell membranes and intracellular structures, e.g. through its phosphatase like enzymatic activity [25]. Possibly such toxic effects outweigh the protective actions when the application time exceeds a certain limit (between 20 and 40 min in our study).

4.1. Critique of the methods

Following preliminary experiments, the duration of car-
dioplegic arrest was set at 50 min to achieve pronounced depression of post-ischemic function as to allow for moni-
toring of treatment effects. Also, a reperfusion period in control hearts and a BDM washout period in treated hearts of 30 min each were selected, because no further recovery of myocardial function was observed beyond this time. Thus differential total periods of reperfusion among groups did not influence the data on recovery of myocardial function. We also preferred to adjust end-diastolic balloon.
pressure to 5 mmHg in all hearts after reperfusion, because control experiments demonstrated that systolic developed pressure was maximal in our heart preparation at this preload in control as well as post-ischemic hearts.

In conclusion, our data show that addition of 20 mM BDM to a normokalemic perfusate during initial reperfusion after cardioplegic ischemic arrest has the potential to attenuate hypercontracture, to markedly reduce ultrastructural myocardial damage, and to improve functional recovery of the heart. However, the optimal duration of such high dose BDM perfusion must be carefully evaluated. In our guinea pig isolated heart preparation 20 min of initial BDM reperfusion were clearly superior to either 5 min or 40 min of treatment. Thus, contraction uncoupling during BDM reperfusion were clearly superior to either 5 min or our guinea pig isolated heart preparation 20 min of initial high dose BDM perfusion must be carefully evaluated. In

References


