"Reperfusion Injury" by Oxygen-Derived Free Radicals?

Effect of Superoxide Dismutase Plus Catalase, Given at the Time of Reperfusion, on Myocardial Infarct Size, Contractile Function, Coronary Microvasculature, and Regional Myocardial Blood Flow

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Do oxygen-derived free radicals, generated at the time of reperfusion, lethally injure viable, previously ischemic myocardium, damage vascular endothelium, and impair recovery of postischemic contractile function? To address these issues, 23 anesthetized open-chest dogs underwent 2 hours of left anterior descending coronary artery occlusion followed by 4 hours of reperfusion. Immediately prior to reflow, each dog was randomized to receive either the free radical scavenging agents superoxide dismutase (SOD)+catalase, or saline alone. SOD+catalase had no significant beneficial effect on infarct size measured by triphenyltetrazolium staining: area of necrosis averaged 38.5±6.1% vs. 46.3±6.2% of the area at risk in treated compared with control animals respectively (p=NS). Furthermore, infusion of SOD+catalase did not alter contractile function of the viable subepicardium: mean segment shortening (measured using sonomicrometry) at 4 hours postreperfusion was -23±5% of baseline, preocclusion values in controls dogs and -24±9% of preocclusion values in animals that received the scavenging agents. However, SOD+catalase treatment preserved the endocardial microvasculature (assessed by semiquantitative electron microscopic analysis) and enhanced regional myocardial blood flow after reperfusion. Specifically, mean score for microvascular injury was 0.41±0.14 vs. 0.10±0.08 (p<0.05) in control compared with SOD+catalase treated groups, and blood flow averaged 0.56±0.11 vs. 1.27±0.33 ml/min/g tissue (p<0.05), respectively, in the previously ischemic endocardium at 2 hours postreflow. Thus, SOD+catalase given at the time of reperfusion had no acute beneficial effect on either the extent of myocyte necrosis or postischemic contractile function in this canine model. SOD+catalase did, however, attenuate both endocardial vascular injury and the "low reflow" phenomenon. These data suggest that microvascular injury and low reflow following prolonged (2 hour) but transient coronary occlusion may be mediated by oxygen-derived free radicals generated at the time of reperfusion. (Circulation Research 1989;64:86–96)

There is no question that timely reperfusion of an occluded coronary artery can salvage previously ischemic myocardium. This in turn can both reduce the extent of ischemia-induced necrosis and enhance long-term recovery of regional contractile function. The benefits of early reperfusion in evolving acute myocardial infarction, first observed in experimental models of transient coronary occlusion, have in recent years become clinically feasible with the advent of techniques such as angioplasty, thrombolysis, and surgical revascularization.

While reduction of infarct size and preservation of left ventricular function are clearly desirable, recent evidence suggests that reperfusion may be a "double-edged sword." That is, restoration of blood flow may salvage one population of previously ischemic myocytes, yet lethally injure other viable, previously ischemic cells. Specifically, this phenomenon—termed "reperfusion injury"—is
thought to be a consequence of cytotoxic and highly reactive oxygen-derived free radicals (OH and O₂·), generated at a greatly accelerated rate upon reintroduction of O₂ to previously ischemic tissue. Increased concentrations of oxygen-centered radicals have been documented in reperfused myocardium by spin resonance spectroscopy. Furthermore, prolonged exposure of isolated perfused heart preparations to free radical generating systems has been shown to result in contractile dysfunction and severe ultrastructural damage to both the myocytes and vascular endothelium. In spite of this in vitro evidence, the concept of reperfusion injury in in vivo models of prolonged coronary occlusion (i.e., >40 minutes) followed by reperfusion remains controversial.

In the present study, we administered the potent free radical scavenging agents superoxide dismutase (SOD)+catalase immediately prior to reflow in an attempt to determine whether oxygen-derived free radicals, generated at the time of reperfusion, 1) lethally injure viable, previously ischemic myocardium, 2) damage the coronary vasculature, and/or 3) impair recovery of postischemic contractile function in an anesthetized, open-chest canine model of transient (2 hour) coronary artery occlusion.

Materials and Methods

Surgery

Forty-five mongrel dogs of either sex, weighing an average of 20.4±5.0 kg, were anesthetized with sodium pentobarbital (30 mg/kg i.v.), intubated, and ventilated with room air. Cannulae were inserted into the left external jugular vein for administration of drugs and fluids and into the left common carotid artery for measurement of heart rate and arterial pressures. A left thoracotomy was performed through the fifth intercostal space, the ribs were retracted, and the heart was exposed and suspended in a pericardial cradle. The left atrial appendage was then cannulated for later infusion of either SOD+catalase or saline and for injection of radioactive microspheres for measurement of regional myocardial blood flow (RMBF). A Millar (Houston, Texas) micro-tipped pressure transducer was positioned within the left ventricular (LV) cavity via the left atrial appendage for measurement of LV pressure and its first derivative, LV dP/dt.

A small segment of the left anterior descending coronary artery (LAD) was dissected free of surrounding tissue and isolated, usually just distal to its first major diagonal branch. The artery was then briefly occluded for less than 5 seconds to delineate the extent of cyanosis.

Regional contractile function in the soon-to-be ischemic subepicardium was assessed using sonomicroscopy, the details of which have been presented previously. One pair of ultrasonic crystals, used to measure segment shortening (SS), was inserted via small scalpel incisions into the superficial subepicardial layer at a depth of 2–4 mm. The crystals were positioned in the center of the LAD bed at a separation of 5–12 mm and were oriented parallel to the minor axis of the heart. Segment shortening, arterial and LV pressures, and dP/dt were monitored continuously throughout the experiment on a Gould recorder (Cleveland, Ohio).

Protocol

After initial baseline measurement of hemodynamic parameters and SS, each dog received a bolus dose of lidocaine (1.5 mg/kg i.v.). The LAD was then abruptly occluded with atraumatic vascular clamps. Hemodynamic parameters and SS were assessed at 30 minutes, 1 hour, and 2 hours postocclusion, while RMBF was measured by injection of radioactive microspheres at 30 minutes postocclusion.

Before reperfusion at 2 hours postocclusion, each dog was randomized to receive one of the following treatments: 1) SOD (Sigma Chemical, St. Louis, Missouri; 3,200 IU/mg; 5 mg/kg)+catalase (Sigma; 2,500 IU/mg; 5 mg/kg) dissolved in 5 ml of saline as an intra-atrial bolus given 1 minute before reperfusion. This was followed by a continuous intra-atrial infusion of SOD (5 mg/kg/hr)+catalase (5 mg/kg/hr) dissolved in saline at a rate of 1 ml/min. This infusion was maintained throughout the 4 hours of reperfusion. 2) Saline (5 ml) given into the left atrium at 1 minute before reperfusion, followed by a continuous intra-atrial infusion at a rate of 1 ml/min for the remainder of the protocol.

A second measurement of RMBF was made at 2 hours postreperfusion, while hemodynamic parameters and SS were assessed at 30 minutes, 1 hour, 2 hours, 3 hours, and 4 hours postreperfusion.

Immediately before the animals were killed at 4 hours postreperfusion, in vivo transmural needle biopsies were obtained from the center of the previously ischemic LAD bed, subdivided into endocardial and epicardial halves, and rapidly fixed by immersion in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for subsequent electron microscopic analysis. The LAD was then reoccluded and monastral blue pigment (0.5 ml/kg) injected into the coronary circulation via the left atrium to delineate the in vivo occluded bed size, or area at risk (AR). Immediately after injection of the dye, the dogs were killed by intracardiac injection of potassium chloride and the hearts excised.

After marking the positions of the ultrasonic crystals, the hearts were cut into six to eight transverse sections, parallel to the atrioventricular groove. Epicardial and endocardial contours of the basal surfaces of the heart slices, and the margins of the occluded LAD bed, were traced onto acetate sheets. Correct placement of the crystals (i.e., within the center of the area at risk at a depth of approximately 2–4 mm) was confirmed at this time. To distinguish necrotic from normal tissue, the heart slices were then incubated for 10 minutes in a 1% solution of triphenyltetra-
zolium chloride (TTC) at 37° C. The heart slices, and boundaries of the area of necrosis (AN) within each heart slice, were retracted onto the acetate sheets, and the hearts were then fixed by immersion in 10% neutral buffered formalin.

Analysis

After fixation, right ventricular tissue was trimmed off each heart slice, and the slices were weighed. Contours of the LV, AR, and AN were cut from the tracing of the heart slices and weighed; AN and AR were corrected for the weight of the tissue slice and summed for each heart. AN was then expressed as both a percentage of the AR (i.e., AN/AR) and percentage of the LV (AN/LV).

For measurement of RMBF, blocks of tissue from both a percentage of the previous ischemic LAD bed and remote, normal myocardium were cut and subdivided into endocardial, midmyocardial, and epicardial segments. RMBF was then quantified by the method of Domenech et al.16

Tissue samples obtained for electron microscopic analysis were cut into 1 mm3 blocks, fixed overnight in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at a pH of 7.2, and then postfixed with 1.0% osmium tetroxide in 0.1 M sodium cacodylate (pH 7.2). The samples then underwent dehydration in graded ethanol and propylene oxide, infiltration, and were embedded in plastic resin (Embed 812, Electron Microscopy Science, Fort Washington, Pennsylvania). Semithin sections (1 µm) were cut and stained with toluidine blue and examined under light microscopy. Thin sections (approximately 70 nm) were then cut from selected blocks, stained with uranyl acetate followed by lead citrate, and examined on a Zeiss EM 10 CA electron microscope.

Analysis was performed on photographs obtained at magnifications of approximately ×2,000-4,000. Myocytes were graded in a semiquantitative fashion from 0 to 4 using the following accepted grading (I bands, glycogen loss, nuclear clumping, and margination); 2=moderate ischemic changes (the findings in 2 plus early intermyofibrillar and sarcoplasmic reticular edema, mitochondrial edema); 3=moderately severe ischemic changes (findings in 2 plus subsarcolemmal blebs, gaps, marked edema, presence of mitochondrial dense bodies); and 4=severe architectural disruption, sarcolemmal breaks, contraction bands and mitochondrial disruption.

The coronary microvasculature was similarly graded on a scale of 0 to 1, where 0=normal; 1=severely injured (decreased pinocytotic vesicles, presence of endothelial gaps, endothelial blebs, fibrin deposition, and microscopic hemorrhage); 0.5=mildly injured (some but not all of the above abnormalities). All electron microscopic analysis was performed by a trained observer (R.A.K.) who was blinded with respect to the treatment group of any given specimen. Three to seven independent fields of view were graded from both the endocardium and epicardium of each dog.

Heart rate, mean arterial pressure, and LV dP/dt were measured and averaged over five continuous cardiac cycles for each sample period. In addition, dP/dt was used to define the separation between the ultrasonic crystals at specific points in the cardiac cycle: end diastolic lengths (EDL) were measured at the onset of the rapid rise of dP/dt, while end systolic lengths (ESL) were measured at peak negative dP/dt.12,13 EDL and ESL were measured from three well-separated cardiac cycles in each sample period, averaged, and used to compute segment shortening (SS), defined by equation (12,13)

\[ SS = \frac{EDL - ESL}{EDL} \times 100\% \]

Values of SS measured during each sample period were then normalized and expressed as a percentage of their normal, baseline value measured before LAD occlusion (i.e., %SS).

Statistics

Dogs with high collateral blood flow during coronary occlusion and a small AR (i.e., animals that were not sufficiently ischemic to develop myocyte necrosis) were excluded from the final analysis. Specifically, our exclusion criteria, established prior to the onset of the protocol, were values of RMBF in the “ischemic” endocardium greater than 0.10 ml/min/g tissue during LAD occlusion, and/or an AR less than 10% of the LV. No attempt was made to revive dogs that fibrillated during occlusion or during reperfusion.

Mean values of AN/AR, AN/LV, AR/LV, RMBF, and electron microscopic score were compared between the control and SOD+catalase treated groups by t tests for unpaired data. Hemodynamics and absolute values of SS were compared between groups preocclusion, at 120 minutes postocclusion (i.e., immediately before reperfusion), and at 30, 120, and 240 minutes postreperfusion; similarly, %SS (normalized to baseline, preocclusion values) were compared at 120 minutes postocclusion and at 30, 120, and 240 minutes postreperfusion. Bonferroni’s correction for multiple comparisons was then applied.18 All values are expressed as mean±SEM and are considered to differ significantly if p<0.05.

Results

Of the 45 dogs entered into the study, 15 animals died before randomization: 14 died due to ventricular fibrillation within the first 3–30 minutes postocclusion, and one animal died because of respirator failure. One control animal fibrillated upon reperfusion, and one control died 30 minutes postreperfusion. Five animals were excluded from the study because they were not sufficiently ischemic during occlusion (i.e., endocardial RMBF during occlusion >0.10 ml/min/g tissue and AR/AR<10%). Specifi-
TABLE 1. Hemodynamic Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pre</th>
<th>Occlusion</th>
<th>Reperfusion</th>
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<tr>
<td>Heart rate (beats/min)</td>
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<tr>
<td>Control (n=11)</td>
<td>149±6</td>
<td>143±5</td>
<td>141±7</td>
</tr>
<tr>
<td>SOD+CAT (n=12)</td>
<td>145±7</td>
<td>139±7</td>
<td>132±7</td>
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<tr>
<td>Mean arterial pressure (mm Hg)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=11)</td>
<td>126±5</td>
<td>127±5</td>
<td>123±5</td>
</tr>
<tr>
<td>SOD+CAT (n=12)</td>
<td>122±5</td>
<td>124±7</td>
<td>114±8</td>
</tr>
<tr>
<td>Peak positive LV dP/dt (mm Hg/sec)</td>
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</tr>
<tr>
<td>Control (n=11)</td>
<td>1,716±59</td>
<td>1,596±68</td>
<td>1,618±62</td>
</tr>
<tr>
<td>SOD+CAT (n=12)</td>
<td>1,808±88</td>
<td>1,684±118</td>
<td>1,629±132</td>
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</tbody>
</table>

SOD, superoxide dismutase; CAT, catalase.

-3.6±1.1% (or -27±9% vs. -23±8% of baseline, preocclusion values) in control compared with treated groups at 2 hours postocclusion (p=NS; Table 3).

Contractile function in the salvaged subepicardium remained markedly "stunned" or dyskinetic throughout the 4 hours of reperfusion and treatment. SOD+catalase had no apparent beneficial effect on postischemic contractile function: SS after 2 hours of reperfusion averaged -2.4±0.6% vs. 0±1.7% (or -18±5% vs. -2±11% of preocclusion values) in control versus treated dogs respectively (p=NS).

Infarct Size and Area at Risk
In vivo area at risk (AR/LV) was essentially equal in both control animals (19.0±0.8%) and those that received SOD+catalase (18.7±1.8%; p=NS; Figure 2). No significant difference in infarct size (expressed as a percentage of the AR) was observed between

Regional Myocardial Blood Flow
Both groups of dogs were equally ischemic at 30 minutes postocclusion (Figure 1). For example, mean RMBF in the ischemic midmyocardium was 0.07±0.02 and 0.05±0.02 ml/min/g tissue in control and treated animals respectively (p=NS).

SOD+catalase given at the time of reperfusion significantly increased blood flow to the previously ischemic endocardium and midmyocardium: in the previously ischemic midwall, RMBF averaged 0.42±0.05 vs. 0.82±0.19 ml/min/g tissue in control versus treated dogs respectively (p<0.05; Figure 1). In contrast, RMBF in the normally perfused circumflex bed did not differ between the two groups following reperfusion: 0.87±0.06 vs. 0.74±0.11 ml/min/g in the midmyocardium (p=NS; Table 2). These data indicate that SOD+catalase selectively enhanced blood flow after reperfusion in the previously ischemic tissue.

Regional Contractile Function
Baseline values of segment shortening averaged approximately 16% in both control animals and those later assigned to receive SOD+catalase. In addition, all dogs were equally dyskinetic during 2 hours of ischemia: mean SS was -3.4±0.9% vs. -3.6±1.1% (or -27±9% vs. -23±8% of baseline, preocclusion values) in control compared with treated groups at 2 hours postocclusion (p=NS; Table 3).

Figure 1. Regional myocardial blood flow (RMBF) in the center of the left anterior descending coronary artery (LAD) bed measured 30 minutes postocclusion and at 2 hours postreperfusion in control and superoxide dismutase (SOD)+catalase treated dogs. *p<0.05 vs. the corresponding control value. ENDO, endocardium; MID, midmyocardium; EPI, epicardium.
control and SOD+catalase treated groups: AN/AR averaged 46.3±6.2% in control animals compared with 38.5±6.1% in animals that received SOD+catalase (p=NS; Figure 2). Similar results were obtained when AN was expressed as a percentage of the total LV mass: AN/LV was 9.1±1.5% vs. 8.2±1.7% for control compared with treated dogs, respectively (p=NS; Figure 2). These data indicate that SOD+catalase, initiated at the time of reperfusion, had no significant beneficial effect on the extent of necrosis produced by 2 hours of LAD occlusion and 4 hours of reperfusion.

The relation between AN/AR and collateral flow to the ischemic midmyocardium during LAD occlusion for both control and treated groups is illustrated in Figure 3. The regression line for dogs that received SOD+catalase fell below that obtained for the saline controls (suggesting a possible reduction in infarct size); however, the y intercepts of the two lines did not differ when analyzed statistically (p>0.20). Similar plots were obtained when either endocardial, epicardial, or weighted mean transmural flow was used as the dependent variable. While these data imply that SOD+catalase given at the time of reperfusion may have limited infarct size in some animals, this did not approach statistical significance because of the considerable variability in both collateral flow and AN/AR in the canine model.22,23

Electron Microscopic Analysis

Biopsies for electron microscopic analysis were obtained from the center of the previously ischemic LAD bed in 20 of the 23 dogs that successfully completed the protocol.

Injury to the vascular endothelium was attenuated by infusion of SOD+catalase: mean score for microvascular injury in the previously ischemic endocardium was significantly less in treated animals (0.10±0.08) than in the saline controls (0.41±0.14; p<0.05; Table 4 and Figure 4). This marked preservation of the endocardial vascular endothelium by SOD+catalase is illustrated in the electron micrographs shown in Figure 5. In contrast, no differences in the degree of epicardial vascular injury or myocyte damage were noted between control and treated groups (Table 4).

**Discussion**

Do oxygen-derived free radicals, generated at the time of reperfusion, lethally injure viable, previously ischemic myocardium? To address this issue, several investigators have administered free radical scavenging agents or antioxidants in an attempt to reduce infarct size in the canine model of transient coronary occlusion. Allopurinol,19,20 oxypurinol,21 SOD+catalase22 and SOD alone,19,23 given before or during coronary occlusion, have all been shown to reduce the extent of necrosis produced by 60–90 minutes of ischemia and 4–24 hours of reflow. These data appear to support the concept of "reperfusion injury"; however, recent evidence indicates that oxygen-centered radicals are also formed during occlusion in species (such as dog and man) in which native collateral vessels provide residual blood flow (and therefore low levels of molecular oxygen) to the ischemic tissue.24,25 As free radicals formed during prolonged periods of coronary occlusion appear to contribute to myocyte necrosis,26,27 studies which address reperfusion injury by initiating treatment before or during occlusion must be interpreted with caution.

In contrast, other studies have failed to document a reduction in infarct size in response to early treatment (before or during occlusion) with free radical scavengers or antioxidants. Allopurinol,21,28 oxypurinol,29,30 and SOD31–33 in these studies did not reduce the extent of myocyte necrosis produced during ischemia.

**Table 3. Regional Contractile Function: Segment Shortening in the Subepicardium of the LAD Bed**

<table>
<thead>
<tr>
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<th>Pre</th>
<th>Occlusion</th>
<th>Reperfusion</th>
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<tbody>
<tr>
<td></td>
<td>30 min</td>
<td>120 min</td>
<td>30 min</td>
</tr>
<tr>
<td>I. % of preocclusion values</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=11)</td>
<td>100%</td>
<td>-27±8%</td>
<td>-27±9%</td>
</tr>
<tr>
<td>SOD+CAT (n=12)</td>
<td>100%</td>
<td>-20±8%</td>
<td>-23±8%</td>
</tr>
<tr>
<td>II. Absolute values</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=11)</td>
<td>15.6±1.2%</td>
<td>-3.6±0.8%</td>
<td>-3.4±0.9%</td>
</tr>
<tr>
<td>SOD+CAT (n=12)</td>
<td>16.3±1.0%</td>
<td>-3.1±1.2%</td>
<td>-3.6±1.1%</td>
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SOD, superoxide dismutase; CAT, catalase.
ALL DOGS

AN/AR (X)  CD CONTROLS  AN/LV (X) AR/LV (X)

SOD + CATALASE  (n=12)

FIGURE 2. Infarct size data (i.e., area of necrosis [AN] expressed as a percentage of the area at risk [AR], AN expressed as a percentage of the total left ventricular weight [LV], and AR expressed as a percentage of the LV) for control and treated dogs.

by 40 minutes to 3 hours of occlusion and 6 hours to 4 days of reperfusion.

Only two other studies have addressed the topic of reperfusion injury by administering a free radical scavenger at the time of reperfusion. Ambrosio et al. found that recombinant human SOD given "only at the moment" of reflow significantly reduced infarct size produced by 90 minutes of ischemia and 48 hours of reperfusion. These data provide evidence in support of reperfusion injury mediated by oxygen-derived free radicals. In contrast, Nejima et al., who also studied the extent of necrosis produced by 90 minutes of coronary occlusion, found no difference in infarct size, measured 1 week postreperfusion, in dogs treated with SOD+catalase compared with controls.

In the present study, we found that SOD+catalase administered 1 minute before reperfusion did not reduce infarct size associated with 2 hours of coronary artery occlusion and 4 hours of reflow when all dogs were considered. However, further retrospective analysis of the data suggested that the efficacy of SOD+catalase treatment in any given animal in our study may have been influenced by the degree of collateral blood flow during LAD occlusion. Mean RMBF to the midmyocardium for the 22 dogs in which flow measurements were obtained was 0.06 ml/min/g tissue. In the subgroup of animals with higher than average collateral flow (RMBF in the ischemic midmyocardium >0.06 ml/min/g), AN/AR was lower in SOD+catalase treated dogs (18.3±6.2%) than in the comparable subgroup of saline controls (40.0±6.7%; p<0.05; Figure 6, left panel). This apparent reduction in infarct size could not be attributed to significant differences in either area at risk (AR/LV, 17.7±2.7% vs. 13.5±2.2% in control compared with treated high-flow subgroups; p=NS) or RMBF during occlusion (i.e., in the midmyocardium, mean RMBF was 0.11±0.01 vs. 0.16±0.03 ml/min/g tissue in control compared with treated subgroups; p=NS). In contrast, in the subgroup of dogs with lower than average collateral flow during occlusion (RMBF in the ischemic midmyocardium <0.06 ml/min/g), infarct size was similar in control (57.4±9.8%) compared with treated animals (48.6±5.9% of the area at risk; p=NS; Figure 6, right panel).

Although these data were obtained from retrospective analysis, are based on an arbitrary subdivision of "higher than average" and "lower than average" flows, and involved small sample sizes, the results imply that the distribution of individual flow values may be of importance in determination of the efficacy of free radical scavengers given at the

<table>
<thead>
<tr>
<th>Control (n=10)</th>
<th>SOD+CAT (n=10)</th>
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<tbody>
<tr>
<td>Myocytes</td>
<td>Microvasculature</td>
</tr>
<tr>
<td>Endo  Epi</td>
<td>Endo  Epi</td>
</tr>
<tr>
<td>3.19±0.36  0.84±0.38</td>
<td>0.41±0.14  0.20±0.11</td>
</tr>
<tr>
<td>2.47±0.46  1.71±0.50</td>
<td>0.10±0.08*  0.23±0.11</td>
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*p<0.05 vs. corresponding controls.

Endo, endocardium; Epi, epicardium; SOD, superoxide dismutase; CAT, catalase.
The effect of free radical scavenging agents on infarct size, few studies have obtained in studies in which a large proportion of the dogs had moderately high collateral blood flow, while negative results may have been obtained in studies in which a large proportion of the animals were severely ischemic during coronary occlusion.

Ambrosio et al34 also concluded that collateral blood flow during occlusion was a crucial determinant of the efficacy of SOD treatment. However, in contrast to our retrospective observations, Ambrosio and coworkers found the hearts that showed the greatest benefit from SOD treatment were those with the lowest (as opposed to the highest) collateral flows during occlusion. This is similar to the apparent, flow-dependent effect of the calcium-channel blocking agent verapamil observed in a limited subset of dogs that were severely ischemic during coronary occlusion.30 In addition, Gallagher et al35 found no reduction in infarct size with SOD or catalase in a population of dogs with moderately high values of RMBF during occlusion. The possible role of collateral flow in influencing the efficacy of treatment with free radical scavenging agents is inconclusive: the effect and importance of "high" versus "low" RMBF during occlusion must be clarified from carefully designed prospective studies.

While several studies have examined the effects of free radical scavenging agents on infarct size, few have assessed the concomitant effect of the scavengers on contractile function of the viable subepicardial tissue salvaged by reperfusion. Considerable evidence indicates that recovery of regional contractile function of myocardium stunned by prolonged (≥40 minute) periods of ischemia, however, has received little attention to date.

As anticipated, all dogs in the present study were severely dyskinetic during the 2 hours of coronary occlusion. A slight, transient improvement in contractile function, similar to that described by Buda et al42 was observed at 30 minutes postreperfusion in both treated and control dogs. However, all dogs again became dyskinetic in the final 2 hours of the protocol. These data are in agreement with several previous studies43-45 that demonstrate that myocardium salvaged by reperfusion after 2 hours of ischemia remains severely depressed for days following reflow.

SOD+catalase, given at the time of reflow, had no apparent beneficial effect on contractile function of the viable subepicardium during the initial 4 hours of reperfusion. These data are similar to those obtained by Puett et al,29 who subjected dogs to 90 minutes of regional ischemia and administered either oxypurinol (an inhibitor of xanthine oxidase) or saline at 60 minutes postocclusion: at 3 hours postreperfusion, percent radial shortening (assessed by echocardiography) was comparable in both oxypurinol-treated and saline control groups. At 24 hours postreperfusion, however, animals that had received oxypurinol exhibited improved contractile function when compared with saline controls.29 Although SOD+catalase and oxypurinol did not have an acute beneficial effect on contractile function, results obtained by Puett et al suggest that antioxidants and scavengers may accelerate recovery of regional contractile function when assessed at or beyond 24 hours postreperfusion. In contrast, Nejima et al33 found that SOD+catalase, given 5 minutes before reperfusion, did not improve regional contractile function assessed 1 week postreperfusion in conscious dogs that underwent 90 minutes of transient coronary occlusion. Thus, the long-term effect of free radical scavenging agents and antioxidants on recovery of regional contractile function following prolonged periods of transient ischemia remains uncertain.

When reperfusion is carried out following prolonged (≥90 minute) periods of coronary occlusion, areas of no reflow,46-47 low reflow,47 and microvascular injury17 are often observed within the area of myocardial necrosis. Are these perfusion defects mediated by oxygen-derived free radicals generated at the time of reperfusion? The effect of free radical scavenging agents on regional myocardial blood flow and microvascular damage have received little attention in in vivo models of transient, regional myocardial ischemia.

As anticipated, control animals in the present study exhibited 'low reflow' following reperfusion: regional myocardial blood flow was restored to only 0.42–0.67 ml/min/g tissue in the previously ischemic LAD bed, or approximately 40–70% of values measured in the normally perfused circumflex bed.
FIGURE 5. Top: Electron micrograph obtained from the previously ischemic endocardium of a saline control animal. Both the myocytes (m) and the vascular endothelial cells (e) are severely damaged (approximate magnification, ×5,100). Bottom: Electron micrograph obtained from the previously ischemic endocardium of an animal that received SOD+catalase at the time of reperfusion. The myocytes (m) are irreversibly injured, but the vascular endothelium (e) has been preserved (approximate magnification, ×3,100).

SOD+catalase administered at the time of reflow enhanced postreperfusion blood flow to the endocardium and midmyocardium of the previously ischemic LAD bed. These data are comparable to the increased values of coronary flow associated with addition of free radical scavenging agents and antioxidants to cardioplegic solutions in models of transient global ischemia. In addition, a trend
toward a similar hyperemic effect was noted by Ambrosio et al at 10 minutes postreperfusion in animals treated with human recombinant SOD. In both our study and that of Ambrosio et al, the increase in flow postreperfusion occurred only in regions of the myocardium that had been ischemic; no hyperemic effect was observed in the normally perfused tissue.

How can this improved blood flow in the previously ischemic myocardium of SOD+catalase-treated animals be explained? The improved values of regional myocardial blood flow probably cannot be attributed to vasodilation, as no effect was observed within the normally perfused circumflex bed. In addition, this increased flow does not appear to be related to improved contractile function, as segment shortening in the previously ischemic LAD bed measured 2 hours postreperfusion did not differ between control and treated groups.

Considerable evidence indicates that oxygen-derived free radicals are responsible for vascular injury in the cerebral circulation following brain injury. Specifically, free radicals have been shown to increase vascular permeability in the brain, leading to edema and tissue damage. Similar observations have, more recently, been made in isolated heart preparations. For example, Jackson et al found an increase in vascular permeability and vascular dysfunction in isolated rabbit hearts perfused with buffered solutions containing oxygen-derived free radicals. Morphologic evidence of vascular injury (by electron microscopy) was observed both in isolated rabbit septae exposed to free radical-generating solutions and in isolated septal preparations subjected to 1 hour of ischemia followed by reperfusion. In each case, the addition of free radical scavenging agents (dimethyl sulfoxide, SOD, and catalase) to the perfusate provided significant protection against this free radical-mediated vascular injury.

Electron microscopic analysis of biopsy samples obtained in the present study clearly indicate that the morphology of the endocardial vascular endothelium was preserved in animals that received SOD+catalase at the time of reflow. This would suggest that oxygen-derived free radicals generated upon reperfusion may play a role in mediating microvascular injury in this model. Because both low reflow and vascular injury were completely ablated in the endocardium of dogs treated with SOD+catalase, it appears likely that the enhanced flow postreperfusion in these animals could be attributed, at least in part, to the preservation of the vascular endothelium by the free radical scavenging agents.

These data may further suggest that the vascular endothelium, thought to be rich in xanthine oxidase, may represent a major source of oxygen-derived free radicals in this canine model of prolonged coronary occlusion followed by reperfusion. However, free radicals may also be generated within the previously ischemic myocytes by either the xanthine oxidase reaction or by other enzymatic pathways known to produce reactive oxygen species. In addition, recent evidence indicates that polymorphonuclear neutrophils, known to rapidly infiltrate previously ischemic myocardium following reperfusion, also represent a significant source of oxygen-derived free radicals in the canine model of prolonged but transient ischemia. The precise source(s) or mechanism(s) of free radical production in the present study is uncertain.

In summary, we observed that SOD+catalase, initiated at the time of reperfusion, did not reduce infarct size produced by 2 hours of coronary artery occlusion and 4 hours of reflow in the anesthetized, open-chest dog. Furthermore, treatment with these

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**Figure 6.** Left: Infarct size data for the subgroup of control and treated animals with higher than average collateral blood flow during left anterior descending coronary artery (LAD) occlusion. *p<0.05 and **p<0.01 vs. the corresponding control values. Right: Infarct size data for the subgroup of control and treated animals with lower than average collateral blood flow during LAD occlusion. AN/AR, area of necrosis as a percentage of area at risk; AN/LV, area of necrosis as a percentage of left ventricle; AR/LV, area at risk as a percentage of left ventricle.
potent scavenging agents did not attenuate posts ischemic contractile dysfunction of the viable, previously ischemic subepicardium. However, SOD+catalase given 1 minute before reperfusion effectively ablated both microvascular damage and the low reflow phenomenon in the endocardium and midmyocardium of the previously ischemic LAD bed. Thus, microvascular injury and low reflow after prolonged but transient coronary occlusion in the canine model may be mediated by oxygen free radicals generated at the time of reperfusion.

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"Reperfusion injury" by oxygen-derived free radicals? Effect of superoxide dismutase plus catalase, given at the time of reperfusion, on myocardial infarct size, contractile function, coronary microvasculature, and regional myocardial blood flow.

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