Reperfusion therapy for acute myocardial infarction reduces myocardial dysfunction and mortality. However, reperfusion can also injure myocardium, and adjunctive agents have been sought to limit reperfusion injury. After laboratory reports of successful reduction in infarct size by superoxide dismutase (SOD) after ischemia and reperfusion, SOD became the first of these agents to be used in clinical trials in patients undergoing reperfusion by thrombolysis or percutaneous transluminal angioplasty of acute myocardial infarction. Because of recent conflicting results in laboratory animals, it seemed appropriate to review the available preclinical studies involving SOD and the rationale for its use during acute myocardial infarction.

SOD, discovered by McCord and Fridovich, is an enzyme that catalyzes the conversion of superoxide (univalently reduced oxygen) to the less toxic hydrogen peroxide. Hearn et al described the paradoxical sudden release of enzymes from isolated, perfused hearts triggered by the restoration of oxygen after hypoxia. Granger et al and McCord proposed that this oxygen paradox was due to the formation of superoxide anion or other partially reduced oxygen species by xanthine oxidase. Xanthine oxidase uses oxygen as an electron acceptor to produce two superoxide anions upon conversion of hypoxanthine to uric acid. The normal endothelial enzyme xanthine dehydrogenase, which uses NAD⁺ as an electron acceptor, undergoes proteolytic conversion to xanthine oxidase by activation of a calcium-dependent protease upon reperfusion. The superoxide, formed by the catabolism of ATP to hypoxanthine and then to uric acid, could generate the more toxic hydroxyl radical and lead to lipid peroxidation, membrane damage, and cell death.

In isolated, buffer-perfused hearts, reperfusion with fluid containing oxygen, but not with anoxic fluid, caused creatine kinase release. SOD and catalase attenuated this injury, and the major source of superoxide was xanthine oxidase. Supportive evidence from other laboratories led to experimental attempts to reduce ischemic and reperfusion injury in animal models of myocardial infarction. In several studies, the addition of catalase was not felt to be necessary in vivo because of the presence of significant amounts in erythrocytes.

Myocardial Infarct Size: Independent Variables

To test the hypothesis that SOD will reduce infarct size in an animal model, all other significant independent variables must be known and measured. The Animal Models for Protecting Ischemic Myocardium (AMPIM) cooperative study in dogs found that the anatomic area at risk, collateral blood flow, and hemodynamic determinants of myocardial oxygen demand (measured as the rate-pressure product) could explain most of the variance in infarct size with the protocol used. However, indexes of contractility were not measured. There were small, unexplained differences in infarct size between participating laboratories, and there was lower collateral blood flow in anesthetized than in conscious dogs. The unexplained differences in infarct size and collateral flow suggest the existence of other unmeasured independent variables that may alter collateral flow and thereby explain some of the residual variance. Also, the preparatory surgery may have induced collateral development in the conscious model, in which 80% of the variability could be explained by the measured indexes (including collateral blood flow). The AMPIM study concluded that a 10% (in unconscious animals) to 13% (in conscious animals) reduction in infarct size (p<0.05) would be detected only 50% of the time with 15 animals each in the control and treatment groups.

Statistical Considerations

The AMPIM study clearly shows that in the dog model considerable variation exists in area at risk, collateral flow, and hemodynamic indexes (rate pressure product); thus, any intervention trial should measure and include these variables in the analysis of the results. Several published SOD trials involving animals did not measure collateral blood flow, whereas in pigs and rabbits, flow measurements...
are not necessarily due to uniformly low collateral flow. Other studies performed one-way analysis of variance or t test analyses of differences in blood pressure and heart rate between groups to establish baseline comparability. The use of this paradigm could lead to erroneous results because a difference attributed to the intervention could have been due to another independent variable (i.e., collateral flow, heart rate, or blood pressure) distributed unequally by chance just enough to bias the result but not quite significantly by separate analysis. An interesting example of such a result is a "randomized" study of 1,073 patients from Duke University that found a significant treatment effect between group 1 and group 2 on survival in a subset of 397 patients with three-vessel coronary artery disease and abnormal left ventricular contraction. In fact, the "treatment" was the same in both groups; the only difference was random selection. Proper multivariate adjustment procedures revealed that the difference resulted from small imbalances in other independent prognostic factors, which were not statistically different in the populations as a whole. The International Studies of Infarct Survival-2 (ISIS-2) trial of streptokinase and aspirin found a favorable survival effect of aspirin (28% ± 5%) for patients whose birth signs were other than Gemini and Libra, but patients with these two astrological signs experienced an adverse survival effect. The lesson is that randomization alone does not assure equivalent distribution of independent variables; proper statistical techniques are the best defense against the perversity of random events. As applied to the studies of SOD, the main concern regarding statistical methods can be illustrated as follows. Suppose there was a trend toward lower blood pressure and lower heart rate in animals given SOD and that neither trend was univariately significant. A difference in slope of the collateral flow and infarct size regression line could have been due to the blood pressure and heart rate trend, but without appropriate statistical techniques, the relation between treatment, heart rate, and blood pressure cannot be determined.

An analysis of covariance model can adjust for known independent covariates, and it allows the hypothesis of no difference in infarct size among groups to be tested.

In equation 1,

\[ Y_{ij} = T_j + \beta_1 X_{ij1} + \beta_2 X_{ij2} + \beta_3 X_{ij3} + \ldots + \beta_k X_{ijk} + e_{ij} \] (1)

the infarct size \( Y_{ij} \) of the \( i \)th animal in the \( j \)th treatment group (i.e., control or SOD) is determined by: \( T_j \), the effect due to treatment \( j \); \( X_{ij1} \), risk area; \( X_{ij2} \), collateral flow; \( X_{ij3} \), hemodynamic index (i.e., rate-pressure product); and up to \( k \) other factors, and \( e_{ij} \), the residual variance. Each coefficient \( \beta_i \ldots \beta_k \) is tested for significance by straightforward regression techniques. Analysis of variance (ANOVA) is one application of this more general equation. The equation also reduces to a linear regression of infarct size against collateral flow when all other covariates are ignored. In the equation, the relation of the covariates to infarct size is assumed to be additive and linear. However, modifications to the model are possible for some alternatives to these assumptions, that is, nonlinear relations such as an infarct size proportional to the square root of area at risk.

Only a few published reports on SOD have attempted a limited regression adjustment for the known covariates. These studies used covariate analysis for some but not all of the known covariates. Other studies expressed infarct size as a percentage of the area at risk in an attempt to control for differences in area at risk. This assumes that infarcts involving 10% of the small area at risk and 10% of a large area at risk are equivalent; this may not be the case. In the AMPIM study, \( r^2 \) for regression of infarct size and risk area was 0.77 and 0.66 for anesthetized and conscious dogs, respectively. However, visual inspection of the published graph suggests that a nonlinear model may be better. The few studies that have attempted regression adjustment for a known covariate have, in each case, used collateral flow. In other studies, the investigators argued that the linear regression between infarct size and leukocyte accumulation, area at risk, or hemodynamic indexes was not significant, or did not statistically differ between control and SOD groups, and therefore could be ignored. The risk in this argument is the same as for nonsignificant baseline differences. In two studies, the control group animals exhibited a linear relation between collateral flow and infarct size, but there was no relation in the SOD group. The large range in infarct size within individual studies (13-41% and 20-40%) in the SOD group is unexplained.

Also, when the hypothesis of equal infarct size in treatment and control groups cannot be rejected, reporting the \( \beta \) error would be helpful, for it reflects the chance of missing a treatment effect. For example, a chosen level of treatment effect (percent infarct size reduction) can be excluded at \( p < \beta \).

Why the Disparity Between Previous Studies?

Ten published and two abstracted studies can be reviewed (Table 1). Several possible reasons exist for the disparate results. As discussed above, only two canine studies with positive results lack collateral flow measurement. Adjustment for collateral flow by covariate analysis was performed in only three other studies. In addition, the experimental protocols used in the studies differed, and there is a possibility that other independent covariates exist that need to be taken into account.

Differences in Protocol

If results are "false-positive," they may be due to inadequate reperfusion time; SOD may delay rather than prevent injury. In a preliminary report, Shirato et al. suggested such a result with SOD in a rabbit.
ischemia-reperfusion model, in which SOD-treated animals analyzed after 3 and 24 hours showed smaller infarcts than did matched controls, whereas SOD-treated animals analyzed at 3 days did not. Triphenyl tetrazolium chloride (TTC) stain for dehydrogenase enzyme activity was used to demarcate viable tissue, as in most other infarct size studies (Table 1). In another preliminary report,29 these investigators found the triphenyl tetrazolium positive tissue at 3 and 24 hours of reperfusion showed some coagulation necrosis on histologic examination. One possible conclusion is that SOD delayed degradation of TTC positive material in dead cells; in any case, TTC staining did not appear to be an adequate marker of cell viability in the treatment group early after reperfusion, that is, 24 hours in rabbits; this has not been studied in dogs.29 Thus, brief reperfusion studies with TTC staining as the only marker of cell viability may be subject to artifact when SOD is used. One study that had positive results with SOD6 allowed 48 hours of reperfusion, and the remaining studies with positive results (Table 1) were at 24 hours or less. In the 48-hour study,6 unmodified t tests were used to test the effect of SOD and other variables on infarct size. Would appropriate adjustment for covariates have resulted in a treatment effect? In contrast to the reperfusion times in the studies with positive results, five of six studies with negative results used at least 2 days of reperfusion (Table 1). The study that had negative results with 1 day of reperfusion used 3 hours of ischemia8; this duration of ischemia per se decreases reperfusion salvage to about 9%.30

The duration of reperfusion in these laboratory studies is likely to be a significant factor in the disparate results for other reasons as well. In the experimental studies,2-13 the duration of effective SOD blood levels was quite short. Of the several sources of superoxide other than xanthine oxidase in reperfused myocardium (NADPH oxidase of neutrophils, mitochondrial leak from ubiquinone, arachidonate metabolism, and cytochrome P-450), the infiltration of neutrophils lasts several days31; the duration of mitochondrial leak and arachidonate metabolism are not precisely known. Adequate levels of SOD might have protected against the “burst” of superoxide from xanthine oxidase, mitochondrial leak, and early neutrophil-produced superoxide, but SOD levels might have been insufficient for more sustained sources of superoxide. The half-life of SOD is less than 30 minutes,8,10,11 and the SOD administration in these studies was at most 1 hour during reperfusion. Longer-acting polyethylene glycol SOD appears to reduce infarct size after both 4 days and 6 hours of reperfusion.32 In addition, there are possibly multiple mechanisms of reperfusion injury (i.e., degranulation of neutrophils, capillary no-reflow, and edema), each with its own time course, which would be expected to add up in nonlinear ways. Even if SOD could clean up “all” of the superoxide from all of the sources, other mechanisms with later action such as neutrophil degranulation, progressive capillary plugging,33,34 edema, etc. may cause an equivalent degree of reperfusion injury. Thus, reduction of one early component of reperfusion injury due to superoxide may unmask other later-acting mechanisms. It has

<table>
<thead>
<tr>
<th>Study</th>
<th>Species</th>
<th>n</th>
<th>Covariates analysis</th>
<th>Regional flow</th>
<th>Ischemia duration (min)</th>
<th>Reperfusion duration</th>
<th>Method of infarct size</th>
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<tr>
<td><strong>Positive: Superoxide dismutase reduced infarct size</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ambrosio et al6</td>
<td>Dog</td>
<td>16</td>
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<td>Yes</td>
<td>90 48 hr</td>
<td>G, H*</td>
<td></td>
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<tr>
<td>Werns et al7</td>
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<td>Limited</td>
<td>Yes</td>
<td>90 6 hr, 24 hr</td>
<td>TTC</td>
<td></td>
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<td>18</td>
<td>No</td>
<td>Yes</td>
<td>60 5 hr</td>
<td>TTC</td>
<td></td>
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<tr>
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<td>19</td>
<td>No</td>
<td>Yes</td>
<td>60 4 hr</td>
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<td>29</td>
<td>No</td>
<td>No</td>
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<td>TTC</td>
<td></td>
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<tr>
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<td>No</td>
<td>No</td>
<td>90 6 hr</td>
<td>TTC</td>
<td></td>
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<tr>
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<tr>
<td>Uraizee et al9</td>
<td>Dog</td>
<td>25</td>
<td>Limited</td>
<td>Yes</td>
<td>40 4 day</td>
<td>H</td>
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<td>Gallagher et al8</td>
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<td>No</td>
<td>Yes</td>
<td>180 1 day</td>
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<tr>
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<td>Yes</td>
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<td>H, TTC</td>
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<td>34</td>
<td>No</td>
<td>Yes</td>
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<td>??</td>
<td>Yes</td>
<td>45 3 hr, 24 hr, 3 day</td>
<td>TTC, H</td>
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<td>Richard et al10</td>
<td>Dog</td>
<td>24</td>
<td>Limited</td>
<td>Yes</td>
<td>90 4 day</td>
<td>H</td>
<td></td>
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</table>

Regional flow, regional coronary blood flow measured; ischemia duration, duration of coronary occlusion before reperfusion; Reperfusion duration, duration of coronary reperfusion before sacrifice and infarct size determination; Method of infarct size, method used to measure infarct size; Limited, analysis of covariates limited to collateral flow, other variables analyzed separately; H, histologic; TTC, triphenyl tetrazolium chloride staining for dehydrogenase and gross planimetry; G, gross appearance, unstained sections.

*Histologic confirmation of sample sections.
†Histologic confirmation of sample sections.

Pig and rabbit models do not require flow measurements as a covariate.

Table 1. Summary of Studies Concerning the Effect of Superoxide Dismutase on Myocardial Infarct Size
been proposed that an early burst of superoxide from endothelial cell xanthine oxidase produces a chemotactic effect for neutrophils in intestinal ischemia. The study by Werns et al suggests that this mechanism may be relevant to dog myocardial ischemia as well. Previous studies show that in untreated canine myocardial ischemia without reperfusion, leukocytes accumulate in inverse linear proportion to collateral flow. In the data published by Werns et al, a correlation seemed to exist between infarct size and leukocyte accumulation in controls but not in SOD-treated animals (reanalysis of data extracted from Figure 2), suggesting that SOD altered leukocyte accumulation.

The duration of ischemia is unlikely to explain a “false-negative” result because this factor was in the range where reperfusion alone has been shown to salvage ischemic myocardium. Indeed, one group of investigators performed nearly identical studies for 40 and 90 minutes of ischemia.

Reperfusion injury could be model dependent, and subtle species or protocol differences may determine the relative contribution of reperfusion injury to total infarct size. In this regard, reperfusion through a critical stenosis (one that limits reactive hyperemia but does not compromise normal blood flow) has been shown in an attempt to model the situation in patients reperfused with thrombolytic therapy. A critical stenosis was used in three of the six studies with positive results but in none of the studies with negative results. In these three studies, the degree of stenosis was not compared between control and treatment groups. However, the effect of a critical stenosis per se on infarct size is unclear. In two published studies, one found a significant detrimental effect, whereas the other failed to find an effect (a small, insignificant trend for the critical stenosis to increase infarct size). Nonetheless, this is another potential difference between the studies with positive and negative results.

The variety of sources of SOD used seems unlikely to have caused the disparity because there are both positive and negative results from each major source. The contamination of SOD by endotoxin was found not to be a confounding problem by Nejima et al. Potential limitations of sample size in the studies with negative results cannot be adequately addressed because none of these studies provides the \( \beta \) statistic or estimates of the minimum treatment effect that can be excluded.

Residual Variance

One major concern is the apparent large residual variation in infarct size. Some of the unaccounted for variance could be due to imprecise measurement of collateral flow or infarct size. Typical variances (ratio of standard deviation to mean) for microsphere measurement of blood flow are approximately 0.2. The variance for other technical aspects is unclear, that is, the tracing of stained areas on ventricular slices. Whether variance is reduced by magnification of the images or whether more precise histologic techniques have real advantages are not known. In any event, within one experiment, these factors should have equal effects in treatment and control groups, as long as the investigator remains unaware. Table 1 shows type of infarct size measurement; none of the studies with positive results used histologic assessment as the primary measure of infarct size.

Suppose, hypothetically, that hemodynamic indexes and area at risk were not significant covariates. Consider the remaining unanalyzed independent covariate, collateral flow. Within treatment groups and within the lowest range of collateral flow (0.0–0.1 ml/min/g), infarct size (% area at risk) within individual studies shows a large range: 16–43%, 30–70%, 6 18–62%, 8 7–68%, 11 42–68%. The large variation within each of these studies suggests the existence of additional unmeasured independent variables (in Equation 1, \( \beta X_{i1} \ldots \beta X_{ik} \)). Indeed, if SOD reduces infarct size, it would either have to work by modifying an independent variable that is known to affect infarct size or an additional undefined variable, but which one? The superoxide produced by xanthine oxidase was believed to be the affected variable. The early burst of superoxide is only one component of the inflammatory reaction to reperfusion. One likely source of the unaccounted for variation resides in other inflammatory responses to acute myocardial infarction. In 1983, Romson et al reported that leukopenia could reduce infarct size. Despite this clear demonstration of an independent variable determining infarct size, only one of the SOD studies reported any detailed data on leukocytes. Interestingly, this study found a highly significant positive correlation between infarct size and relative leukocyte accumulation in the ischemic area in control animals. However, it is unclear which of these variables (leukocyte accumulation or infarct size) influences the other (i.e., which is the independent variable). Additional preliminary evidence suggesting late “reperfusion” injury mediated by superoxide was obtained by comparing a long half-life preparation of SOD injected every 12 hours throughout 4 days with a single injection of human cloned SOD (short half-life) and with placebo. The sustained level of SOD was associated with a dramatic reduction in the extent of necrosis compared with the single injection or placebo. However, the model that was used was coronary microembolization (25 microspheres), which may be pathophysiologically distinct from ischemia and reperfusion.

Unfortunately, in future studies, the use of total neutrophil count as a variable is unlikely to provide a satisfactory covariate for neutrophil-mediated inflammatory effects for three important reasons. First, it is the neutrophil count within an organ and the degree of activation and not the circulating count that is important in causing injury.
it appears that the state of activation or priming of the circulating neutrophil pool before the ischemia is a more important determinant of injury than the total number of cells. 48 Third, the relation between neutrophils, activation, and myocardial injury is nonlinear; it likely shows thresholds or bifurcations. (A bifurcation, characteristic of a nonlinear system is an abrupt change in the dependent variable.) New nonlinear techniques may be necessary for data analysis. Last, the contributions of other aspects of inflammation is unknown; potential factors include recent health history of an animal, vitamin E and other antioxidants added to food, complement activation, etc.

Conclusion

The answer to the central question of our paper, of whether SOD alters myocardial infarct size, is unknown. Potentially critical systematic differences between studies with positive and negative results could be due to the duration of reperfusion before infarct size determination, the method used to measure infarct size, the presence of a critical stenosis, statistical techniques, consideration of all appropriate covariates, and the possible existence of undefined covariates.

Implications

Reduction in ischemic injury after 2 or more days of myocardial reperfusion has not been convincingly shown with short infusions of SOD; the effect of prolonged infusions or of special preparations of SOD with long half-lives on ultimate infarct size may be beneficial. 32, 46 The design of the early SOD animal experiments was logically based on experiments in the isolated, perfused heart and the paradigm of a burst of superoxide from hypoxanthine metabolism by xanthine oxidase. In light of recent data on additional mechanisms of reperfusion injury, several reasons seem to exist for the disparate results. Maybe, antioxidant therapy should be directed against the several sources of superoxide: xanthine oxidase, neutrophils, mitochondrial leak, catecholamine oxidation, and arachidonate metabolism. The duration of injurious superoxide production is not precisely known; however, neutrophil activation in healing infarction likely lasts for several days. 31 Thus, future laboratory trials of antioxidants should consider these several different sources and their durations. Of concern for application to patients is the lack of significant xanthine oxidase in human myocardium 50 so that animal models in which xanthine oxidase is an important source of injury may not accurately predict results in humans. In the broader view of reperfusion injury, more fundamental data regarding the duration of other aspects of inflammatory injury would add considerable rationale to the experimental design. In patients after undergoing angioplasty or thrombolysis, sustained reperfusion is necessary to achieve clinically important benefits. Ultimately, animal trials of adjuncts to reperfusion must mimic the desired clinical situation of sustained reperfusion throughout the healing phase of myocardial infarction, especially because anti-inflammatory interventions may have undesirable effects on the healing phase. Identification of new methods to quantify variables in the inflammatory system before and during ischemia will be necessary. Recent evidence indicates that reperfusion-induced inflammatory injury involves several sources of superoxide and multiple mechanisms in addition to oxidant injury, and these factors should be considered in future experimental designs.

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Can superoxide dismutase alter myocardial infarct size?
R Engler and E Gilpin

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