Relation Between Myocardial Glutathione Content and Extent of Ischemia-Reperfusion Injury

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The relation between the extent of myocardial injury sustained during reperfusion and total glutathione (GSH) content in the ischemic myocardium was examined in anesthetized open-chest pigs subjected to coronary occlusion for 45 minutes and reperfusion for 2 hours. In pigs infused with saline during reperfusion (n=6) there was a decrease in myocardial GSH content from 380±48 µg/g in normally perfused myocardium to 182±36 µg/g in the ischemic reperfused myocardium (p<0.02). Myocardial infarct size (expressed as a percentage of the ischemic area) was 12.5±0.8%. There was a delay of recovery of contractile function before returning to 60% of preocclusion value. In pigs pretreated with buthionine sulfoximine (BSO) (n=5), an inhibitor of cellular GSH synthesis, there was reduction in GSH content to 215±25 µg/gm in normally perfused myocardium and to 77±8 µg/gm in the ischemic reperfused myocardium. The extent of injury was greater as evidenced by an increase in infarct size to 30.4±4.0% (p<0.001), severe destructive changes in subepicardial ultrastructure, which were absent in saline-infused pigs, and persistence of dyskinesia throughout reperfusion. In pigs infused with glutathione intravenously (0.8 gm/kg) at a rate of 6.5 mg/kg/min (n=6), 5 minutes before and continuously during reperfusion, there was an increase in GSH content to 582±67 µg/g in normally perfused myocardium and to 312±80 µg/g in ischemic reperfused myocardium. The increase in myocardial GSH was associated with a reduction in infarct size to 7.5±1.3% (p<0.05, compared with saline-infused pigs) and an early recovery of contractile function of the ischemic myocardium. GSH infusion into pigs pretreated with BSO (n=4) failed to increase myocardial GSH content and failed to reduce the extent of myocardial injury. Thus, the extent of myocardial injury sustained during reperfusion is very dependent on the effectiveness of its antioxidant defenses. Markedly increased susceptibility to injury occurs when the GSH content in the ischemic myocardium becomes depleted. (Circulation 1989;80:1795–1804)

Considerable evidence suggests that myocardial injury may occur during reperfusion of the ischemic myocardium. Reactive oxygen species such as superoxide, hydrogen peroxide, and hydroxyl radical are generated during reperfusion, which might play an important role in the genesis of myocardial reperfusion injury. Although the myocardium is equipped with antioxidants that are protective against reactive oxygen species injury, recent data suggest that these antioxidants are depleted by the initial ischemic insult.

Glutathione (GSH) is an important antioxidant in the heart. A decrease in myocardial GSH content has been observed during ischemia and reperfusion of the ischemic myocardium. We hypothesized that this depletion of GSH may be detrimental to the ability of the ischemic myocardium to protect itself against reactive oxygen species during reperfusion. We tested this hypothesis by experimental modulation of myocardial GSH in pigs. We have previously demonstrated that depletion of GSH content in cultured endothelial cells renders them more susceptible to oxidant injury, whereas enhancement of endothelial GSH increases their resistance.

In this study, myocardial GSH was experimentally depleted by pretreatment with buthionine sulfoximine (BSO), a potent inhibitor of cellular GSH synthesis, to determine if reperfusion injury was
increased compared with untreated animals. We further evaluated whether myocardial GSH content could be augmented by intravenous administration of GSH and examined whether the extent of damage could be reduced.

**Materials and Methods**

**Preparation of Animals**

Yorkshire pigs of either sex weighing 25–30 kg were preanesthetized with ketamine hydrochloride (300 mg) and acepromazine maleate (20 mg i.m.). Anesthesia was induced with an intravenous injection of methohexital sodium (20 mg/kg). After intubation, surgical anesthesia was maintained with etrhane (0.5–1%), nitrous oxide (65%), and oxygen (35%) using a Harvard respirator (Harvard Apparatus, South Natick, Massachusetts). Ventilation was adjusted to maintain arterial blood gases at physiologic levels. A heating pad was placed under the animal to maintain body core temperature.

The heart was exposed through a left thoracotomy at the fifth intercostal space. The pericardium was cut and a pericardial sling was constructed. A catheter was then inserted through a stab wound at the apex and connected to a pressure transducer to measure left ventricular pressure. A marginal branch of the circumflex artery was isolated, and a ligature was placed loosely around it. (A small artery was chosen because occlusion of a large epicardial artery such as the left anterior descending or circumflex artery frequently results in ventricular fibrillation.) The myocardium supplied by this branch was identified by transient occlusion of the vessel. Within seconds of occlusion, a blue discoloration of the myocardium was apparent that rapidly returned to normal after restoration of blood flow. A pair of 2-mm circular ultrasonic crystals was inserted into the identified region to a depth of 2–3 mm, to measure segment-length changes of the myocardium supplied by the isolated vessel. Maximum segment-length changes were obtained by placing the crystals at an oblique angle as described by Gallagher et al. Measurement of diastolic and systolic segment lengths were timed to the positive and negative dp/dt as described by Theroux et al. The changes in segment-length shortening during intervention were compared with the baseline value obtained before occlusion and expressed as a percentage of the baseline value. An electrocardiogram was obtained from the crystals. Left ventricular pressure and segment-length changes were recorded on a Gould 2000 recorder (Gould Inc, Cleveland, Ohio) at paper speeds of 0.5–100 mm/sec. The output from the recorder was analyzed on an IBM-AT computer by an A-D converter (2800A, Data Translation, Marlboro, Massachusetts), using software developed previously. Before coronary occlusion and reperfusion, an intravenous bolus of lidocaine (40 mg) was administered.

**Experimental Protocol**

Four groups of pigs were subjected to 45 minutes of coronary occlusion and 2 hours of reperfusion using the following protocol: 1) infusion with saline during reperfusion; 2) infusion with GSH intravenously during reperfusion; 3) pretreatment with BSO and infusion with saline during reperfusion; and 4) pretreatment with BSO and infusion with GSH during reperfusion. In this protocol, the saline infusion and GSH infusion studies were completed before the BSO studies were undertaken.

**Depletion of Myocardial GSH**

Depletion of myocardial GSH was achieved by intramuscular injections of BSO (Chemalog, South Plainfield, New Jersey), a potent inhibitor of γ-glutamylcysteine synthetase. One gram of BSO was dissolved in sodium chloride and sterilized by passing through a 0.22-μm Millipore filter (Millipore, Bedford, Massachusetts). Half the dose (0.5 g) was given on the evening (12 hrs) before the study, and the other 0.5 g was given 1 hour before the study. Preliminary studies using chronic instrumented pigs in our laboratory revealed that this dose of BSO had no effect on heart rate, coronary blood flow, left ventricular pressure, or myocardial contractility (using the slope of the end-systolic pressure-diameter relation).

**Enhancement of Myocardial GSH**

Enhancement of myocardial GSH was achieved by continuous intravenous administration of GSH, starting 5 minutes before reperfusion and continuously throughout the 2-hour reperfusion period. Glutathione (Sigma Chemical, St. Louis, Missouri) was dissolved in normal saline, and the pH was adjusted to 7.35 with 1.0 N sodium hydroxide. The final GSH concentration was 25 mg/ml. After sterilization by passing through a 0.22 μm Millipore filter, GSH at a dosage of 0.8 g/kg was infused intravenously at a rate of 6.5 mg/kg/min using a Harvard constant infusion pump (Harvard Apparatus). Preliminary studies conducted in our laboratory showed that infusion of GSH at this dosage in chronic instrumented pigs did not alter left ventricular pressure, heart rate, coronary blood flow, or myocardial contractility.

**Quantification of the Myocardial Area at Risk and Infarct Size**

Quantification of the area of myocardium supplied by the occluded artery and myocardial infarct size was done by a modification of the differential stain technique using triphenyltetrazolium chloride (TPT) and Evans blue solution described by Bush et al. At the conclusion of the study, the heart was quickly removed and washed, and the coronary arteries were flushed with normal saline. The ligature used for coronary occlusion was once again tied around the vessel. A small arterial cannula was
carefully inserted into the vessel just distal to the occlusion and firmly secured. Two balloon-tipped catheters were inserted into the right and left coronary ostia, and the balloons were inflated with the tip of the catheters positioned just distal to the ostia. The two catheters were connected to pressurized bags containing Evans blue solution (0.5%). Pressure within the bags was maintained to transmit dye to the coronary ostia at approximately 40 mm Hg, which was confirmed using a mercury manometer. Simultaneously, the catheter in the occluded artery was perfused with TPT (1.5%) in phosphate buffer (10 mM, pH 7.4) at a similar pressure. After perfusion for 15 minutes, the heart was cut from apex to base into slices approximately 0.8 cm thick. With this technique, normally perfused myocardium was identified by the Evans blue. The myocardial area at risk (myocardial bed supplied by the occluded vessel) was stained brick red by TPT, except for infarcted areas that were unable to reduce TPT to red formazan and remained unstained.17 The outlines of normal, ischemic, and infarcted areas of the basal and apical surfaces of the slices were traced on transparent overlays, and the areas were quantified by digital planimetry using commercially available computer software (General Purpose Analysis Systems, Sony, Hackensack, New Jersey). Myocardial infarct size was expressed as a percentage of the area at risk, and area at risk was expressed as a percentage of the left ventricle.

Preparation of Plasma and Tissue for GSH Measurements

Preparation of plasma and myocardial samples for measurement of GSH was performed according to the method described by Puri and Meister.18 Heparinized blood samples (1 ml) were obtained at 5 minutes before occlusion and 30 minutes after occlusion. Additional samples were taken at 30, 60, and 120 minutes of reperfusion. After centrifugation to separate blood from plasma, 0.1 ml of 5% aqueous picric acid (Fisher, Rochester, New York) was added to each 0.4 ml of plasma. The mixture was then centrifuged at 10,000g for 15 minutes at −4°C to precipitate protein. The clear supernate was immediately frozen at −70°C until assay.

Multiple biopsies of myocardial tissue of approximately 200 mg were taken from normal, ischemic, and infarcted areas of the heart slices after differentiation by the stain technique. The tissue was blotted, weighed, and homogenized in 5 vol 1% aqueous picric acid. It was then centrifuged, and the clear supernate was frozen as described previously. Experiments in our laboratory revealed that there was no difference in GSH contents from myocardium obtained before and after the stain.

Measurement of GSH

Measurement of total GSH was done according to the enzymatic method of Tietze.19 The tissue extract was diluted (50–100 times) in phosphate buffer (0.1 M, pH 7.5, containing 5 mM EDTA). The following were combined in a cuvette: 0.2 ml 3 mM DTNB (5,5′-dithio-bis-[2-nitrobenzoic acid]), 0.2 ml NADPH (1 mg/ml), 0.05–0.1 ml sample, 0.05 ml GSH reductase (25 units/ml), and phosphate buffer to a final volume of 1 ml. GSH reductase is added last to initiate the reaction. Absorbance at 412 nm was recorded for 3 minutes at room temperature (22°C). Standard curves were generated by substituting known amounts of GSH and equal amounts of picric acid in the sample. Internal standards, in which known amounts of GSH were added to the sample, were used to ascertain that samples were free of substances known to interfere with GSH reductase activity.

Ultrastructural Assessment

Multiple subepicardial biopsies 3-mm deep were taken from myocardium supplied by the occluded artery from two animals in each group. Biopsies were taken at 45 minutes of occlusion and 2 hours of reperfusion. The biopsies were immediately cut into approximately 1-mm square pieces and fixed in 4% formaldehyde and 1% glutaraldehyde in Millonig’s phosphate buffer, pH 7.3.20 The tissues were post-fixed in 2% OsO₄ in Millonig’s phosphate buffer, dehydrated, and embedded in Polybed 812. Thin sections were stained with uranyl acetate and lead citrate and examined with a Philips EM 300 electron microscope (Philips Corp., Mount Vernon, New York). The sections were examined by a pathologist (C.Y.L.), who was blinded to the treatment groups.

In Vitro Assessment of the Effect of GSH on TPT Reduction by Myocardium

In vitro studies were conducted to determine whether GSH infusion affected the formazan reaction between the TPT stain and myocardial dehydrogenases. The studies were performed as described by Green and Narahara21 with a slight modification. A piece of myocardium, approximately 300 mg, was homogenized using a tissue homogenizer (Tissumizer, Tekmar Corp., Cincinnati, Ohio) in 5 volumes of 10 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA. After centrifugation at 800g for 10 minutes at 4°C, the supernatant was used for the enzyme assay. The basic assay condition consists of 0.05 ml 20 mM TPT, 0.05 ml 10 mM NaN₃, 0.1 ml 0.25 M Na succinate (or GSH), 0.2 ml tissue extract, and 20 mM phosphate buffer, pH 7.5, to a final volume of 0.5 ml. The mixtures were incubated for 60 minutes in a water bath at 37°C. At the end of the incubation, 1.5 ml of 95% ethanol was added to each tube. After mixing thoroughly, they were centrifuged at 2,500g for 10 minutes at 4°C. The supernatants were passed through a 0.22-μm Millipore filter. The absorbance at 458 nm of the clear filtrates was recorded.
Table 1. Hemodynamic Data

<table>
<thead>
<tr>
<th></th>
<th>Preocclusion</th>
<th>30-Minute occlusion</th>
<th>5-Minute reperfusion</th>
<th>30-Minute reperfusion</th>
<th>90-Minute reperfusion</th>
<th>120-Minute reperfusion</th>
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<tr>
<td>Saline infusion (n=6)</td>
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<td>82±4</td>
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<td>81±4</td>
<td>80±3</td>
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<td>9,008±554</td>
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<td>BSO pretreatment (n=5)</td>
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<td>78±3</td>
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<tr>
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<td></td>
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<tr>
<td>HR</td>
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<td>RPP</td>
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<td>11,889±406</td>
<td>11,440±1,229</td>
<td>12,090±1,394</td>
</tr>
</tbody>
</table>

HR, heart rate (beats/min); LVP, left ventricular systolic pressure (mm Hg); RPP, rate-pressure product.

Considerations for Animal Inclusion Into the Study

Six to eight pigs were entered into each study group. If ventricular fibrillation developed, direct current cardioversion using a maximum energy level of 20 J was attempted. Animals were included for analysis if no more than two defibrillation attempts were necessary and if there was prompt restoration of sinus rhythm.

Data Analysis

Time varying data were analyzed for changes by two-way analysis of variance followed by Scheffe's test.22 Comparison of myocardial GSH levels and infarct size were made by the Student's paired and unpaired t test where necessary. Results are expressed as mean±SEM. A p value less than 0.05 was considered significant. This study was approved by the Institutional Animal Care and Use Committee.

Results

Hemodynamic Data of Animals Subjected to Ischemia and Reperfusion

Hemodynamic data were obtained from six saline-infused pigs, five pigs pretreated with BSO, six pigs infused with GSH during reperfusion, and four BSO-pretreated pigs given GSH infusion during reperfusion. Left ventricular pressure, heart rate, and rate-pressure product are presented in Table I. There were no differences between the groups before occlusion and during occlusion or reperfusion.

Plasma GSH

Figure 1 shows the plasma GSH levels in the four groups. Compared with the saline-infused pigs, BSO pretreatment resulted in persistently lower plasma GSH throughout the study period (p<0.05). GSH infusion initiated 5 minutes before reperfusion resulted in an increase in plasma GSH levels by three orders of magnitude during the reperfusion period in both non-BSO- and BSO-pretreated animals.

Myocardial GSH

In saline-infused pigs, the GSH level in normally perfused myocardium was 380±48 µg/g (Figure 2). After 45 minutes of coronary occlusion, there was a decrease in GSH in the ischemic myocardium to 173±38 µg/g (p<0.02). At 2 hours of reperfusion, the GSH level in the ischemic myocardium remained low at 182±36 µg/g. In BSO-pretreated pigs, the GSH level in the normally perfused myocardium was 215±25 µg/g (p<0.01, compared with normally perfused myocardium of saline-infused pigs). After 45 minutes of coronary occlusion the GSH level in the ischemic myocardium was reduced to 74±4 µg/g (p<0.01, compared with ischemic myocardium of saline-infused pigs). At 2 hours of reperfusion, the level in the ischemic myocardium remained low at 77±8 µg/g.

In pigs infused with GSH before and during reperfusion, the GSH level in the normally perfused myocardium was increased to 582±67 µg/g (p<0.05, compared with GSH in normal myocardium of saline-infused pigs). The GSH level in the ischemic myocardium was also increased to 312±80 µg/g (p<0.01, compared with GSH level in ischemic myocardium of saline-infused pigs).

In pigs pretreated with BSO and infused with GSH during reperfusion, no increases in myocardial GSH levels were noted. The levels in the normally perfused and ischemic myocardium were identical to that of BSO-pretreated pigs not infused with GSH.

GSH levels from infarcted tissue were extremely low in all animals (7 µg/g in pigs infused with saline, 26.5 µg/g in BSO-pretreated pigs, 32.5 µg/g in GSH-infused pigs, and 18.4 µg/g in pigs pretreated with BSO and infused with GSH).
Myocardial Infarct Size

There was no significant difference in the area at risk among the four groups of pigs studied (23.3±2%, saline infusion; 19.8±1.8%, BSO pretreatment; 24.5±3.0%, GSH infusion; 21.3±2.5%, GSH infusion into BSO-pretreated pigs).

Myocardial infarct in our pig model tended to be patchy, particularly in the saline-infused and GSH-infused pigs. Myocardial infarct size was 12.5±0.8% of the area at risk in the saline-infused pigs. In pigs pretreated with BSO, infarct size was significantly increased to 30.4±4% (p<0.001). Pigs infused with GSH exhibited significantly smaller infarct sizes (7.5±1.3% p<0.05, compared with those infused with saline). Infarct size remained increased at 29.3±6.3% in BSO-pretreated pigs infused with GSH during reperfusion.

Figure 3 shows the relation between myocardial infarct size and GSH levels in the ischemic myocardium. An inverse relation is evident; low myocardial GSH was associated with a large myocardial infarct. Ischemic myocardium enhanced with GSH during reperfusion was associated with a reduction in the size of myocardial infarct.

Effect of GSH on Triphenyltetrazolium Reduction by Myocardium

The TPT–staining technique is a histochemical stain of the dehydrogenase activities in which TPT is reduced to formazan. To determine whether cellular levels of GSH affected the TPT reaction and, thus, affected measurements of myocardial infarct size within the area at risk, we studied the effect of GSH on reduction of TPT by myocardial dehydrogenases. Schaper and Schaper17 have reported that succinate dehydrogenase and NAD-dependent dehydrogenases of the myocardium play important roles in the reduction of TPT. As shown in Table 2, GSH alone or in the presence of NAD did not cause reduction of TPT by myocardial extract. In contrast, in the presence of succinate, TPT was reduced by myocardial extract consistent with the observation of Schaper and Schaper.17 However, GSH did not affect TPT reduction by myocardial succinate dehydrogenase. Thus, the

![Graph showing effect of BSO pretreatment and GSH infusion on plasma GSH levels. Blood samples were taken at 5 minutes before occlusion, 30 minutes of occlusion, and 30, 60, and 120 minutes of reperfusion. After extraction with picric acid, plasma GSH was determined. BSO pretreatment resulted in significant reduction in plasma GSH (p<0.05 compared with saline-infused animals) at each sampling time. GSH infusion resulted in large increase (three orders of magnitude) in plasma GSH levels. Increase was also seen on infusion of GSH into BSO-pretreated animals. Note that in animals infused with GSH, 30-minute occlusion and 30-minute reperfusion points were connected by dotted lines because GSH infusion was given 5 minutes before reperfusion and first blood sample was not obtained until 30 minutes after reperfusion.](http://circ.ahajournals.org/doi/abs/10.1161/01.CIR.89.6.1792?journalCode=circ)

**Figure 1.** Graph showing effect of BSO pretreatment and GSH infusion on plasma GSH levels. Blood samples were taken at 5 minutes before occlusion, 30 minutes of occlusion, and 30, 60, and 120 minutes of reperfusion. After extraction with picric acid, plasma GSH was determined. BSO pretreatment resulted in significant reduction in plasma GSH (p<0.05 compared with saline-infused animals) at each sampling time. GSH infusion resulted in large increase (three orders of magnitude) in plasma GSH levels. Increase was also seen on infusion of GSH into BSO-pretreated animals. Note that in animals infused with GSH, 30-minute occlusion and 30-minute reperfusion points were connected by dotted lines because GSH infusion was given 5 minutes before reperfusion and first blood sample was not obtained until 30 minutes after reperfusion.

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observed differences in infarct size could not be due to the effect of GSH on the TPT stain.

**Ultrastructural Evaluation**

Table 3 summarizes the myocardial ultrastructural changes due to coronary occlusion and reperfusion. Biopsies taken at 45 minutes of coronary artery occlusion from the subepicardium of pigs infused with saline or pretreated with BSO revealed occasional contraction bands of the myofibrils and mild intracellular edema. The mitochondria showed slight reduction of matrix density. At 2 hours of reperfusion, the ultrastructural changes were similar in saline-infused pigs except that occasional swelling of the mitochondrial cristae was present (Figure 4b, as compared with Figure 4a, normal myocardium). This was also seen in pigs infused with GSH during reperfusion. In contrast, in pigs pretreated with BSO and subjected to occlusion and reperfusion, marked abnormalities were present (Figures 4c and 4d). Nuclear swelling or shrinkage was seen. The myocardial cells were edematous and there was distortion and disorganization of the sarcomeres. There was destruction of mitochondrial cristae and membranes, and extreme clearing of mitochondrial matrix with inclusion of large dense mitochondrial granules. Glycogen was absent. The changes were identical in BSO-pretreated pigs that were infused with GSH during reperfusion.

**Recovery of Contractile Function**

Coronary occlusion resulted in dyskinesia (paradoxical motion) of the ischemic myocardium in all pigs (Figure 5). In pigs infused with saline, no return of contractile function of the ischemic myocardium occurred during the initial 30 minutes of reperfusion. At 2 hours of reperfusion, there was return to approximately 60% of the preocclusion value. In pigs pretreated with BSO, no recovery of function was observed during the 2-hour reperfusion period even though the severity of dyskinesia was less than that of saline-infused pigs. In pigs infused with GSH, there was an enhanced rate of recovery of contractile function with segment-length shortening being observed within 5 minutes of reperfusion. At 2 hours of reperfusion, the extent of recovery was the same as that of saline-infused pigs. In BSO-pretreated pigs infused with GSH during reperfusion, no enhancement of functional recovery was noted.

**Discussion**

The results of this study demonstrate that myocardial glutathione plays an important role in protecting the ischemic myocardium against reperfusion injury. There was an inverse relation between the myocardial GSH content and the extent of myocardial injury sustained during reperfusion.

Considerable evidence implicates reactive oxygen species generated during reperfusion as important in the genesis of reperfusion injury.1,5,6 The origin of these reactive oxygen species remains incompletely understood but appears to arise from sources both intrinsic and extrinsic to the myocardium. The major intrinsic source consists of the xanthine oxidase pathway,6,23 whereas activated neutrophils have been implicated as the important extrinsic source.24–26 Similar to cells of other organs, myocytes are equipped with antioxidant enzyme systems that offer protection against damage by reactive oxygen species. These antioxidant systems include superoxide dismutase, catalase, and the GSH redox system.23 Recent evidence suggests that ischemia results in a considerable reduction in these intracellular oxidant scavenging agents,7 thereby providing the milieu for increased oxidant injury during reperfusion. In this study, we focused on the role of GSH in protecting against myocardial reperfusion injury and examined the effects of augmented GSH as an approach to maintaining intracellular antioxidant defenses.

The effectiveness of GSH as an antioxidant is a result of its ability to donate hydrogen atoms from its thiol group to most carbon-, oxygen-, and

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**Table 2. Effect of GSH on the Reduction of Triphenyltetrazolium by Myocardium**

<table>
<thead>
<tr>
<th>Tissue extract</th>
<th>TPT reduction (absorbance at 458 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue extract alone (control)</td>
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<tr>
<td>Plus GSH 5 mM</td>
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<tr>
<td>Plus GSH 20 mM</td>
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<tr>
<td>Plus GSH 50 mM</td>
<td>0.037</td>
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<tr>
<td>Plus NAD (1 mg/ml) and GSH 50 mM</td>
<td>0.021</td>
</tr>
<tr>
<td>Plus succinate 50 mM</td>
<td>0.124</td>
</tr>
<tr>
<td>Plus succinate 50 mM plus GSH 50 mM</td>
<td>0.128</td>
</tr>
</tbody>
</table>

Results are the mean of two experiments.
TABLE 3. Ultrastructural Changes During Ischemia and Reperfusion and the Effects of BSO and GSH Treatments

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Matrix density reduction</th>
<th>Swollen cristae</th>
<th>Broken membrane or cristae</th>
<th>Dense granules</th>
<th>Intracellular edema</th>
<th>Myofilaments</th>
<th>Glycogen</th>
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</thead>
<tbody>
<tr>
<td>45 min of coronary occlusion</td>
<td>*</td>
<td>†</td>
<td>†</td>
<td>Absent</td>
<td>†</td>
<td>Focal contraction bands</td>
<td>Present</td>
</tr>
<tr>
<td>45 min of coronary occlusion plus 2 hr of reperfusion</td>
<td>*</td>
<td>*</td>
<td>†</td>
<td>Absent</td>
<td>†</td>
<td>Focal contraction bands</td>
<td>Present</td>
</tr>
<tr>
<td>BSO pretreatment and 45 min of coronary occlusion</td>
<td>*</td>
<td>†</td>
<td>†</td>
<td>Absent</td>
<td>†</td>
<td>Focal contraction bands</td>
<td>Present</td>
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<tr>
<td>BSO pretreatment and 45 min of coronary occlusion plus 2 hr of reperfusion</td>
<td>‡</td>
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<td>Present</td>
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<td>Marked distortion</td>
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<tr>
<td>45 min of coronary occlusion plus 2 hr of reperfusion with GSH infusion</td>
<td>*</td>
<td>†</td>
<td>†</td>
<td>Absent</td>
<td>†</td>
<td>Focal contraction bands</td>
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<tr>
<td>BSO pretreatment and 45 min of coronary occlusion plus 2 hr of reperfusion with GSH infusion</td>
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<td>‡</td>
<td>Present</td>
<td>‡</td>
<td>Marked distortion</td>
<td>Absent</td>
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* slight; † absent; ‡ severe.

nitrogen-centered radicals. Hydrogen peroxide is removed by GSH, a reaction catalyzed by GSH peroxidase, a selenium-dependent enzyme. The oxidized glutathione (GSSG) is reduced back to GSH by GSH reductase, a reaction using NADPH as a cofactor. In addition, GSH is capable of reducing lipid membrane damage by inhibition of lipid peroxidation and might also be effective against such highly reactive species as the hydroxyl radical and singlet oxygen.

The influence of ischemia on myocardial GSH content has been recently investigated. Ischemia results in depletion of myocardial GSH content. Further depletion has been observed during reperfusion. In our study, a precipitous decrease in GSH was observed during ischemia without further changes during reperfusion. The decrease in GSH during ischemia and reperfusion is thought to be due to a combination of increased metabolic use and leakage of GSH from the myocardium. Ferrari et al suggested that this depletion of myocardial GSH, along with other antioxidants during ischemia, contributes to myocardial reperfusion injury. Our results support this concept. There was a noticeable increase in damage to reperfused myocardium with very low GSH induced by BSO pretreatment. The myocardial infarct size was significantly increased and there were severe subepicardial ultrastructural abnormalities. These findings were absent in reperfused myocardium with higher GSH levels. Further, the ultrastructural abnormalities were not evident before reperfusion in ischemic myocardium with very low GSH content. These findings support the concept of injury to viable myocardium during reperfusion. Moreover, they strongly suggest that the extent of injury depends on the level to which myocardial GSH is depleted.

Because depletion of myocardial GSH during ischemia promotes injury during reperfusion, we examined whether augmentation of myocardial GSH during reperfusion would be beneficial. Recently we have shown that cultured endothelial cell GSH content could be enhanced by exogenous GSH. The plasma half-life of GSH after a bolus injection is extremely short. Therefore, we decided to administer GSH by continuous intravenous infusion attempting to enhance myocardial GSH content. Intravenous infusion of a high concentration of GSH before and during the entire reperfusion period was associated with an increase in GSH content both in the normally perfused and ischemic reperfused myocardium. The possibility that this increase was a contaminant from high plasma GSH levels was excluded by the following reasonings: First, before biopsy, the coronary circulation was flushed with saline to remove any blood present in the coronary vessels. Second, very low levels of GSH were present in infarcted tissue even in pigs infused with GSH. Finally, in pigs pretreated with BSO and infused with GSH, the myocardial GSH content remained low. It is possible that flushing the coronary arteries with saline might not completely remove GSH present in the interstitial space, especially in the less injured myocardium where brief saline exchange with the interstitial fluid might have limited washout. This might account for the higher myocardial GSH content in GSH-infused pigs. However, this is unlikely because the GSH contents of normal and ischemic myocardium of BSO-pretreated and GSH-infused pigs were similar to those of BSO-pretreated pigs. In this study, we have reported the myocardial GSH contents in μg GSH per gram of myocardial wet weight. Because the ischemic and infarcted myocardium is likely to be edematous, the true GSH concentrations in these tissues might be higher than reported here.

The results of this study demonstrated that enhanced myocardial GSH by GSH infusion is
beneficial in protecting against reperfusion injury. Myocardial infarct size was significantly smaller compared with that of animals with lower myocardial GSH. The possibility that the histochemical reaction used to evaluate infarct size was affected by the high glutathione content was examined. Our in vitro studies demonstrated that the formazan reaction between TPT and myocardial dehydrogenases was unaffected by the addition of GSH.

Further evidence suggesting a relation between myocardial reperfusion injury and myocardial GSH content is provided by the differences in functional recovery of the posts ischemic myocardium. Recent evidence has implicated oxidant injury in the genesis of posts ischemic myocardial dysfunction.\textsuperscript{32-34} In this study, there was no recovery of function when myocardial GSH content was low, whereas there was a faster rate of recovery when GSH content was increased. It could be argued that functional recovery was influenced by differences in infarct size in the experimental groups. Similar results have been observed by others, however, when only reversible injury was produced. Using isolated heart preparations, Ceconi et al\textsuperscript{35} have shown that

![Figure 4](image-url)

**Figure 4.** Microphotographs of effect of ischemia and reperfusion on subepicardial ultrastructure. (a) Subepicardium of contralateral, normally perfused myocardium of saline-infused pigs and (b) ischemic reperfused subepicardium of saline infused pigs. There is intracellular edema and mitochondria are swollen (c) in ischemic reperfused myocardium of pigs pretreated with BSO. There is striking intracellular edema and myofilaments exhibit prominent contraction bands. There is severe edema of mitochondria with clearing of matrix and disruption of cristae. Glycogen is absent (d), higher magnification of (c). Swollen mitochondria show dissolution of membranes, disruption of cristae, and presence of dense granules. Each bar represents 1 \mu m.

![Figure 5](image-url)

**Figure 5.** Graph of functional recovery of ischemic myocardium. Coronary occlusion is associated with paradoxical motion (dyskinesia) in all cases. In BSO-pretreated animals, an absence of functional recovery is observed. GSH infusion hastens rate of recovery compared with control animals. GSH infusion has no effect on functional recovery in BSO-pretreated animals.
enhancement of myocardial GSH with N-acetylcysteine was beneficial to functional recovery after a short period of global ischemia. Foreman et al. who infused N-acetylcysteine into dogs during coronary occlusion and on reperfusion, noted an improvement in regional myocardial dysfunction even though there was no change in the GSH content of the ischemic myocardium. By contrast, Chatham et al. have shown that depletion of myocardial GSH in rats given intraperitoneal BSO resulted in a depression of functional recovery.

In this study, neither the BSO pretreatment nor GSH infusion had any effect on heart rate or left ventricular systolic pressure that could alter myocardial oxygen demand. Preliminary studies in our laboratory showed that neither intervention altered myocardial contractility. Although we did not measure coronary collateral blood flow, pigs have been shown to have virtually no collateral blood supply. In addition, the beneficial effects observed from GSH infusion cannot be attributed to possible extracellular scavenging effects of high plasma GSH levels because infarct size, ultrastructural changes, and recovery of function were identical in BSO-pretreated pigs infused with GSH and those given only BSO.

There are several possible limitations of this study. First, left ventricular systolic pressure was lower than that observed in chronic swine preparations in our laboratory. This could be due either to prolonged ethane anesthesia or to prolonged exposure of the heart, or to a combination of both. The extent to which these conditions influenced other measurements in this study is unclear. Second, myocardial infarct size in saline-infused pigs was much smaller than that observed by others after a similar period of coronary occlusion. Most previous studies evaluating infarct size in pigs used a major epicardial artery such as the left anterior descending coronary artery or the proximal portion of the circumflex artery, which results in rapid onset of transmural ischemia and a large area of infarction. Our attempts to use a large artery in the open-chest preparation invariably resulted in irreversible ventricular fibrillation. This prompted the use of a marginal branch of the circumflex artery, which is smaller and produces ventricular fibrillation less frequently. The area of myocardium supplied by this vessel was comparatively small. It is likely that the small infarct was the result of a small area at risk. However, this variable was constant among all treatment groups. Third, the myocardial infarct size was determined by the tetrazolium stain after a 2-hour reperfusion. Recent evidence suggests that early measurements of infarct size using this technique might be affected by interaction with agents such as superoxide dismutase, leading to an erroneous conclusion of infarct size reduction. In addition, washout of dehydrogenases, which are necessary for the reduction of tetrazolium, might not have been completed at 2 hours of reperfusion. Therefore, whether the results presented in this study using a 2-hour reperfusion actually reflect the ultimate infarct size after prolonged recovery requires further investigation.

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References
42. Downey JM, Shirato G, Miura T, Toyofuku T: Tetrazolium is unreliable as an index of drug-induced salvage (abstract). J Mol Cell Cardiol 1988;S70
43. Shirato C, Miura T, Downey JM: Superoxide dismutase (single dose) delays rather than prevents necrosis in reperfused rabbit heart. FASEB J 1988;A918

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A Singh, K J Lee, C Y Lee, R D Goldfarb and M F Tsan

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