Evidence Against the “Early Protection–Delayed Death” Hypothesis of Superoxide Dismutase Therapy in Experimental Myocardial Infarction

Polyethylene Glycol–Superoxide Dismutase Plus Catalase Does Not Limit Myocardial Infarct Size in Dogs

Masaru Tanaka, Robert C. Stoler, Gregory P. FitzHarris, Robert B. Jennings, and Keith A. Reimer

We previously found that superoxide dismutase (SOD) did not limit myocardial infarct size after 40 or 90 minutes of ischemia and 4 days of reperfusion in dogs. Because some other studies have shown limitation of infarct size after shorter periods of reperfusion, we postulated that our negative results might be due to late reperfusion injury mediated by superoxide anions produced after excretion of SOD. To test this “early protection–delayed death” hypothesis, we have examined whether SOD, conjugated to polyethylene glycol (PEG-SOD) to prolong its circulating half-life, limited myocardial infarct size. The circumflex artery was occluded for 90 minutes followed by 4 days of reperfusion. PEG-SOD (total dose, 10,000 units/kg) and catalase (55,000 units/kg) were given during the 30 minutes before reperfusion. Plasma SOD levels in the treated group were 330±20 units/ml at the onset of reperfusion and 140±10 units/ml on day 4 (circulating half-life, 75±5 hours) versus 5±1 units/ml in controls. Histological infarct size was 37.1±4.2% of the area at risk in the treated group (n=11) versus 44.5±6.2% in controls (n=10) (p=NS). Infarct size and collateral blood flow were inversely related in controls; PEG-SOD and catalase did not shift this regression (p=NS by analysis of covariance). Thus, infarct size was not limited when measured after 4 days of reperfusion, even though plasma SOD exceeded 100 units/ml throughout this reperfusion period. These results do not support the early protection–delayed death hypothesis, but do support our previous conclusion that myocyte death (lethal reperfusion injury) is not caused by superoxide anions accessible to SOD in the circulation. (Circulation Research 1990;67:636–644)

Timely reperfusion of ischemic myocardium limits the size of a myocardial infarct by interrupting the wave front of cell death that progresses from the subendocardium to the subepicardium.1 However, reperfusion itself may kill some myocytes that are still viable at the moment of reperfusion. The most popular hypothetical mechanism of such a reperfusion injury involves myocardial or vascular damage caused by oxygen free radicals. Electron spin resonance techniques have shown increased free radical production during reperfusion of ischemic myocardium.2–4 Moreover, it has been demonstrated that oxygen free radicals produced by an exogenously administered free radical–generating system can damage myocytes and vascular endothelium.5–7 However, it remains uncertain whether oxygen free radicals cause myocardial cell death during ischemia and reperfusion in vivo. Conflicting results have been reported from experimental animal studies from various laboratories in which the efficacy of superoxide dismutase (SOD) with or without catalase to limit myocardial infarct size have been tested.8–20 The explanation for these discrepant results is unknown. However, in all of the studies with native SOD, SOD has been administered to cover only the superoxide radicals produced in the
early reperfusion period. Moreover, most of the studies demonstrating limitation of myocardial infarct size have had an experimental protocol in which infarcts were evaluated after a short period of reperfusion (less than 6 hours). Studies with protocols including longer reperfusion times (more than 3 days) usually have had negative results. Thus, since the biological half-life of SOD is very short (6–10 minutes), these discrepant results could be explained if myocytes, initially salvaged by SOD therapy, are killed later by continued production of free radicals after the administered SOD has been eliminated.

The present study was designed to test the hypothesis that continuous treatment with SOD throughout a 4-day reperfusion period could achieve a sustained limitation of myocardial infarct size resulting from 90 minutes of ischemia in dogs. To achieve sustained SOD treatment, we used polyethylene glycol–conjugated SOD (PEG-SOD), which has a plasma half-life reported to exceed 30 hours. The results of the present study demonstrate that, despite a prolonged high plasma concentration of SOD, treatment with PEG-SOD and catalase failed to limit myocardial infarct size when quantitated 4 days after reperfusion.

Materials and Methods

In general, animal selection, surgical preparation, and postmortem analysis were performed according to the criteria described in the multicenter AMPIM study. All dogs received humane care in accordance with the guidelines of the American Physiological Society for the use of laboratory animals.

Animal Selection

Twenty-nine adult mongrel dogs of either sex weighing 14.5–20.5 kg were used. No dogs with a hematocrit below 35%, clinically evident infection, circulating heartworm filariae, or pregnancy were accepted in the study.

Experimental Design

The experimental protocol is summarized in Figure 1. Infarcts were produced by 90 minutes of left circumflex coronary arterial occlusion followed by reperfusion. Myocardial blood flow was measured before occlusion and at 10 and 80 minutes during occlusion by the microsphere technique. Animals were randomly assigned to one of two experimental groups. 1) In the treatment group (n = 16), dogs received an intra-atrial infusion of 10,000 units/kg body wt PEG-SOD (lot E8804008, Enzon, Inc., South Plainfield, N.J., kindly donated to us by the Sterling Research Group, Rensselaer, N.Y.) and 55,000 units/kg bovine liver catalase (Sigma Chemical Co., St. Louis). These enzymes were administered over a 30-minute period starting 60 minutes into occlusion and ending at reperfusion. 2) Corresponding controls (n = 13) received an equivalent volume of saline through the same route and in the same time frame.

On the fourth day after the experiment, infarct size was measured by histological methods, expressed as a percentage of ischemic vascular bed, and analyzed with respect to the collateral blood flow to the ischemic bed (see below). Venous blood samples for determination of plasma concentration of SOD were drawn immediately before the treatment, at 3 minutes before reperfusion, at 30 minutes after reperfusion, and at the moment the dogs were killed. Plasma was separated and frozen for later analysis.

Surgical Preparation

Dogs were anesthetized with sodium pentobarbital (30–40 mg/kg i.v.), and additional doses were given as needed throughout the experiment. They were intubated and ventilated with 200 ml/kg/min of room air supplemented with a low flow of oxygen by a Harvard respirator (South Natick, Mass.). The right femoral artery and vein were cannulated to monitor peripheral arterial pressure and administer additional anesthetic, respectively. A thoracotomy was performed in the fourth left intercostal space, and the heart was suspended in a pericardial cradle. The left circumflex coronary artery was isolated distal to the atrial branch but proximal to the first large marginal branch. A pneumatic occluder and ultrasonic flow probe (Transonic Systems, Inc., Ithaca, N.Y.) were placed around the isolated artery. Occlusion and reperfusion of the artery were accomplished by inflation and deflation of the occluder, respectively. Occlusion and reperfusion were confirmed by the flowmeter. Three catheters then were passed into the left atrium for measurement of atrial pressure; injection of microspheres; and infusion of PEG-SOD.
catalase, or saline. After the initial surgical preparation but before coronary occlusion, animals were assigned randomly to either the treatment or control group. All dogs were allowed at least 20 minutes after completion of the surgical preparation to reach steady state before the first microsphere injection.

Regional Myocardial Blood Flow Measurement

The regional distribution of myocardial blood flow was assessed with 10±2 μm radioactive microspheres by techniques described previously.14,15,21 At each measurement time, two to three million spheres labeled with 153Gd, 46Sc, or 113Sn were injected via the left atrial catheter. A reference arterial blood sample was obtained from the femoral artery at 7.75 ml/min for 2.5 minutes beginning just before injection of spheres.

Postmortem Studies

The anatomic boundaries of occluded and unoccluded coronary beds were determined by simultaneously injecting two different dyes, triphenyltetrazolium chloride (TTC) (1%, Sigma) and monastral blue dye (4%, Sigma), at 120–140 mm Hg to the previously occluded left circumflex coronary artery and left main coronary artery, respectively. The perfusion fluid was sodium phosphate buffer (0.09 M, pH 7.4) with 8% dextran (molecular weight 82,000; Sigma) added to prevent edema during perfusion. After fixation in 10% phosphate buffered formalin, hearts were cut into eight transverse slices, each of which was weighed and photographed. The area at risk was identified and traced from enlarged projections (magnification, ×8) of the photographic slide of each ventricular slice. Microscopic slides were prepared from the ischemic region of five of eight transverse ventricular slices as described previously.14 Two slides from each tissue block were cut, and one was stained with hematoxylin and eosin and the other with Heidenhain’s variant of Mallory’s connective tissue stain. Infarcts were traced on projections (magnification, ×8) of the microscopic slide with the connective tissue stain. The area at risk and infarct area were quantified by retracing these tracings on a digitizing tablet interfaced to a personal computer.

Myocardial blood flow was measured in the central portion of the ischemic vascular bed and the nonischemic bed, as reported previously.14,15,21 Tissue on both sides of the ischemic and nonischemic interface was excluded to avoid cross-contamination of samples. Myocardial blood flow was expressed as milliliters per minute per gram wet weight. Corrections were made for swelling of the infarct as evidenced by a preocclusion ischemic-nonischemic regional flow ratio of less than 0.9.22

Measurement of Plasma Superoxide Dismutase Concentration

Plasma SOD levels were determined with the method of McCord and Fridovich.23 The assay was performed in 3 ml of 0.05 M potassium phosphate buffer at pH 7.8 containing 10−4 M EDTA, 10−5 M cytochrome c, 5×10−5 M xanthine, and sufficient xanthine oxidase to produce a rate of reduction of cytochrome c at 550 nm of 0.025 absorbance unit/min. The concentration of xanthine oxidase to achieve this rate varied, but it averaged 10−5 M. One unit of SOD was defined as the amount of SOD required to inhibit the reduction of cytochrome c by 50% (i.e., to a rate of 0.0125 absorbance unit/min).

Statistical Analysis

Data are expressed as group mean±SEM. Statistical comparisons of group means were made with Student’s t test. The effects of treatments on hemodynamics or collateral blood flow were analyzed with a paired t test. To control for variability in infarct size caused by differences in collateral blood flow, the regression of infarct size versus collateral blood flow was evaluated, and an analysis of covariance was performed using collateral flow as an independent variable and infarct size as a dependent variable. A value of p<0.05 was considered statistically significant.

Results

Animal Survival

Twenty-nine dogs were used and randomly assigned to either group in this study. Sixteen were assigned to the treated group and 13 to the controls.

The incidence and time of ventricular fibrillation and survival rate are summarized in Table 1. Five dogs (three from the treated group, two from the controls) developed ventricular fibrillation within 7 minutes after coronary occlusion. One dog from the treated group was defibrillated successfully. Three dogs in the treated group developed ventricular fibrillation early in the period of reperfusion. Two of them were defibrillated successfully and survived to
the completion of the study. No dog in the control group developed ventricular fibrillation after reperfusion. One dog in the control group and two in the treated group died during the first postoperative night. Thus, overall survival was 77% (10/13) in the control group and 69% (11/16) in the treated group.

**Plasma Superoxide Dismutase Concentration**

Plasma concentrations of SOD in the 11 surviving treated dogs (Figure 2) averaged 6±1 units/ml before treatment, 310±20 units/ml at 3 minutes before reperfusion, 330±20 units/ml at 30 minutes into reperfusion, and 140±10 units/ml on the fourth day (93 hours). From these measurements, the circulating half-life of PEG-SOD in this study was 75±5 hours. In the control group, endogenous plasma SOD concentrations averaged 4±1, 5±2, 5±1, and 5±2 units/ml at each respective time point. Thus, the plasma concentrations of SOD in the treated group were elevated markedly throughout 4 days of reperfusion.

**Baseline Predictors of Infarct**

The size of the area at risk. Area at risk (Table 2, Figure 3) was similar between the two groups, averaging 41.0±1.6% of the left ventricle in the controls and 38.4±1.4% in the treated group (p=NS).

Collateral blood flow. The mean myocardial blood flow in the circumflex region before occlusion (Table 2) was similar between the two groups; 1.12±0.15 ml/min/g wet wt in controls and 1.32±0.11 ml/min/g wet wt in the treated group (p=NS).

Although transmural collateral blood flow to the ischemic region at 10 and 80 minutes into occlusion (Table 2) was slightly higher in the treated group compared with controls (0.19±0.04 versus 0.17±0.03 ml/min/g wet wt at 10 minutes and 0.24±0.06 versus 0.16±0.03 ml/min/g wet wt at 80 minutes into occlusion, respectively), the difference was not statistically significant. In the group treated with PEG-SOD and catalase, there was a trend toward increased collateral blood flow between the 10-minute (pretreatment) and 80-minute (during treatment) measurements. However, the difference did not reach statistical significance by paired t test.

**Hemodynamics**

Throughout the experiments, heart rate, blood pressure, and rate pressure product (Figure 4) were similar between the two groups. Treatment with PEG-SOD and catalase did not cause any significant changes in hemodynamic parameters.

Thus, the two groups were comparable regarding the major determinants of myocardial infarct size such as collateral blood flow, area at risk, and hemodynamic determinants of myocardial oxygen demand.

**Infarct Size**

There was no difference in infarct size, as a percentage of the left ventricle (Table 2, Figure 3), between the two groups: 18.2±2.6% in the control group and 14.4±1.8% in the treated group. To control for variation in the area at risk, infarct size also was expressed as a percentage of the area at risk. This was also similar between the groups: 44.5±6.2% in the control group and 37.1±4.2% in the treated group.

Because direct group comparison of infarct size with Student’s t test does not control for the influence of collateral blood flow, a major determinant of myocardial infarct size, the relation of infarct size to collateral blood flow was examined (Figure 5). There was an inverse relation between these two variables in the controls. Treatment with PEG-SOD and catalase did not shift this relation; this was confirmed by analysis of covariance, with collateral blood flow as an independent variable and infarct size as a dependent variable (F=1.14, p=NS). The same analysis of covariance excluding two treated dogs that required defibrillation did not change the conclusion (F=1.44, p=NS). Thus, PEG-SOD plus catalase did not limit infarct size, even when the variation caused by collateral flow was controlled.

**Discussion**

There have been a substantial number of experimental studies done to test the efficacy of various anti–free radical therapies on myocardial infarct size, but the results have led to conflicting conclusions.8–20 Although several experimental variables have been proposed to explain the conflicting results, the correct explanation remains unknown.24 The present study was designed to test whether sustained limitation of myocardial infarct size with SOD depends on continued treatment throughout the reperfusion period. The basis for this question is as follows.

In previous studies from this laboratory,14,15 native SOD with a much shorter biological half-life was used, and no infarct size limitation after 40 or 90 minutes of ischemia followed by 4 days of reperfusion was observed. On the other hand, several investigators8–13 using shorter durations (6 hours or less) of reperfusion have reported that infarct size was limited by treatment with native SOD alone or com-
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PEG-SOD + catalase

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\( p \) values refer to nonpaired \( t \)-test analyses of treated group vs. control group means. AAR, area at risk; LV, left ventricle; Control LCx flow, transmural mean left circumflex vascular bed flow before occlusion; LCx CBF, collateral blood flow to the left circumflex vascular bed; I, M, and O, inner, middle, and outer third, respectively; HR, heart rate; SBP and DBP, systolic and diastolic blood pressure, respectively; RPP, rate pressure product (SBP×HR) measured in mm Hg beats/min.

* Dogs requiring defibrillation.
FIGURE 3. Area at risk (AAR) and infarct size in the control group and the group treated with polyethylene glycol–conjugated superoxide dismutase (PEG-SOD) plus catalase. AAR expressed as a percentage of left ventricle (LV) and infarct size expressed as a percentage of either LV or AAR were similar between the control group and the group treated with PEG-SOD plus catalase. Error bars indicate 1 SEM.

bined with catalase. These discrepant results could be explained by the hypothesis that, although SOD may exert a beneficial effect in the early reperfusion period, the rapid excretion of SOD may be followed by delayed myocardial cell death caused by the late production of oxygen free radicals coupled with depressed cellular defense mechanisms.25–27 For example, leukocytes that progressively accumulate in the damaged myocardium may be a continuing source of oxygen free radicals during the reperfusion period.

This “early protection–delayed death” hypothesis was not supported by the results of the present study. Although the conjugation of SOD to PEG markedly lengthened its biological half-life (plasma SOD analysis showed a high concentration of SOD even after 4 days of reperfusion), no limitation of infarct size was observed in the treated group. Because chronic maintenance of high plasma SOD concentrations throughout the reperfusion period did not limit infarct size in the present study, the failure to continue treatment in previous studies with native SOD is unlikely to be a sufficient explanation for the negative results of those studies.14–16 Thus, the explanation for conflicting results among studies of the effects of SOD on myocardial infarct size remains unknown.

FIGURE 4. Hemodynamic parameters vs. time in the control group and the group treated with polyethylene glycol–conjugated superoxide dismutase (PEG-SOD) plus catalase. There was no significant difference between the two groups in heart rate, systolic or diastolic blood pressure (SBP or DBP), or rate pressure product (RPP) at any time. BL, baseline measured before the first myocardial blood flow measurement; R, reflow measured at 30 minutes after reflow; Minutes, minutes of coronary occlusion. Error bars indicate 1 SEM.

FIGURE 5. Relation of infarct size to transmural collateral blood flow. There was an inverse relation between infarct size, expressed as a percentage of area at risk, and transmural collateral blood flow in controls. It is readily apparent that treatment with polyethylene glycol–conjugated superoxide dismutase (PEG-SOD) and catalase did not alter this relation. This impression was confirmed by analysis of covariance (F=1.14, p=NS). The same analysis, excluding two treated dogs that required defibrillation (arrows) did not change the conclusion (F=1.44, p=NS).

Results of several other studies also are not explained by the early protection–delayed death hypothesis. For example, Matsuda et al18 and Przyklenk and Kloner20 reported that SOD failed to limit infarct size when infarcts were assessed after only 4 hours of reperfusion. Conversely, the positive results reported by Jolly et al18 and Werns et al12 were observed after 24 hours of reperfusion, by which time the SOD administered at the time of reperfusion should have been excreted.

On the other hand, some studies have supported the early protection–delayed death hypothesis. For example, Shirato et al23 reported that a single dose of SOD at the time of reperfusion limited myocardial infarct size in rabbits when infarcts were assessed after either 3 or 24 hours of reperfusion, but therapy was ineffective when the duration of reperfusion was 72 hours. The duration of ischemia was the same, 45 minutes in all three groups.

Recently, Tamura et al28 reported that PEG-SOD limited canine myocardial infarct size. Their study deserves detailed comparison with the present study because opposite conclusions were drawn, even
Table 3. Major Similarities and Differences Between This Study and the Study by Tamura et al

<table>
<thead>
<tr>
<th></th>
<th>This study</th>
<th>Study by Tamura et al</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>Dog</td>
<td>Dog</td>
</tr>
<tr>
<td>Duration of occlusion</td>
<td>90 min</td>
<td>90 min</td>
</tr>
<tr>
<td>Duration of reperfusion</td>
<td>4 days</td>
<td>4 days</td>
</tr>
<tr>
<td>Measurement of CBF</td>
<td>21/21 (100%)</td>
<td>11/26 (42%)</td>
</tr>
<tr>
<td>Quantitation of IS</td>
<td>Light microscopy</td>
<td>Macrochemistry (TTC)</td>
</tr>
<tr>
<td>Dose of PEG-SOD</td>
<td>10,000 units/kg</td>
<td>1,000 units/kg</td>
</tr>
<tr>
<td>Catalase</td>
<td>55,000 units/kg</td>
<td>Not used</td>
</tr>
<tr>
<td>Critical stenosis</td>
<td>None</td>
<td>Used</td>
</tr>
</tbody>
</table>

CBF, collateral blood flow; IS, infarct size; TTC, triphenyltetrazolium chloride; PEG-SOD, polyethylene glycol-conjugated superoxide dismutase.

though the experimental protocols of the two studies were similar. Major similarities and differences are listed in Table 3. Both studies used an anesthetized open-chest canine model with 90 minutes of left circumflex coronary artery occlusion and 4 days of reperfusion. PEG-SOD was given during the late occlusion period before reperfusion (during the last 15 minutes of the 90-minute occlusion in the study by Tamura et al, during the last 30 minutes of the 90-minute occlusion in the present study). Nevertheless, there are several potentially important differences between the two studies.

First, it is essential to control for the variation in myocardial collateral blood flow in studies with canine models of myocardial infarction. Otherwise, false-positive or false-negative conclusions can be drawn because of undetected baseline differences between groups in the severity of the ischemic insult. We measured collateral blood flow in all dogs, but this was done in only 11 of 26 animals in the study by Tamura et al.

Second, whereas we used histological evaluation of infarct size, Tamura et al measured infarct size by macrochemistry (TTC staining). TTC staining results from enzymatic activity within the myocardium and may not necessarily indicate tissue viability. For example, myocardium that has undergone autolysis for 6 hours and is in full rigor mortis still stains with TTC. Moreover, it is possible that therapies may exist that delay the myocardial loss of dehydrogenase activity without actually delaying or preventing cell death. In this regard, Downey et al reported that the TTC method is an unreliable indicator of tissue salvage from SOD. In addition, infarcts typically have highly irregular boundaries with interdigitating peninsulas of viable and necrotic myocardium; tracing TTC-negative myocardium grossly without magnification may produce imprecise results. Also, TTC negativity may occur because of artifactual inhomogeneity in TTC perfusion rather than true loss of enzymatic activity. Thus, although TTC macrochemistry is much less time consuming than histological quantitation, it may be less reliable.

Third, the therapeutic regimen differed between the two studies. PEG-SOD used in these studies was produced by the same company (Enzon, Inc., now a subsidiary of the Sterling-Winthrop Co.). Tamura et al used 1,000 units/kg PEG-SOD, while we used 10,000 units/kg with 55,000 units/kg catalase. Accordingly, the plasma SOD activity in our study was higher throughout the experiment than it was in the study by Tamura et al (330 versus 23 units/ml at reperfusion and 140 versus 4 units/ml after 4 days). It is conceivable that a protective effect of low levels of SOD may have disappeared at the blood levels achieved with the higher (10,000 units/kg) dose administered in the present study. The possibility that too much SOD may be detrimental is supported by a recent study in which SOD was tested with a rabbit model of infarction. In that study, although 15,000 units/kg SOD limited infarct size, a very high dose of SOD (150,000 units/kg) paradoxically increased infarct size. However, the dose of PEG-SOD used in the present study (10,000 units/kg) was lower than the dose of regular SOD (15,000 units/kg) used in most of the earlier positive studies in dogs. Thus, although higher plasma levels are obtained with PEG-SOD versus the same dose of regular SOD, it seems quite unlikely that our negative results with PEG-SOD can be explained by toxicity associated with a markedly excessive dose. Interestingly, negative results were reported recently from a study of rabbit infarcts when a dose of 1,000 units/kg PEG-SOD was used.

Fourth, the manner through which reperfusion was established differed between the two studies. Whereas Tamura et al used a critical stenosis to prevent reactive hyperemia, we abruptly reperfused the hearts, allowing full reactive hyperemia to develop. It is conceivable that reactive hyperemia might accentuate production of oxygen metabolites and overwhelm the scavenging capacity of administered SOD. Whether the presence or absence of a critical stenosis influences the myocardial response to reperfusion and/or the efficacy of SOD is unknown. However, we have recently observed that the number of neutrophils that progressively accumulate in the reperfused myocardium and are a major proposed source of superoxide radicals, and also of infarct size, was not affected by the presence or absence of a critical stenosis at the onset of reperfusion.

Finally, it must be considered that a small therapeutic effect might not be detected in the relatively small number of dogs used per group in the present study. To evaluate this possibility, we have analyzed our results after enlarging the control group by including the control dogs from our preceding study. In that study, virtually identical methods were used to test the efficacy of regular SOD and oxypurinol on myocardial infarct size. Inclusion of these additional data (Figure 6) does not lend support to the hypothesis that a small protective effect was overlooked.
Thus, at present, we do not know which, if any, of the aforementioned experimental differences account for the opposing results of this study versus the study by Tamura et al.28

Conclusions

Although administration of PEG-SOD resulted in a high plasma concentration of SOD throughout 4 days of reperfusion, no limitation of infarct size was observed. These results suggest that superoxide anions, accessible to circulating SOD, do not contribute to myocardial infarct size by causing either early or delayed myocyte death after 90 minutes of ischemia and reperfusion.

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Figure 6. Relation of infarct size to transmural collateral blood flow, including the results of the present study (same as Figure 5) and results of a previous control group studied by the same methods.15 The possibility that a minor therapeutic effect of polyethylene glycol–conjugated superoxide dismutase (PEG-SOD) might become demonstrable if group sizes were larger is not supported by our retrospective data.
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Key Words • PEG-SOD • catalase • myocardial infarct size • reperfusion injury • free oxygen radicals
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