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SUPEROXIDE DISMUTASE THERAPY FOR MYOCARDIAL ISCHEMIA

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Oxygen derived free radicals have been shown to be generated during reperfusion of ischemic myocardium by a variety of approaches including spin trap probes. Three levels of injury have been described for the reperfused heart. Periods of ischemia of only several minutes can trigger lethal arrhythmias on reperfusion. Anti-oxidants including SOD and or catalase, as well as iron chelators reduce the incidence of these arrhythmias in both dog and rat. Xanthine oxidase inhibitors are equipotent with SOD in this model suggesting that xanthine oxidase is the source of the radicals. Periods of occlusion lasting 10-15 minutes produce a recoverable defect in contractility termed "stunning". SOD plus catalase has been shown to reduce the incidence of stunning in a variety of models including the xanthine oxidase deficient rabbit. Neither agent on its own seemed to be effective against stunning in either the rabbit or the dog. Stunning is more difficult to demonstrate in the rabbit heart, presumably due to its lack of xanthine oxidase. Periods of ischemia in excess of 20 minutes will result in some irreversible cell death (infarction) with reperfusion. While studies using histochemical methods suggested that SOD plus catalase given at the time of reperfusion could limit necrosis in the dog model, histological studies reveal that infarct size was not modified but rather, SOD appears to interfere with the ability of tetrazolium to histochemically discriminate between living and dead cells. While PEG SOD with its extended plasma half life was reported to reduce infarct size in the dog, it was unable to protect the reprefused rabbit heart. To date, none of the scavengers have been proven to limit infarction suggesting that free radicals contribute to arrythmias and stunning, but do not kill cells in the reperfused heart.

KEY WORDS: SOD, myocardial infarct size, reperfusion injury, PEG SOD.

The superoxide anion and its derivatives have been implicated as an underlying etiology of many clinical disorders. Prominent among these has been ischemic heart disease.[21,45] The bulk of this evidence has been based on the ability of superoxide dismutase (SOD) to reduce injury in animal models of myocardial ischemia. Unfortunately, quantifying mycocardial injury is still an imprecise science and, as a result, the performance of SOD and the other various antioxidant interventions has varied widely in the different models. Because of the discrepant data it has not been possible to determine the exact role that superoxide or other free radicals play in the ischemic heart or what long-term clinical benefit might be derived from an anti-free radical intervention in the ischemic patient. Although it is well established that some free radicals are produced on reperfusion of the ischemic heart, it is the magnitude of the injury that superoxide and its reaction products produce which remains in question. Although the *in vitro* work has established a theoretical framework for the free radical hpothesis, in the final analysis, this question will only be answered by measuring the protection derived in a whole animal by SOD.

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Three levels of ischemic cardiac injury are currently recognized and all three have proposed to involve, at least in part, a free radical mechanism. The first detectable level of injury is the generation of reperfusion arrhythmias. Reperfusion after an ischemic period of only several minutes can result in, ventricular tachycardia or fibrillation.[3] Secondly, increasing the length of the ischemic period to between 5 and 15 minutes will result in a prolonged deficit in contractility following reperfusion. This state has been called the "stunned" myocardium. In stunned myocardium all of the ischemic myocytes are still viable and the heart will completely recover its function but such recovery may require several days.[4,17,55] Finally, when the ischemic period is extended to 20 minutes or longer, some of the heart cells will be irreversibly injured and become infarcted.[58] The greater the depth and duration of the ischemic insult, the more widespread the cell death. It is the latter form of injury, infarction, which has attracted the greatest attention from the clinical community. Because infarcted tissue is not contractile, pump function of the heart is greatly diminished in these patients contributing to mortality and morbidity. Infarcted tissue is not regenerated and any deficit in contractile function due to infarction will be permanent. If one intervenes early after the onset of ischemia, survival of tissue which would otherwise have died can be accomplished. This will have the effect of limiting the amount of infarction present in the heart and will preserve pump function. The most obvious such intervention is the restoration of blood flow to the ischemic zone and several strategies have been devised for effecting early reperfusion of the ischemic heart. Those strategies included emergency angioplasty and the thrombolytic agents. Clinical trials confirmed that both mortality and myocardial pump function is improved by early reperfusion,[23,67] and few would doubt that the benefit was derived from infarct size reduction[7,11] although technical limitations still prevent direct measurement of infarct size in man.

IS REPERFUSION INJURY A REAL PHENOMENON?

Reperfusion itself, however, may bring with it a component of injury. In the seventies Hearse and colleagues demonstrated that the reintroduction of oxygen to a hypoxic heart was accompanied by a rapid and profound disruption of the tissue as assessed by both release of cytosolic enzymes[30] and ultrastructure.[31] Apparently some component of reoxygenation was responsible for the injury. Among the several possible explanations which they considered were the oxygen derived free radicals.[29] Several investigators subsequently found that a variety of free radical scavengers including SOD could reduce the injury in the hypoxic heart model which argued in favor of a free radical mechanism.[27,28,46,50] It should be noted that not all investigators found the antioxidants to be protective in the hypoxic heart mode.[69]

Reperfusion of ischemic tissue also triggers a similar abrupt appearance of tissue disruption.[6,34,36] It is currently a matter of intense debate as to whether the ischemic tissue is actually injured by reperfusion per se or whether reperfusion is simply causing cells previously killed by the ischemia to suddenly undergo abrupt morphological changes, perhaps through an osmotic effect.[35,69] Proof of reperfusion injury has been elusive because the only reliable test of viability currently available for an ischemic myocyte is to observe whether it recovers on reperfusion, a test that itself would invoke any reperfusion injury. It has, therefore, been impossible to tell whether reperfused tissue died before or after reperfusion. Unless that problem can be solved,

proof of free radical-mediated reperfusion injury will require the unequivocal demonstration that an anti-free radical intervention administered only at the reperfusion period can promote cell survival. Finally, injury may result from reperfusion but could involve a mechanism unrelated to free radicals such as sodium mediated calcium entry.[25]

DOES THE REPERFUSED HEART MAKE FREE RADICALS AND WHAT IS THEIR SOURCE?

Recent studies with spin-trap probes convincingly demonstrate that at least some free radicals are generated in the reperfused heart.[2,5,22,72,73] It is not known whether the free radicals which are produced contribute to infarction since it is not known how much of a free radical load the heart can tolerate. It is possible that the production is simply insignificant. Mitchell and colleagues[47] examined glutathoine in hearts reperfused after varying ischemic times and found that the reduction form GSH, never approached depletion. Their conclusion was that the oxidant stress at reperfusion never exceeded the limits of the endogenous scavengers.

The source of the free radicals in reperfused myocardium is currently a subject of debate. Several sources have been proposed. One such source is xanthine oxidase.[8,45] Hypoxanthine released from the ischemic heart is oxidized by xanthine oxidase upon reperfusion making both superoxide and hydrogen peroxide. The xanthine oxidase system is a highly mobilizable radical source, in that both purine and enzyme will be in place and active after occlusions as short as 5 minutes. Free radicals from this source seem to account almost fully for early reperfusion arrhythmias seen in the rat[43] as well as stunning in the dog.[10] Unfortunately, xanthine oxidase seems to be absent from the human heart.[26]

Other sources of radicals could include oxidation of catecholamines, either through autoxidation,[37] or enzymatic oxidation. Prostaglandin pathways also can generate radicals[41] and certainly are active during ischemia/reperfusion. Mitochondria have long been suspected of being a source of free radicals within the reperfused myocyte. Ischemia may induce a defect in the mitochondrion causing it to produce excess radical[63] or pehaps the loss of endogenous antioxidants during ischemia may leave the myocyte unable to cope with the normal mitochondrial "leak" of radical.[18,63] The mitochondrial hypothesis has been the most difficult to test simply because blockade of mitochondrial radical producing pathways also blocks oxidative phosphorylation killing the heart. This obviates the use of improved viability as an end point for any such studies.

Currently many investigators are proposing leukocytes as the source of free radical in the reperfused heart. Leukocytes, when activated, can generate oxyradicals as part of their bacteriocidal mechanism via their NADPH oxidase pathway. It has been proposed that early after the onset of ischemia, leukocytes enter the ischemic tissue and attack viable myocytes[64] primarily through the release of radicals. There is no dispute that leukocytes concentrate in infarcted myocardium. The only question is, are leukocytes attacking viable cells at the time of reperfusion? Leukocytes are known to serve a role in scar healing by clearing the debris from tissue and their presence may be totally appropriate. While many doubt that sufficient leukocytes could be in place early enough to account for injury incurred at the time of reperfusion, they could contribute to a free radical-mediated injury in the hours following reperfusion. The

major problem with the leukocyte theory is that much of the data describing a free radical-mediated injury has been collected in the crystaloid perfused isolated heart which is leukocyte free.

MEASUREMENT OF INFARCT SIZE IN AN ANIMAL MODEL

The traditional approach to testing an agent's ability to limit necrosis has been to expose a heart to a standard ischemic insult and measure the resulting infarct size in both the presence and the absence of the agent. The problem lies both in achieving a standard insult and in measuring the infarct size. Consider the latter problem first. Infarction is a dynamic process with cells making the transition from living to dead in stages. Distinct changes in the myocyte's ultrastructure, with sarcolemmal defects and mitochondrial swelling with dense bodies can be seen within an hour of coronary occlusion.[33,60] Under the light microscope, however, that tissue may appear perfectly normal. After 24 hours of occlusion coagulation necrosis can be seen with the light microscope. In addition, there is often a heavy infiltration with leukocytes and petechial hemorrhages may be present. The exact borders of where dead tissue meets living may still be indistinct, however.

Because histological evaluation of infarct size is tedious and requires that at least a day, and preferably 3, be included between the onset of ischemia and the evaluation, most investigators have sought an alternative which could be employed in a non-recovery type of animal model. Tetrazolium (TTC) staining soon emerged as the method to replace ST segment analysis. Dehydrogenase enzymes and the cofactor NADH react with TTC salts in living tissue to produce a formazan pigment which is intensely colored.[51] Since enzyme and especially co-factor [40] are lost from the heart cells early after infarction, it was believed that the stain specifically differentiated living from dead tissue. In models of permanent occlusion a coronary artery was typically occluded for 6 hours, the heart removed, sectioned and the slices incubated in TTC. While some reports found that this protocol accurately differentiated living from dead tissue,[19,54] others reported that some necrotic tissue retained the ability to react with the stain at the 6 hour mark.[16] More importantly, it was found that early evaluation of at least one drug in this model gave misleading results in that infarct size limitation found 6 hours after occlusion was no longer apparent when the evaluation was performed a full 24 hours after occlusion.[9]

When the focus of research shifted from permanent occlusion models to reperfusion, it was noted that reperfusion caused a much more rapid expression of cell death including the loss of TTC stainability.[42] It has generally been found that TTC staining several hours after reperfusion yields essentially the same infarct size as seen by histology several days after reperfusion.[32] Based on those observations, TTC staining has become the method of choice for infarct size studies in many laboratories including ours. This may have been unfortunate, however, in that a recent observation from this laboratory suggests that even in the setting of reperfusion, TTC may be prone to artifacts. Figure 1 shows that infarct size by histology was much larger than that by TTC following SOD treatment in rabbits.[13] Thus, although early evaluation with TTC reveals the ultimate infarct size in an untreated heart, drugs may interfere with that relationship. Because any intervention which actually does limit infarct size should give a positive TTC result, we still believe that TTC still should be employed as a screen for cardioprotective agents but that follow up tests must be performed to

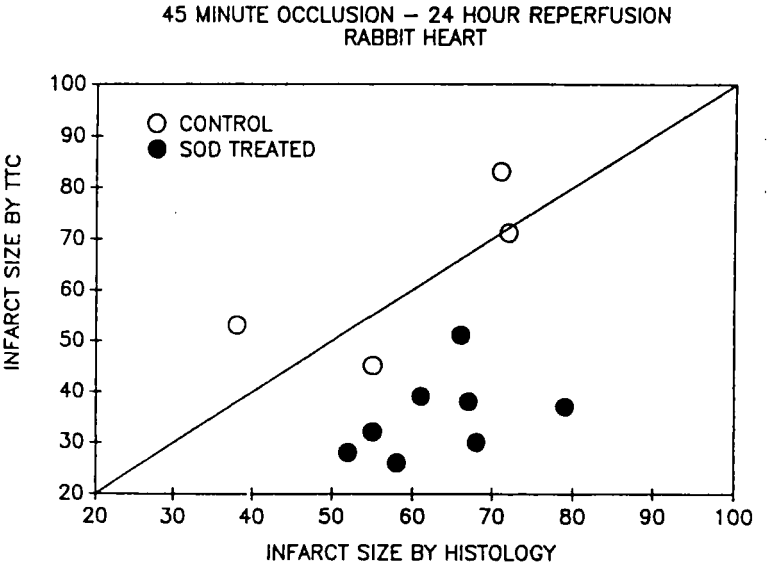

FIGURE 1 Rabbit hearts treated with SOD were analyzed by both histology and tetrazolium (TTC). Similar values were obtained by both methods in the untreated animals (open circles). In the SOD treated animals, however, infarcts were much smaller by TTC than by histology. It was concluded that SOD caused much dead tissue to retain the ability to react with tetrazolium. How many other agents possess the ability to cause a false positive result with TTC is not known at present. Source.[13]

eliminate false positives. Agents that fail to limit infarct size in an early TTC evaluation can probably be considered inactive at that point.

COLLATERAL FLOW CAN BE A MAJOR DETERMINANT OF INFARCT SIZE

A second problem which has plagued investigators has been that of administering a standard ischemic insult. Dog hearts are quite variable in terms of their infarct size following a coronary occlusion.[49] That variability both reduces the sensitivity of the model and increased the possibility of spurious conclusion. Reimer and Jennings,[57] studying the dog model found that infarcts begin at the subendocardium and progress toward the subepicardium as the duration of ischemia increases. While the lateral border of the infarct was superimposed on the lateral boundary of the perfusion field, the transmural involvement was determined by two factors: the duration of ischemia and the level of collateral flow. Figure 2 reveals that naturally occurring collateral flow significantly contributes to ischemic cell viability in both permanently occluded and reperfused dog hearts. Another feature of the dog heart is the wide variation of collateral flow from dog to dog. Figure 2 reveals that collateral flow may range from less than 5% to as high as 80% of the preocclusion value. The 20 to 1 range makes it virtually impossible to achieve a normal distribution of collateral flows with 10 or less animals in a group. Thus, the investigator can not assume that differences in collateral flow between groups will average themselves out nor are the standard parametric tests for significance appropriate when a normal distribution has not been

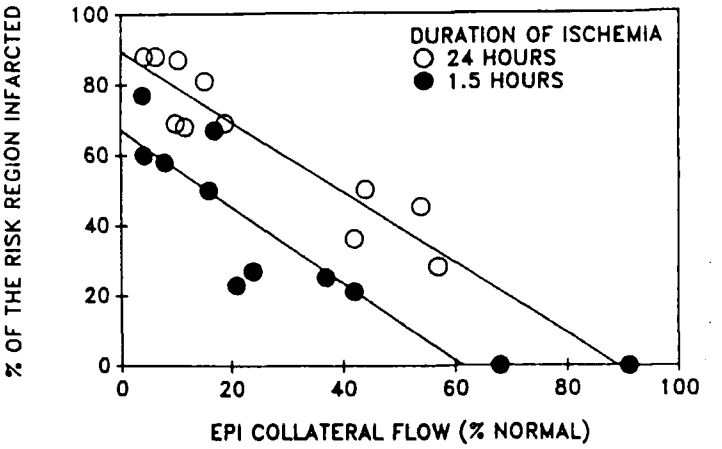

FIGURE 2 The extent of infarction of the ischemic zone (risk region) is plotted against collateral flow for both permanently occluded (open circles) and reperfused hearts (solid circles). Note that infarct size is inversely related to collateral flow and that early reperfusion tends to shift that relationship down in a parallel fashion. Note the wide range of collateral flows seen in the dog population and the variability in infarct size it introduces. A cardioprotective drug should also cause a downward shift of the relationship. Source: unpublished data from the author's laboratory.

achieved. Most studies performed in the early eighties, including many from the author's laboratory, did not measure collateral flow and only expressed infarct size as a percentage of the field of the occluded artery. It is likely that many of those early studies were corrupted by unaccounted differences in collateral flow between the groups, especially where there was considerable overlap of data between the two groups. Collateral flow artifacts can be avoided by using an analysis of variance on the infarct sizes with collateral flow (measured by microspheres) as a covariate.

Unfortunately collateral flow does not account for all of the variability between animals either. Figure 2 shows that in the rabbit, a species which has only a sparse native collateral circulation in its heart,[44] collateral flow is too low to influence viability but there is still unexplained variability in infarct size. We find that a 45 minute occlusion will cause an average of 60% of the risk region to infarct but with a standard deviation of $\pm$ 12%. This variability, which cannot be accounted for by any hemodynamic parameter such as heart rate, blood pressure or double product in our data, puts severe limits on the resolution of the method.

IS SOD A CARDIOPROTECTANT?

The most common approach to removing free radicals in the animal models has been to employ a scavenger against one of the radical species. A scavenger removes free radicals directly and does not discriminate as to the source of the radical. Superoxide dismutase scavenges superoxide by dismutating it to oxygen and hydrogen peroxide. Catalase, reduced glutathione and glutathione peroxidase similarly scavenge hydrogen peroxide. The hydroxyl radical is the most difficult to scavenge. Because of its high reactivity, its probability of encountering a molecule other than the scavenger is high and the efficiency of a scavenger like DMSO or dimethylthiourea[20] to remove it

TABLE I

Study	Catalase present	Species	Col fl as covariate	Ischemia duration	Reperfuse duration	Infarct method
			Positive Studies			
Ambosio *et al.*		Dog	No	90	48h	Gross
Werns *et al.*		Dog	Yes	90	6,24h	TTC
Naslund *et al.*		Pig	N/A	60	5h	TTC
Chambers *et al.*	*	Dog	No	60	4h	TTC
Jolly *et al.*	*	Dog	No	90	20h	TTC
Wernes *et al.*		Dog	No	90	6h	TTC
Downey *et a.*	*	Rabbit	N/A	45	4h	TTC
			Negative Studies			
Urazee *et al.*		Dog	Yes	40	96h	HIST
Gallagher *et al.*	*	Dog	No	180	24h	TTC
Nejima *et al.*		Dog	Yes	90	7h	HIST
Patel *et al.*		Dog	Yes	120	4,48h	TTC
Shirato *et al.*		Rabbit	N/A	45	3,24,72h	TTC
Miura *et al.*	*	Rabbit	N/A	45	72h	HIST
Richard *et al.*	*	Dog	Yes	90	48h	HIST

Adapted from: Engler, *Circulation*, **79**, 1137, 1989

Abbreviations: COL FL = collateral flow, HIST = conventional histology, TTC = tetrazolium staining, N/A = Notapplicable to this species, *indiicates that catalase was present with SOD. Ischemic times are in minutes.

is thought to be small at best. Therefore the strategy has been to try to eliminate the hydroxyl radical's precursors, hydrogen peroxide and superoxide or to chelate the iron which is a necessary catalyst for hydroxyl radical formation. To be effective the scavenger must get to the appropriate site of either production or attack. That site might be inaccessible to a macromolecule such as inside the myocyte or in a cleft between a leukocyte and the sarcolemma. The non-enzymatic scavengers are more adept at reaching intracellular sites due to their low molecular weights but since scavenging consumes them they may be prematurely exhausted.

SOD and catalase have been the most widely studied of all of the antioxidant species. Table I is adapted from the recent review by Engler[15] and summarizes 13 infarct size trials using SOD with and without catalase. Jolly *et al.*[38] were the first to employ SOD in an infarct size trial. They used open chest dogs, occluding a coronary branch for 90 minutes and reperfusing for 24 hours. SOD and catalase together were infused over a two hour period. Infarct size, which was expressed as a fraction of the region at risk, was smaller in groups which received the drug at the time of reperfusion over those that either did not receive drug or received it only 40 minutes after reperfusion. Shortly thereafter Chambers *et al.*[8] reported a positive effect in open chest, nephrectomized dogs receiving SOD only. The observation that SOD without catalase could reduce infarct size in the dog model was confirmed by Werns *et al.*[70] and Ambrosio *et al.*[1] Finally, SOD plus catalase was reported to limit infarct size in a rabbit[12] and a porcine model[52] of ischemia/reperfusion. Except for the one study by Werns *et al.*[71] TTC staining in the first 24 hours following reperfusion was the end point for all of these studies.

The lower half of Table I reveals at many studies with these agents have been negative. Uraizee *et al.*[68] found that the SOD + catalase mixture did not affect infarct

size in their model where the coronary artery was reperfused for 4 days with infarcts sized by histology and analyzed according to their collateral flow. While the authors ascribed their discrepant data to the refined methodology, particularly the attention to collateral flow, critics argued that the 40 minutes of occlusion which Uraizee *et al.* employed may have been too short to evoke a free radical burst. In response they repeated their study using a 90 minute occlusion period[59] but again no effect on infarct size was seen. In a similar study Nejima *et al.*[53] also failed to demonstrate protection with SOD alone. Interestingly they also used histology as the end point and included collateral flow in their analysis.

CAN WE EXPLAIN WHY THE RESULTS HAVE BEEN SO DISCREPANT?

It is not fully understood why the early positive studies stand in such contrast to the latter negative studies but several explanations appear likely. One of the most obvious explanations is that an erroneous result might have resulted from the failure to take collateral flow into account in the first dog studies. That potential artifact is illustrated in the study of human recombinant SOD by Ambrosio *et al.*[1] Although a significantly smaller percent of the ischemic zone infarcted in the SOD group, the plot of percent infarction against collateral flow revealed that the SOD treated animals tended to have higher collateral flows. Analysis of variance with collateral flow as a covariate revealed no differences between the groups. It seems quite possible that there was not any real drug effect in the Ambrosio *et al.* study. Because of the expense of such trials, virtually all of the canine infarct size studies have had very few animals in each group which greatly increases the chance of false interpretation. As a result 5 canine studies

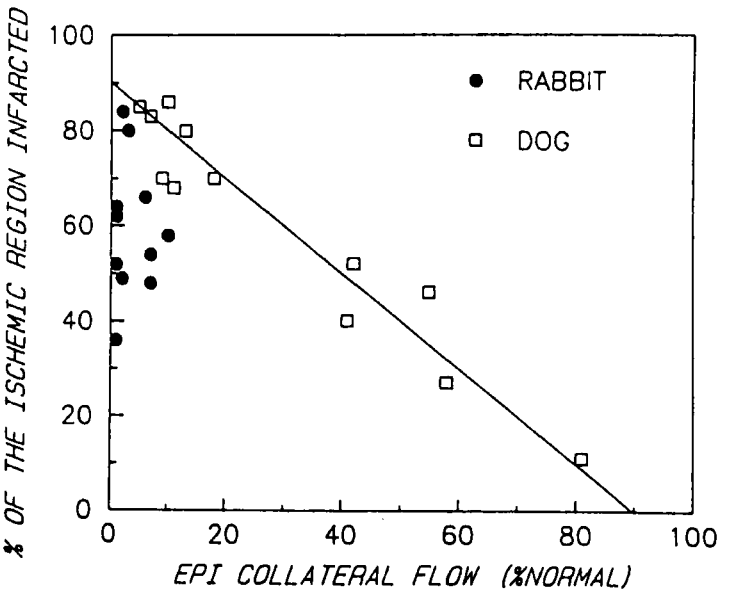

FIGURE 3 Unlike the dog, rabbits (and pigs) have very few collateral channels in their coronary circulation. As a result collateral flow is too low to influence infarct size. Source: unpublished data from the authors laboratory.

from Table 1, including one form our laboratory, must be deemed unreliable due to the omission of a collateral flow analysis.

While the collateral flow argument might be sufficient to dismiss the early dog studies as unreliable, that argument can hardly be applied to rabbit[12] or pig[52] trials as neither of these species have significant coronary collateral circulations.[42,44] Further more the dog study of Werns *et al.*[71] still shows significance when collateral flow is analyzed. A possible source of the discrepancy could be the method used to estimate the infarct size. All of the positive studies involved TTC staining and, with one exception, 24 hours or less of reperfusion. Indeed when we represented our original SOD plus catalase study,[12] but this time analyzing infarct size 3 days after reperfusion with histology, protection was no longer seen.[48] We recently examined SOD alone in a rabbit model with three different reperfusion times. After 3 and 24 hours of reperfusion, TTC indicated very small infarcts but at 72 hours no differences were found by TTC.[62] Apparently SOD greatly retards the rate of loss of enzyme and cofactor from dead tissue to give the appearance of viability for a full day after reperfusion. SOD might be delaying the washout of enzymes by preserving capillary permeability as it does in other organs.[24] Although one report where cardiac permeability was measured following an ischemic insult failed to find any protection from SOD plus catalase,[65] Przyklenk and Kloner[56] did see electron micographic evidence of SOD-induced preservation of capillary endothelium in dogs. The mechanism for the induced artifact with the tetrazolium method remains unclear.

THE DOSE AND SCHEDULE FOR SOD HAS VARIED WIDELY BETWEEN THE STUDIES

A final reason for discrepancy between the studies may be the dose and schedule with which SOD was given. Virtually no dose response studies have been performed on SOD and as a result the studies in Table 1 represent a wide range of doses and schedules. How critical is the dose and schedule in those studies? To answer that question we have recently explored the dose response relationship for SOD in the *in situ* rabbit heart. A coronary branch was occluded for 45 minutes and SOD was given as a bolus in all cases 15 minutes prior to reperfusion. To lower the possibility of a SOD-induced false positive, we have found that freezing and thawing the tissue prior to TTC staining improves the discrimination between living and dead tissue even in the presence of SOD. We, therefore, performed the dose-response studies using the frozen protocol as that method tends to reduce (but not necessarily eliminate) the incidence of false positives.

Rabbits were anesthetized and subjected to 45 min coronary arterial branch ligation for 45 min followed by 3 h of reperfusion. The volume of the risk zone was assessed with fluorescent microspheres and infarct size was assessed by tetrazolium staining after freezing and thawing the tissue. Human recombinant SOD was given as a bolus dose 10 minutes before reperfusion. Figure 4 shows that in control animals (no SOD) 63 $\pm$ 2.8% (mean $\pm$ standard error) of the risk zone infarcted. SOD at 2 mg/kg resulted in 58 $\pm$ 6% of the risk zone infarcting, which was not different from the control. SOD at 5 mg/kg resulted in infarct size of 45 $\pm$ 5% which was a small but significant reduction in indicated infarct size ($p < .05$% by ANOVA against all groups). 15 mg/kg yielded an infarct size of 52 $\pm$ 7% respectively which was not different from control. In rabbits treated with 50 mg/kg SOD infarct size was signifi-

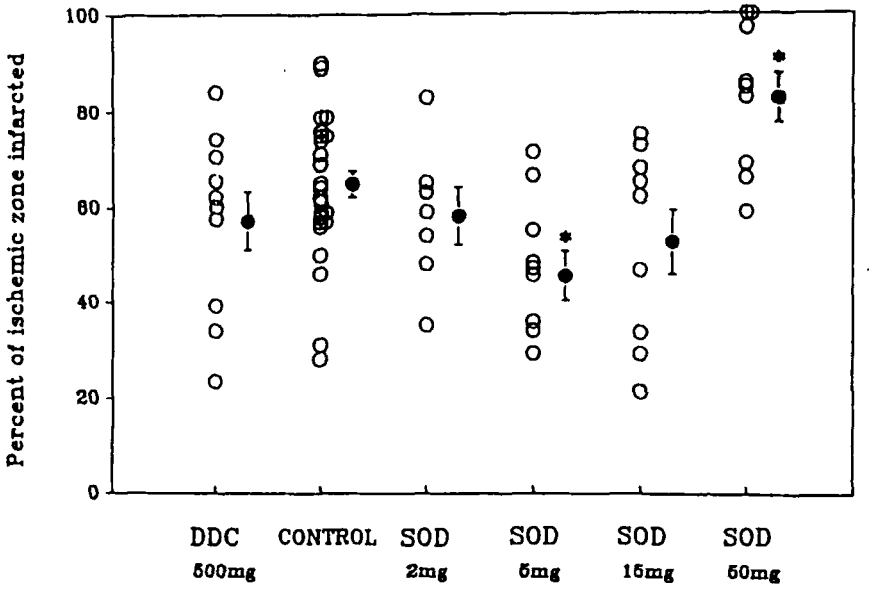

FIGURE 4 Infarct size as a function of SOD dose in the open chest rabbit. Note: an optimal effect is seen at 5 mg/kg.

cantly increased over the control value to 82.8 ± 5%. Thus a peak effect was seen at 5 mg/kg and a toxic effect was seen at 50 mg/kg.

Rabbits are unusual in that they have a high level of EC SOD in their plasma.[39] While humans and dogs have less than 1 U/ml of SOD in their plasma, our rabbits had 12 ± 3 U/ml. To see if the high level of extracellular (EC) SOD present in the rabbits's blood may have masked any protection, we inhibited EC SOD with DDC (diethyldithiocarbamate) as a 500 mg bolus IV before ischemia. DDC is a copper chelator and will inactivate all Cu, Zn SOD in the rabbit. There was no obvious hemodynamic effect of the DDC. Plasma SOD activity was lowered to 0.2 ± 0.02 U/ml at the time of occlusion and had recovered to only 1.6 ± 1 U/ml at the end of the study. The infarct size of 57.1 ± 6% was not different from control. The high levels of endogenous EC SOD seems to offer little protection to the reperfused rabbit heart either.

Next we examined the dose-response relationship for SOD on reoxygenation injury in the rabbit heart. Hearts were mounted on a Langendorff apparatus and were perfused with Krebs-Henseleit buffer. After 60 min hypoxic perfusion, hearts were reoxygenated. The coronary effluent was assayed for creatine kinase lactate dehydrogenase (LDH). Except for controls, SOD was present in the buffer throughout the experiment. The data appear in Figure 5. Control rabbit hearts released 284 ± 75 U/kg bw of LDH. Rabbit hearts receiving 5 mg/l inactive SOD (the triangles) had a release of 303 ± 64 U/kg bw. Hearts receiving 0.5, 1 and 5 mg/l had LDH release of 192 ± 83, 148 ± 78 and 132 ± 49 U/kg bw respectively which were all significantly less than that for the control. Notice that 50 mg/l failed to protect the rabbit heart. We don't think that the failure to protect was due to a non-specific effect of the enzyme either because when the SOD was inactivated to an activity equivalent to the

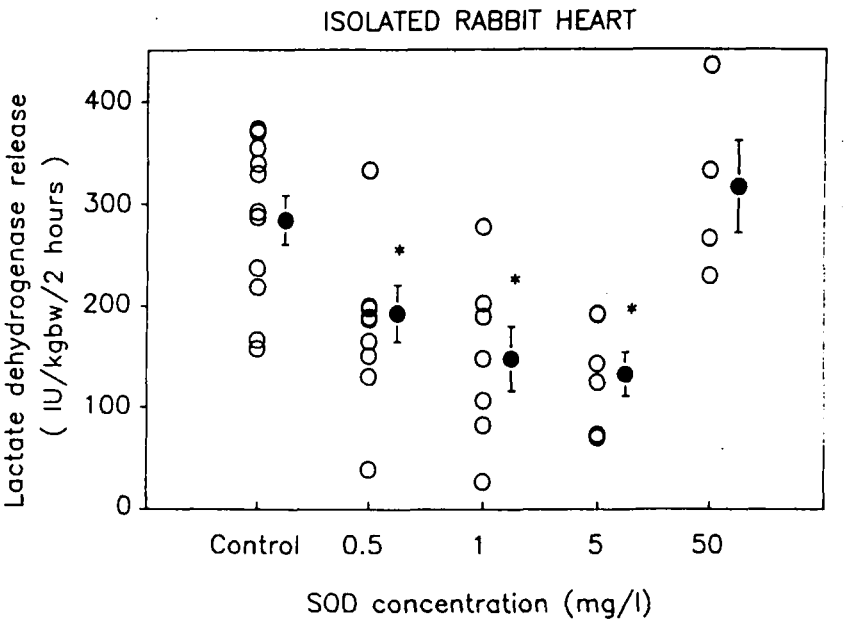

FIGURE 5 Lactate dehydrogenase release as a function of dose in the isolated hypoxic rabbit heart. Note: an optimal range is seen.

5 mg/l dose protection again was present (filled circles). Thus, an optimal dose again was seen.

SOD was effective over a wide range of concentrations in the oxygen paradox model in both species but that effect may be lost at excessive doses. No exacerbation of injury was seen in any dose examined but it should be appreciated that 50 mg/l in the perfusate is considerably less than 50 mg/kg *in vivo*. In the latter case the 50 mg of SOD is confined to the plasma compartment, about 50 ml/kg, giving a concentration of 1000 mg/l. Such high doses could not be tested in the isolated heart due to economic considerations.

Infarct size was increased in the animals receiving 50 mg/kg. The reason we believe that this is a real finding is because TTC positive tissue may or may not be viable but TTC negative tissue is unambiguously thought to be dead as it is completely depleted of either dehydrogenase enzymes and/or NADH. Several investigators have reported a negative effect of high dose SOD. Bernier *et al.* found that SOD lost its effect at high doses in the rat arrhythmia model.[15] Similarly, Myers *et al.* found 30 mg/l SOD to be ineffective in an isolated rabbit heart model which was similar to that reported here.[50] Elroy-Stein *et al.*[14] found that over production of SOD in transfected cells actually resulted in enhanced lipid peroxidation. Scott *et al.* have also shown that bacteria with elevated SOD levels are much more susceptible to a free radical generating system than those with normal SOD levels.[61] Because catalase restored normal resistance, Scott *et al.* ascribed the detrimental effect of high SOD to excess hydrogen peroxide production. We propose an alternative mechanism. A critical balance of the hydroperoxyl radical (HO_2. - the protonated form of superoxide) may be important for the termination of lipid peroxidation. The reaction would be as follows:

$$OH\cdot + LH \rightarrow H_2O + L\cdot \qquad \text{initiation}$$

$$L\cdot + O_2 \rightarrow LOO\cdot \qquad \text{propagation}$$

$$LOO\cdot + LH \rightarrow LOOH + L\cdot$$

$$LOO\cdot + HO_2\cdot \rightarrow LOOH + O_2 \qquad \text{termination}$$

The over-scavenging of superoxide could remove this important "chain breaker" from the system. Whatever the mechanism, it is interesting to note that many investigators have paid little attention to the dose and that excessive doses are just as ineffective as too little.

Along with dose the schedule is also important. Figure 6 shows a simulation of the plasma and the cardiac interstitial fluid concentration of SOD in the bolus injection studies. SOD only has a 10 minute half life in the rabbit plasma but because it enters the cardiac interstitium with about a 20 minute half time, the 5 mg/kg dose on the left quickly raises the interstitial space into the therapeutic range which was identified in the isolated heart studied (the shaded area). The kinetics keep it there for an hour or more. Contrast that to the 50 mg/kg bolus on the left. In this case interstitial fluid SOD levels skyrocket into the toxic range soon after reperfusion and it is not surprising that infarct size was extended in those studies. The simulation studies would indicate that it is the interstitial concentration of SOD that is critical. That means that in a constant infusion protocol, a 30 or 40 minute pretreatment should be allowed before the heart is reperfused. While some of the studies have made allowances for that, notably that of Werns *et al.*,[71] most others did not.

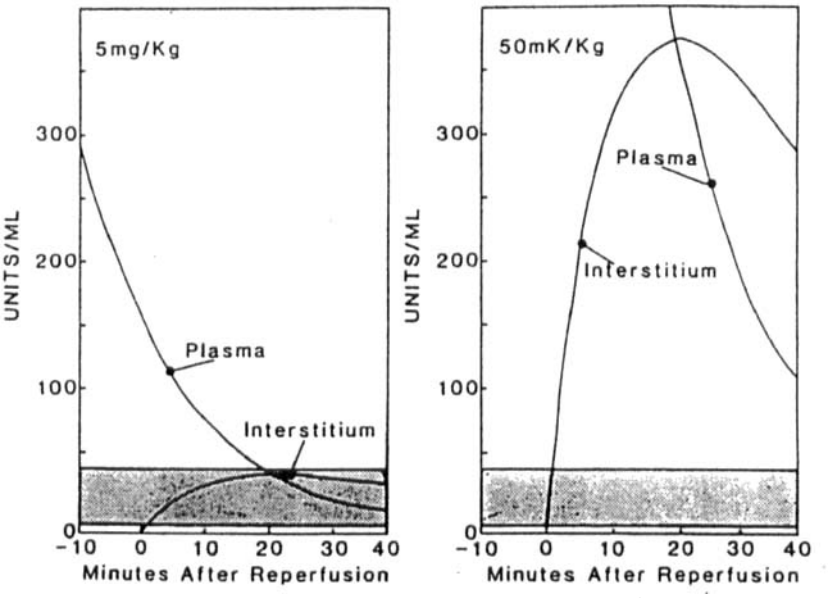

FIGURE 6 A simulation of plasma and cardiac interstitial levels of SOD following a bolus injection. The shaded area is the effective dose range derived from Figure 5. Enzyme release as a function of SOD dose in rabbit hearts exposed to 60 minutes of hypoxia. Note that the SOD concentration was maintained constant in the perfusate throughout the study.

MUST SOD'S RETENTION TIME BE PROLONGED TO CONFER PROTECTION?

One explanation as to why SOD has failed in so many of the infarct models was that its half life was too short. In all of the studies in Table I the enzyme was withdrawn soon after reperfusion. Tamura *et al.*[66] propose that free radicals may be generated long after the initial reperfusion phase and that SOD must be present for hours or even days after the reperfusion in order to confer protection. Their PEG SOD trials[66] supported that claim. SOD was conjugated to polyethelyne glycol to prevent it from being filtered at the kidney which extended its plasma half life to several days. Using a dog model they showed a marked reduction of infarct size. Althoug TTC was their endpoint, they did account for collateral flow and did reperfuse for 4 days. It should also be pointed out that in the study by Chambers *et al.*[8] the kidneys were ligated and thus SOD levels were maintained throughout the study which may account for the extremely small infarcts seen in that study as well. Based on this evidence we recently tried to confirm the results of Tamura *et al.*[66] in the rabbit model. To avoid any possibility of an artifact with tetrazolium we chose to use histology as the endpoint for that study.

The PEG-SOD that we used had 16 PEG-3000 units per SOD molecule giving it a molecular weight of 78 kD. Gel chromatography showed that the molecule actually behaved as if it had a molecular weight of 540 kD. We believe that this is due to long side chains which give the molecule a very large radius of gyration. Cytochrome-*c* assay revealed an activity of 3000 U/mg. The compound was found to have a *t*1/2 in rabbits of about 50 hours.

We investigated the ability of this PEG SOD to limit infarct size in a rabbit model

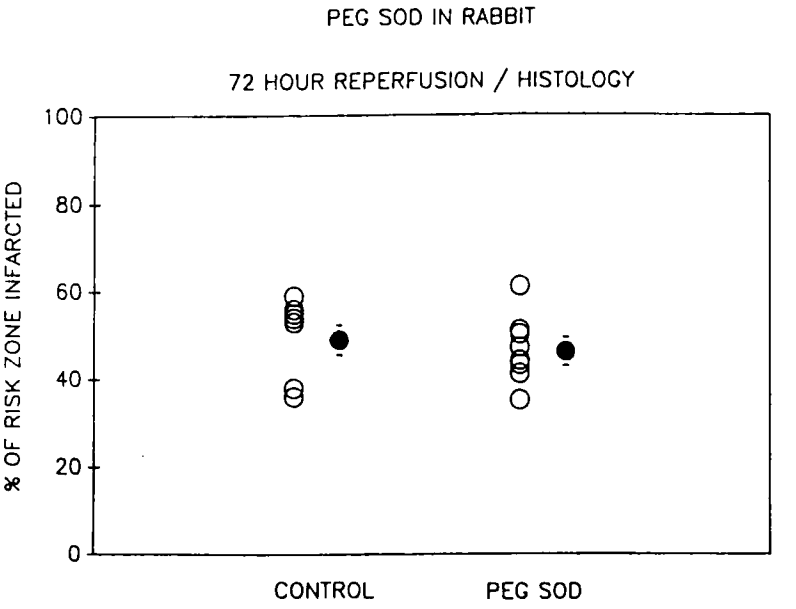

FIGURE 7 Infarct size in rabbits receiving 1000 U/kg of PEG-SOD and reperfused for 72 hours. Infarcts were sized by histology.

of ischemia-reperfusion. To avoid any ambiguity over TTC determinations as discussed above, we used histology to size the infarcts on the 3rd day of reperfusion. Rabbits were anesthetized with pentobarbital and the hearts exposed under sterile conditions. An anterior branch of the left coronary was occluded for 30 minutes and reperfused. A single dose of 1000 U/kg of PEG SOD was given IV 15 minutes prior to coronary occlusion (50 h half life in rabbit). After reperfusion was begun the chest wound was closed and the animal was returned to the cage. Three days later the heart was removed, the risk zone marked with fluorescent microspheres, and infarct size was measured by histology (H&E and Azan's stain). Figure 7 reveals that 48.9 ± 3.1% and 46.5 ± 2.7% of the risk zone was infarcted in the control (n = 8) and the treatment (n = 8) group respectively. There was no significant difference between the treatment and the control groups in this study.

WHY DIDN'T PEG-SOD LIMIT INFARCT SIZE IN THE RABBIT TRIAL?

PEG SOD is reported by Tamura *et al.*[66] to limit infarct size in dogs at this dose and schedule when tetrazolium is used to assess infarct size. We do not know whether our discrepant finding is due to species differences, differences in the PEG-SOD (they were not from the same manufacturer), or due to the method used to visualize the infarct. While the contact time was increased with PEG-SOD, increasing the size of the molecule decreases its access to certain critical compartments and decreases its efficacy. Using the isolated Krebs perfused heart, preliminary studies in our laboratories indicate a direct relationship between apparent protection by SOD and its access to the interstitial compartment. Sheep Cu, Zn SOD, which carries the opposite net charge from human Cu, Zn SOD at pH 7.4, equilibrates in cardiac lymph within 15

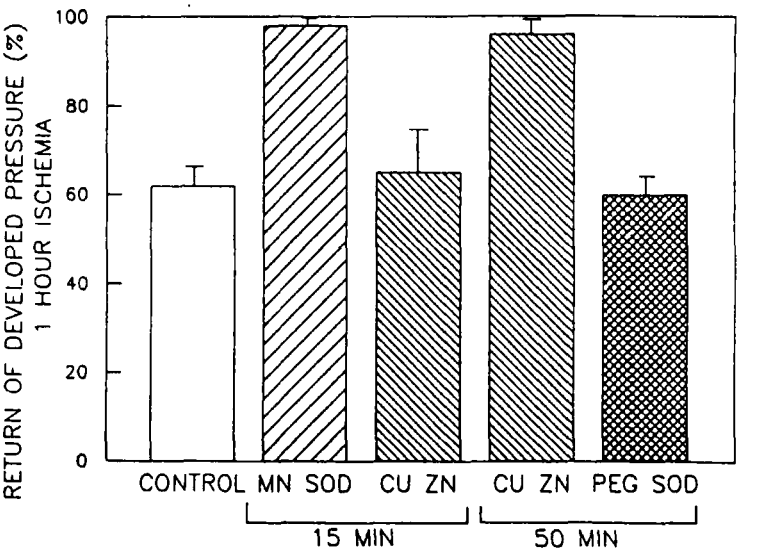

FIGURE 8 The protection afforded by several SODs in the isolated rabbit heart depends on their size and the duration of pretreatment. The presence or absence of effect correlated with the appearance of the SOD in the cardiac lymph.

minutes. Human Cu, Zn SOD required 60 minutes to equilibrate and PEG SOD showed only trace activity in the lymph after 60 minutes. Mn SOD, which also has a long plasma half life, has a more favorable net charge causing it to enter the lymph almost as quickly as the Sheep SOD. Figure 8 shows that Mn SOD preserved tension development in the isolated heart after ischemia while human Cu, Zn SOD did not. When the pretreatment was extended from 15 minutes to 50 minutes, human Cu, Zn SOD was as protective as the Mn SOD but PEG SOD which does not enter the lymph still failed to protect.

In light of current evidence, we conclude that increasing the size of SOD is detrimental rather than beneficial. On the other hand the original theory may still be correct in that continued treatment long after reperfusion with an SOD which does enter the interstitial space may be a requirement for a sustained protection. None of the previous studies have attempted to maintain SOD levels for any extended period after reperfusion.

DOES ENDOGENOUS EC SOD OFFER PROTECTION TO THE REPERFUSED HEART?

If the optimal plasma concentration for SOD is 3–30 U/ml for the rabbit heart, then does that mean that EC SOD, which averages 16 U/ml in the rabbit, fully protects the heart? We don't think so. Eliminating EC SOD with DDC did not extend the infarct in Figure 4 above. EC SOD, like PEG SOD may be too large to cross the capillary to any appreciable extend and thus be excluded from the cardiac interstitium. Human Cu, Zn SOD may still offer considerable protection against infarction in the reperfused heart if the proper tissue concentration could be achieved and maintained.

In summary, we have attempted to show that many of the SOD trials to date suffer from a variety of methodological problems which could not have been anticipated at the time those trials were instituted. As a result there is little evidence that infarct size was actually limited in any of existing studies there is also emerging evidence that few of those studied employed a dose and schedule which would have been appropriate for salvage. As dose and schedule data become better understood, a clinical efficacy for myocardial infarction patients may yet be shown for SOD.

References

1. G. Ambrosio, L.C. Becker, G.M. Hutchens, H.F. Weisman and M.L. Weisfeldt (1986) Reduction in experimental infarct size by recombinant human superoxide dismutase: insights into pathophysiology of reperfusion injury. *Circulation*, **74**, 1424–1433.
2. J.E. Baker, C.C. Felix, G.N. Olinger, and B. Kalyanaraman (1988) Myocardial ischemia and reperfusion: Direct evidence for free radical generation by electron spin resonance spectroscopy. *Proceedings of the National Academy of Science USA*, **85**, 2786–2789.
3. M. Bernier, J.D. Hearse and A.S. Manning (1986) Reperfusion-induced arrhythmias and oxygen-derived free radicals. Studies with "anti-free radical" interventions and a free radical generating system in the isolated perfused rat heat. *Circulation Research*, **58**, 331–340.
4. R. Bolli (1988) Oxygen-Derived Free Radicals and Postischemic Myocardial Dysfunction ("Stunned Myocardium"). *JACC*, **12(1)**, 239–249.
5. R. Bolli, B.S. Patel, M.O. Jeroudi, E.K. Lai and P.B. McCay Demonstration of Free Radical Generation in "Stunned" Myocardium of Intact Dogs with the Use of the Spin Trap alpha-Phenyl N-Tert-Butyl Nitrone. *Journal of Clinical Investigation*, **82**, 476–485.
6. E.K. Braunwald and R.A. Kloner (1985) A Myocardial reperfusion: a double-edged sword? *Journal*

of Clinical Investigation, **76**, 1713–1719.
7. C.A. Campbell, K.J. Alker and R.A. Kloner (1988) Does dissue plasminogen activating factor reduce infarct size in an experimental model of mechanical coronary artery occlusion and reperfusion in dogs. *Journal of American Colica Cardiology*, **11**, 54A.
8. D.E. Chambers, D.A. Parks, G. Patterson, R. Roy, J.M. McCord , S. Yoshida, L.F. Parmley, and J.M. Downey (1985) Xanthine oxidase as a source of free radical damage in myocardial ischemia. *Journal of Molecular Cellular Cardiology*, **17**, 145–152.
9. D.E. Chambers, D.M. Yellon, and D.J. Hearse (1983) Effects of flurbiprofen in altering the size of myocardial infarcts in dogs: reduction or delay? *American Journal of Cardiology*, **51**, 884–890.
10. M.L. Charlat, P.G. O'Neill, J.M. Egan, D.R. Abernathy, L.H. Michael, M.L. Myers, R. Roberts and R. Bolli (1987) Evidence for a pathogenetic role of xanthine oxidase in the "stunned" myocardium. *American Journal of Physiology*, **252**, H566–H577.
11. H. Darius, A. Yanagisawa, M.E. Brezinski and A.M. Lefer (1986) Direct cardioprotective effect of tPA in feline ischemic myocardium. *Fed proc*, **45**, 808 (abs).
12. J.M. Downey, T. Miura, L.J. Eddy, D.E. Chambers, T. Mellert, D.J. Hearse, and D.M. Yellon (1987) Xanthine oxidase is not a source of free radicals in the ischemic rabbit heart. *Journal of Molecular Cellular Cardiology*, **19**, 1053–1060.
13. J.M. Downey, C. Shirato, T. Miura, and T. Toyofuku, (1988) Tetrazolium is unreliable as an index of drug induced salvage. *Journal of Molecular Cellular Cardiolog*, **20** *Supp*. V: S 70, abstract.
14. O. Elroy-Stein, Y. Bernstein and T. Groner (1986) Over production of human Cu/Zn-superoxide dismutase in transfected cells: extention of paraquat-mediated cytotoxicity and enhancemant of lipid peroxidation. *EMBO Journal*, **5**, 615–622.
15. R. Engler and E. Gilpin (1989) Can superoxide dismutase alter infarct size?. *Circulation*, **79**, 1137–1142.
16. S.M. Factor, S. Cho and E.S. Kirk (1982) Non-specificity of triphenyl tetrazolium chloride (TTC) for the gross diagnosis of acute myocardial infarction. *Circulation*, **66**, *(Supp* 1: II–333, abstract.
17. N.E. Farber, G.M. Vercellotti, H.S. Jacob, G.M. Pieper and G.J. Gross (1988) Evidence for a Role of Iron-Catalyzed Oxidants in Functional and Metabolic Stunning in the Canine Heart. *Circulation Research*, **63**, 351–360.
18. R. Ferrari, C. Ceconi, S. Curello, A. Cargnoni and D. Medici (1986) Oxygen free radicals and reperfusion injury; the effect of ischemia and reperfusion on the cellular ability to neutralise oxygen toxicity. *Journal of Molecular Cellular Cardiology*, **18** *sup* 4, 67–69.
19. M.C. Fishbein, S. Meerbaum, J. Rit, *et al.* (1981) Early phase acute myocardial infarct size quantification: validation of the triphenyl tetrazolium chloride staining technique. *American Heart Journal*, **101**, 593–599.
20. R.B. Fox, R.N. Harada, R.M. Tate and J.E. Repine (1983) Prevention of Thio-urea induced pulmonary edema by hydroxyl-radical scavengers. *Journal of Applied Physiology*, **55**, 1456–1459.
21. T.J. Gardner, J.R. Stewart, A.S. Casale, J.M. Downey and D.E. Chambers (1988). Reduction of myocardial ischemic injury with oxygen derived free radical scavengers. *Surgery*, **94**, 423–427.
22. P.B. Garlick, M.J. Davies, D.J. Hearse and T.F. Slater (1987) Direct detection of free radicals in the reperfused rat heart using electron spin resonance spectroscopy. *Circulation Research*, **61**, 757–760.
23. GISSI, Effectiveness of intravenous thrombolytic treatment in acute myocardial infarction. *Lancet*, **1**, 402–1986, 397.
24. D.N. Granger, M.E. Hollwarth, and D.A. Parks (1986) Ischemia reperfusion injury: role of oxygen-derived free radicals. *Acta Physiolica Scand. Supp* **548**, 47–63.
25. P.M. Grinwald (1982) Calcium uptake during post-ischemic reperfusion in the isolated rat heart; influence of extracellular sodium. *Journal of Molecular Cellular Cardiology*, **14**, 359–365.
26. C.M. Grum, K.P. Gallagher, M.M. Kirsh and M. Shlafer (1989) Absence of detectable xanthine oxidase in human myocardium. *Journal of Molecular Cellular Cardiology*, **21**, 263–267.
27. Y. Guaduel and M.A. Duvelleroy (1984) Role of oxygen radicals in cardiac injury due to reoxygenation. *Journal of Molecular Cellular Cardiology*, **16**, 459–470.
28. C. Guarnieri, R. Ferrari, O. Visioli, C.M. Caldarera and W.G. Nayler (1978) Effect of alpha-tocopheral on hypoxic-perfused and reoxygenated rabbit heart muscle. *Journal of Molecular Cellular Cardiology*, **10**, 893–906.
29. D.J. Hearse and S.M. Humphrey (1975) Enzyme release during myocardial anoxia: a study of metabolic protection. *Journal of Molecular Cellular Cardiology*, **7**, 463–482.
30. D.J. Hearse, S.M. Humphrey and E.B. Chain (1973) Abrupt reoxygenation of the anoxic potassium arrested perfused rat heart: a study of myocardial enzyme release. *Journal of Molecular Cellular Cardiology*, **5**, 395–407.
31. D.J. Hearse, S.M. Humphrey, W.G. Nayler, A. Slade and D. Border (1975) Ultrastructural damage

associated with reoxygenation of the anoxic myocardium. *Journal of Molecular Cellular Cardiology*, **7**, 315–324.
32. P.J. Horneffer, B. Healy, V.L. Gott and T.J. Gardner (1987) The rapid evolution of a myocardial infarction in an end-artery coronary preparation. *Circulation*, **76**, v39–v42.
33. R.B. Jennings and C.E. Ganote (1974) Structural changes in myocardium during acute myocardial ischemia. *Circulation Research*, **34** *(sup* III): iii156–iii168.
34. R.B. Jennings and K.A. Reimer (1981) Lethal myocardial ischemic injury. *American Journal of Pathology*, **102** 241–255.
35. R.B. Jennings and K.A. Reimer (1983) Factors involved in salvaging ischemic myocardium: effect of reperfusion of arterial blood. *Circulation*, **68** (*suppl.*: 125–136.
36. R.B. Jennings, H.M. Sommers, G.A. Smyth and H.A. Flack (1960) Myocardial necrosis induced by temporary occlusion of a coronary artery in the dog. *AMA Archives of Pathology*, **70**, 68–78.
37. S.L. Jewett, L.J. Eddy and P. Hochstein (1989) Is the autoxidation of catecholamines involved in ischemia-reperfusion injury?, *Free Radical Biology and Medicine*, **6**, 323–326.
38. S.R. Jolly, W.J. Kane, M.B. Bailie, G.D. Abrams and B.R. Lucchesi (1981) Canine myocardial reperfusion injury: its reduction by the combined administration of superoxide dismutase and catalase. *Circulatory Research*, **54**, 277–285.
39 K. Karlsson and S.L. Marklund (1988) Extracellular superoxide dismutase in the vascular system of mammals. *Biochemical Journal*, **255**, 223–228.
40. H.H. Klein, S. Puschmann, J. Schaper and W. Schaper (1981) The mechanism of the tetrazolium reactiom in identifying myocardial infarction. *Virchows Archives (Pathol. Anat.)* **393**, 287–297.
41. H.A. Kontos, E.P. Wei, E.F. Ellis, L.W. Jenkins, J.T. Polvilshock, T. Rowe M.L. Hess (1985) Appearance of superoxide anion radical in cerebral extracellular space during increased prostaglandin synthesis in cats. *Circulation Research*, **57**, 142–115.
42. R.F. del Maestro, J. Bjork and K.E. Arfors (1981) Increase in microvascular permiability induced by enzymatically generated free radicals. *Microvascular Research*, **22**, 255–270.
43. A. Manning, M. Bernier, R. Crome, S.H. Little and D.J. Heavse (1988) Reperfusion arrhythmias: a study of the role of xanthine oxidase-derived free radicals in the rat heart. *Journal of Molecular Cellular Cardiology*, **20**, 35–40.
44. M.P. Maxwell, D.J. Hearse and D.M. Yellon (1987) Species variation in the coronary collateral circulation during regional myocardial ischemia: a critical determinant of the rate of evolution and extent of myocardial infarction. *Cardiovascular Research*, **21**, 737–746.
45. J.M. McCord (1985) Oxygen-derived free radicals in postischemic tissue injury. *New England Journal of Medicine*, **312**, 159–163.
46. J.M. McCord, R.S. Roy and S.W. Schaffer (1985) Free radicals and myocardial ischemia: the role of xanthine oxidase. *Advances in Myocardiology*, **5**, 183–189.
47. J.R. Mitchell, C.V. Smith, H. Hughes, M.L. Lenz, H. Jaeschke, S. Scott, M.B., L.H., and M.L. Entman (1987) No evidence for reactive oxygen damage in ischemia-reflow injury. *Transactions of the Association of American Physicians*, **100**, 54–61.
48. T. Miura, J.M. Downey, D. Hotta, 11 and O. Iimura (1988) Effect of superoxide dismutase plus catalase on myocardial infarct size in rabbits. *Canadian Journal of Cardiology*, **4**, 407–411.
49. T. Miura, D.M. Yellon, D.J. Hearse and J.M. Downey (1987) Determinants of infarct size during permanent occlusion of a coronary artery in the closed chest dog. *Journal of the American College of Cardiology*, **9**, 647–654.
50. C.L. Myers, S.J. Weiss, M.M. Kirsh and M. Shlafer (1985) Involvement of hydrogen peroxide and hydroxyl radical in the oxygen paradox: reduction of creatine kinase release by catalase, allopurinol or desferoxamine, but not by superoxide dismutase. *Journal of Molecular Cellular Cardiology*, **17**, 675–684.
51. M. Nachlas and T. Schnitka (1963) Macroscopic identification of early myocardial infarcts by alterations in dehydrogenase activity. *American Journal of Pathology*, **42**, 379–406.
52. U. Nasllund, S. Haggmark, G. Johansson, S.L. Marklund, S. Reiz and A. Ogberg (1986) Superoxide dismutase and catalase reduce infarct size in a porcine myocardial ischemia/reperfusion model. *Journal of Molecular Cellular Cardiology*, **18**, 1077–1084.
53. J. Nejima, D.R. Knight, J.T. Fallon, N. Uemura, W.T. Manders, D.R. Canfield, M.V. Cohen and S.F. Vatner (1989) Superoxide dismutase reduces reperfusion arrhythmias but fails to salvage regional function or myocardium at risk in conscious dogs. *Circulation*, **79**, 143–153.
54. C. Nienaber, M. Gottwik, B. Winkler and W. Shaper (1983) The relationship between the perfusion deficit, infarct size and time after experimental coronary occlusion. *Basic Research of Cardiology*, **78**, 210–226.
55. K. Przyklenk and R.A. Kloner (1986) Superoxide dismutase plus catalase improve contractile

function in the canine model of the "stunned myocardium". *Circulation Research*, **58**, 148–156.
56. K. Przyklenk and R.A. Kloner (1989) Reperfusion injury by oxygen free radicals? Effect of superoxide dismutase plus catalase, given at the time reperfusion, on myocardial infarct size, contractile function, coronary microvascular,. *Circulation Research*, **64**, 86–96.
57. K.A. Reimer and R.B. Jennings (1979) The wavefront phenomenon of myocardial ischemic cell death II. Transmural progression of necrosis within the framework of ischemic bed size (myocardium at risk) and collateral flow. *Laboratory Investigation*, **40**, 633–640.
58. K.A. Reimer, J.E. Lowe, M.M. Rassmussen and R.B. Jennings (1977) The wavefront phenomenon of ischemic cell death. 1. Myocardial infarct size vs. duration of coronary occlusion in dogs. *Circulation*, **56**, 786–794.
59. K.A. Reimer, M.M. Rasmussen and R.B. Jennings (1973) Reduction by propranolol of myocardial necrosis following temporary coronary artery occlusion in dogs. *Circulation Research*, **33**, 353–363.
60. J. Schaper, J. Mulch, B. Winkler and W. Schaper (1979) Ultrastructural, functional and biochemical criteria for estimation of reversibility of ischemic injury: a study on the effects of global ischemia in the isolated dog heart. *Journal of Molecular Cellular Cardiology*, **11**, 521–541.
61. M.D. Scott, S.R. Meshnick and J.W. Eaton (1987) Superoxide dismutase-rich bacteria. *Journal of Biological Chemistry*, **262**, 3640–3645.
62. C. Shirato, T. Miura and J.M. Downey (1988) Superoxide dismutase (single dose) delays rather than prevents necrosis inreperfused rabbit hearts. *FASEB Journal*, **2**, A 918, abstract.
63. M. Shlafer, C.L. Myers and S. Adkins (1987) Mitochondrial hydrogen peroxide generation and activities of glutathione peroxidase and superoxide dismutase following global ischemia. *Journal of Molecular Cellular Cardiology*, **19**, 1195–1206.
64. P.J. Simpson and B.R. Lucchesi (1987) Free radicals and myocardial ischemia and reperfusion injury. *Journal of Laboratory Clinical Investigation*, **110**, 13–30.
65. K.P. Sunnergen and M.J. Rovetto (1987) Myocyte and endothelial injury with ischemia reperfusion in isolated rat hearts. *American Journal of Physiology*, **252**, H1211–H1217.
66. Y. Tamura, L. Chi, E.M. Driscoll, Jr., P.T. Hoff, B.A. Freeman, K.P. Gallagher and B. R. Lucchesi (1988) Superoxide Dismutase Conjugated to Polyethylene Glycol Provides Sustained Protection Against Myocardial Ischemia/Reperfusion Injury in Canine Heart. *Circulation Research*, **63**, 944–959.
67. TIMI (1985) The thrombolysis in myocardial infarction (TIMI) trial: Phase one findings. *New England Journal of Medicine*, **312**, 832–936.
68. A. Uraizee, K.A. Reimer, C.E. Murry and R.B. Jennings (1987) Failure of superoxide dismutase to limit infarct size of myocardial infarction after 40 minutes of ischemia and 4 days of reperfusion in dogs. *Circulation*, **75**, 1237–1248.
69. R.S. VanderHeide, P.A. Sobotka and C.E. Ganote (1987) Effect of free radical scavenger DMTU and Mannitol on the oxygen paradox in perfused rat hearts. *Journal of Molecular Cellular Cardiology*, **19**, 615–625.
70. S.W. Werns, M.J. Shea, E.M. Driscoll, C. Cohen, G.D. Abrams, B. Pitt and B.R. Lucchesi (1985) The independent effects of oxygen radical scavengers on canine infarct size reduction by superoxide dismutase and not catalase. *Circulation Research*, **56**, 895–898.
71. S.W. Werns, P.J. Simpson, J.K. Mickelson, M.J. Shea, B. Pitt and B.R. Lucchesi (1988) Sustained limitation by superoxide dismutase of canine myocardial injury due to regional ischemia followed by reperfusion. *Journal of Cardiovascular Pharmacology*, **11**, 36–44.
72. J.L. Zweier (1988) Measurement of Superoxide-derived Free Radicals in the Reperfused Heart. *The Journal of Biological Chemistry*, **263(3)**, 1353–1357.
73. J.L. Zweier, B.K. Rayburn, J.T. Flaherty and M.L. Weisfeldt (1987) Recombinant Superoxide Dismutase Reduces Oxygen Free Radical Concentrations in Reperfused Myocardium. *The Journal of Clinical Investigation*, **80(6)**, 1728–1734.

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