Effects of perfluorooctylbromide emulsions on myocardial function ATP levels and the antioxidant defense system in an in vitro rat model of cardioplegia-induced ischemia/reperfusion injury

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THE EFFECTS OF PERFLUOROOCTYLBROMIDE EMULSIONS ON MYOCARDIAL FUNCTION, ATP LEVELS AND THE ANTIOXIDANT DEFENSE SYSTEM IN AN IN VITRO RAT MODEL OF CARDIOPLEGIA-INDUCED ISCHEMIA/REPERFUSION INJURY.

By

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Protective effects of perfluorooctylbromide emulsions on myocardial ischemia and reperfusion (MI/R) injury were evaluated in a modified Langendorff rat heart preparation. Isolated rat hearts were equilibrated in Krebs-Henseleit solution (KH) for 35 minutes and perfused with cardioplegic solution (CPS) for 3 minutes. Control hearts were bathed in the CPS. Treated hearts were given a single infusion of 100%, 50% or 25% PFOB emulsified in CPS and then bathed in the emulsions. All treatment groups were subjected to 5 minutes of ischemia. Following reperfusion with KH solution, hearts subjected to 100% or 50% PFOB emulsions showed improved recovery of left ventricular function. The 25% PFOB emulsion did not differ from the control. Tissue activities of the antioxidant enzymes glutathione peroxidase, superoxide dismutase, and catalase were not changed by MI/R injury in this model. A 100% PFOB emulsion produced no effect on these enzymes. Levels of ATP and reduced and oxidized glutathione (GSH and GSSG respectively) were also determined in preischemic hearts and after ischemia and reperfusion in both control and 100% PFOB treated hearts. No differences were noted in GSSG levels. However, cardioplegia-induced MI/R injury decreased myocardial ATP and GSH levels. The 100% PFOB emulsion prevented the decline in these substances.

These data suggest that PFOB emulsions protect the myocardium against injury associated with cardioplegia and reperfusion.
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INTRODUCTION

1. Myocardial Ischemia and Reperfusion Injury

The heart is dependent on aerobic metabolism for energy production. Ischemia occurs when coronary flow is insufficient to supply the necessary oxygen for oxidative phosphorylation and to remove cellular metabolites (Jennings 1970). Restoration of the coronary arterial flow after a variable period of ischemia is called reperfusion. Myocardial ischemia and reperfusion (MI/R) injury is produced during ischemia and exacerbated during the reperfusion period (Jennings et al 1985, Gettes et al 1991 and Rao et al 1983).

Three levels of MI/R injury are currently recognized (Downey 1990). The first level of injury produces arrhythmias generated upon reperfusion after an ischemic period of a few minutes (Bernier et al 1986). The second level develops after 5 to 15 minutes of ischemia followed by reperfusion and is characterized by a prolonged decrease in contractility. This state is also referred to as myocardial stunning. Generally, the heart will completely recover after several days (Bolli 1988, Farber et al 1988, Przyklenk et al 1986). The most severe type occurs when the ischemic period is extended to 20 minute or longer. Some of the heart cells will be irreversibly injured and necrotic (Grover et al 1990, Maxwell et al 1989). The third level of injury,
infarction, has attracted the greatest attention because infarcted tissue contributes to mortality and morbidity in the clinic. A lot of research about MI/R has been devoted to establishing the mechanisms involved in the development of irreversible tissue injury. Protective effects of different kinds of substances have been evaluated mainly in models of irreversible MI/R injury (Maxwell et al 1989, Grover et al 1990, Teoh et al 1990).

A number of different experimental models have been employed in such evaluations. These include: (a) regional or global ischemia following coronary artery occlusion or clamping the aorta in vivo (Maxwell et al 1989, Godin et al 1980, Teoh et al 1990), (b) total or low flow ischemia in isolated perfused hearts or septa (Ferrari et al 1985, Grover et al 1990, Downey et al 1987), (c) cardioplegia in experimental animals (Teoh et al 1990, Jones et al 1982). Experiments have also been done in other models such as a cardiac myocyte preparation (Goldhaber et al 1989, Cheung et al 1984).

2. Mechanisms of MI/R Injury

Contractile function of myocardium depends on the hydrolysis of high energy phosphates. The absence of sufficient arterial flow causes a rapid lowering of myocardial PO$_2$ and a loss of aerobic metabolism (Gettes 1986). The maximum rate of ATP production from anaerobic
glycolysis is less than 10% of the rate of oxidative phosphorylation necessary to sustain cardiac function (Kobayashi et al 1979). Insufficient substrate delivery leads to rapid depletion of high energy phosphates. Myocardial contraction will stop in less than one minute after global ischemia (Reimer et al 1986).

Excessive intracellular Ca$^{2+}$ has been implicated as a primary event in irreversible MI/R injury and cell necrosis (Farber 1982). The mechanisms involved in the accumulation are very complex. Depletion of ATP causes increased intracellular Na$^+$. The rise of intracellular Na$^+$ is thought to increase intracellular Ca$^{2+}$ via the Na/Ca exchange mechanism (Reuter and Scholz 1968). The increased Ca$^{2+}$ release and decreased re-uptake by the sarcoplasmic reticulum is also thought to elevate the Ca$^{2+}$ inside the cell (Gettes et al 1991). The third mechanism results from the reduced activity of sarcolemmal Ca$^{2+}$ ATPase which leads to a reduction of Ca$^{2+}$ efflux and net Ca$^{2+}$ uptake. During reperfusion, the enhanced influx of Ca$^{2+}$ through Ca$^{2+}$ channels may also contribute to the large increase of intracellular Ca$^{2+}$ (Tani and Neely 1988). In addition, oxygen free radicals produced primarily upon reperfusion may be involved in Ca$^{2+}$ overloading. Lipid peroxidation of cellular structures by these free radicals can exacerbate the above mentioned ionic perturbations (Hearse et al 1988). The raised cytosolic Ca$^{2+}$ in the prolonged ischemic and
reperfused myocardium results in an irreversible increase in resting tension (contracture) forming rigor complexes (Lowe et al 1979). Shortening of the myofibril associated with contracture-rigor may be due to the loss of structural integrity in lethally injured myocytes (Ganote 1983). Moreover, the contracture-rigor may compress blood vessels and thus restrict reperfusion capacity (Gavin et al 1978).

The lack of venous washout secondary to the diminution of arterial flow causes several extracellular ionic and metabolic changes, such as decreased pH and accumulation of lactate, catecholamines and lysophosphatides (Gettes et al 1991). Some of the alterations have been postulated to disrupt membrane protein function and to change cellular electrophysiology (Corrie and Dobmeyer 1989).

Myocardial edema is another consequence of this myocardial deterioration (Goto et al 1991). Edema results from the increased intracellular concentrations of osmotic particles such as $\text{H}_2\text{PO}_4^-$, lactate and $\text{H}^+$ produced during ischemia. Enhanced tissue osmolality leads to cellular swelling and eventually to membrane disruption.

Oxygen-derived free radicals generated during myocardial ischemia and reperfusion are also responsible for MI/R injury. These radicals are capable of various toxic activities such as inactivation of sulfhydryl enzymes, crosslinking of proteins, DNA breakdown, and lipid peroxidation which causes membrane disruption (Freeman and

Free radicals are compounds that have unpaired electrons in their outer shell. This unpaired state makes them very unstable and prone to react with other molecules to either gain or lose electrons (Downey 1990). Oxygen-derived free radicals include the superoxide anion, hydroxyl radical and hydroperoxyl radical. Hydrogen peroxide, which although technically not a radical, is generally included in this list because it is the most common precursor of the hydroxyl radical. The hydroxyl radical is proposed to be the most reactive of these compounds. It has a short half-life and usually will react within 1 to 5 molecular diameters of its site of formation (Pryor 1986). The primary reactant that serves as the ultimate source for each of the oxidants is superoxide. Superoxide can be easily changed to hydrogen peroxide by superoxide dismutase (SOD). The hydroperoxyl radical is formed in the biological system by protonation of superoxide (Bielski et al. 1983).

Several possible sources of oxyradicals exist:

(A). Xanthine oxidase. Hypoxanthine release from the ischemic heart as a consequence of increased ATP degradation is oxidized by xanthine oxidase, generating superoxide and hydrogen peroxide. An additional important feature of this
hypothesis is the conversion of xanthine dehydrogenase to xanthine oxidase through proteolysis caused by ischemia (Brown et al 1988, McCord 1985, Chambers et al 1985). The enzyme is thought to exist primarily as a dehydrogenase form in normal myocardium and does not generate free radicals.

While ischemic rat and dog hearts have abundant xanthine oxidase, the rabbit and human hearts contain minute amounts of the enzyme (Downey et al 1987, Godin and Bhimji 1987, Muxfeldt and Schaper 1987). This limits the usefulness of xanthine oxidase inhibitors, such as allopurinol, in MI/R injury.

(B). Dissociation of the intramitochondrial electron transport chain, also called mitochondrial "leak" (Turrens and Boveris 1980, Shlafer et al 1987). This is caused by altered phospholipid integrity which inactivates cytochrome oxidase, resulting in release of superoxide radicals.


(D). Activation of Phospholipase A\textsubscript{2} by calcium influx. This initiates production of a fatty acid, arachidonic acid (AA), from cell membrane phospholipids. Major metabolites of AA include prostaglandins. Biosynthesis of prostaglandins from AA can generate radicals (Kontos et al 1985, Mathews and Holde 1990).
(E). Leukocyte mediated free radical generation (Lucchesi 1986 and 1990). Leukocytes were found in infarcted myocardium several decades ago (Mallory et al 1939, Fishbern et al 1978). Recent studies have shown that leukocytes are activated and accumulate in ischemic and nonischemic areas (Mullane et al 1984 Pinckard et al 1980). After the onset of ischemia, the initial damage of the myocardium activates the complement system, which in turn, promotes neutrophil activation and infiltration (McManus et al 1983). Once activated, leukocytes can release a variety of mediators that are bacteriocidal and potentially toxic to tissue. Among these mediators are the free radicals.

Another important neutrophilic enzyme is myeloperoxidase. Its reaction with $H_2O_2$ can oxidize various halides, like $Cl^-$, to produce toxic products such as $HOCl$ (Harrison and Schulz 1976). Other mediators released from leukocytes include platelet-activating factor (Lepran and Lefter 1985), arachidonic acid, arachidonic acid metabolites and lysosomal enzymes.

(F). Ferrous and ferric ion. Iron ions catalyze the Fenton reaction that converts hydrogen peroxide to hydroxyl radical; in phosphate buffered media at neutral pH these ferric and ferrous ions undergo cyclic oxidation-reduction reactions which are coupled with conversion of oxygen into superoxide (Cohen and Sinet 1980). Contaminant iron in the buffer can account for this radical generation in
experimental models in vitro. In vivo the free iron in the blood can also undergo this auto-oxidation. Furthermore, free iron may be released by superoxide from iron-binding proteins during MI/R (Thomas et al 1985).

Although the above mentioned mechanisms involved in free radical generation begin during ischemia, the greatest generation of free radicals occurs during reperfusion (Werns et al 1985, Downey 1990).

An additional mechanism that may contributes to MI/R injury is the decreased defense against free radicals in cells following ischemic damage (Godin et al 1989, Ferrari et al 1985, Julicher et al 1984). Free radicals are produced by normal physiological processes. The biosystem has natural defense mechanisms to protect cells. Endogenous antioxidant molecules such as glutathione (GSH), vitamin E, ascorbic acid, and cysteine prevent oxidative damage (Cotgreave et al 1988). This defense also includes the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPD), cytochrome C peroxidase, ascorbate peroxidase, and GSH transferases. SOD catalyzes a dismutation in which two superoxides are transformed into one \( \text{H}_2\text{O}_2 \) and one \( \text{O}_2 \). CAT converts \( \text{H}_2\text{O}_2 \) to \( \text{O}_2 \) and \( \text{H}_2\text{O} \) while the GPD reduces \( \text{H}_2\text{O}_2 \) to \( \text{H}_2\text{O} \) coincident with the oxidation of glutathione (GSH) to glutathione disulfide (GSSG) (Mathews and Holde 1990 pp. 534-535).
Mechanisms that contribute to MI/R injury do not work independently. Many interrelationships exist between them and generally they enhance each other.

Along with the discoveries about the mechanisms involved in the MI/R injury, a variety of therapeutic interventions have been developed. Antioxidant enzymes such as SOD and CAT and free radical scavengers such as α-tocopherol were demonstrated to reduce the damage in different experimental models (Guarnieri et al. 1978, Bolli et al. 1989, Shlafer et al. 1982). Exposure to endotoxin and hyperthermic stress can induce synthesis of endogenous tissue antioxidants and can attenuate the injury (Bensard et al. 1990, Currie et al. 1988). Calcium antagonists have protective effects against MI/R damage by reducing the Ca\(^{2+}\) overloading (Ferrari et al. 1991). Werns et al. (1988) found that inactivators of neutrophils have the ability to reduce myocardial necrosis. The iron chelator desferrioxamine limits MI/R in some experimental models (Liu et al. 1990). Enhanced oxygen delivery by perfluorocarbons during ischemia and cardioplegia is protective in MI/R injury models (Tomera et al. 1982).

3. **Cardioplegia**

In order to expedite cardiac surgery and transplantation, the heart is arrested with cardioplegic solutions (CPS) which normally contain high potassium
concentrations to stop the heart in diastole. The heart is ischemic during the cardioplegic period and variable MI/R injury occurs (Hearse et al 1981, pp.5-6).

The earliest open-heart surgery primarily employed moderate or profound hypothermia to reduce cardiac damage (Bigelow et al 1950,1954; Drew and Anderson 1959). The severe time limitation and difficulty in maintaining hypothermia led to the development of other methods of myocardial protection. In addition, some surgeons used cardiopulmonary bypass with a pump oxygenator, keeping the coronary arteries perfused and the heart beating which obviously intensified the difficulties of the surgery (Isom et al 1973).

Melrose and colleagues (1955) introduced the concept of "elective cardiac arrest" or cardioplegia. After occluding the aorta, they rapidly injected a 2.5% solution of potassium citrate in whole blood into the aortic root to arrest the heart. However, the Melrose technique was later abandoned because of myocardial injury associated with potassium citrate (McFarland et al 1960). Subsequently, methods such as coronary perfusion, intermittent aortic occlusion, topical hypothermia and ischemia at normal temperature were employed by different groups around the world (Hearse et al 1981, pp.7-12).

The need to protect the myocardium and at the same time to maintain a clear operating field for surgery preserved
interest in cardioplegia. This led to a better understanding of the factors which are necessary to safely induce chemical arrest while preserving cell structure and function. Actually the reported effects of potassium citrate may have been due to tissue ischemia. Gay and Ebert (1973), Holscher (1967) and Tyers et al (1975) suggested that the high concentrations of potassium and citrate were responsible for much of the damage. CPS of the 1960's and 1970's contained components which differed from the initial formula (2.5% potassium citrate) and also differed from each other. Hearse (1974, 1975 and 1976) tested experimentally the individual components of these solutions on the isolated rat heart in the Rayne Institute at St Thomas' Hospital in London. He advocated that CPS should retain as closely as possible extracellular, rather than intracellular, concentrations of ions. He also recommended that additions which were individually effective would be used only in their optimal concentrations. The "St Thomas Solution" he proposed based on this principle contained physiological concentrations of sodium and calcium to which were added 16 mmoles/L KCl to arrest the heart instantly, 16 mmoles/L MgCl which had been shown to have a marked additive effect to the potassium for myocardial protection, and 1 m mole/L procaine hydrochloride. This is the formula of the St. Thomas' Hospital Cardioplegia Solution No.1 which was used continuously between 1975 and 1981 (Hearse et al 1981,
pp.12-14). Worldwide clinical experiences with this or other very similar solutions indicated that they induced cardiac arrest rapidly and, in combination with topical hypothermia, afforded excellent myocardial preservation (Brambridge et al 1977, Conti et al 1978, Cooley 1979, Tyers et al 1977). In 1981, this formulation was modified to include buffering and pH control with a small reduction in sodium and calcium (Hearse et al 1981, pp.159). This modified solution is now called St. Thomas' Hospital Cardioplegia Solution No.2 and is the most widely used CPS in the United States and Europe (Ledingham et al 1990).

The overall protection afforded by cold chemical cardioplegia is contributed by three important components: energy conservation through the rapid chemical induction of cardiac arrest, the slowing of metabolism and degenerative processes by hypothermia, and the prevention of unfavorable cellular changes by the inclusion of specific protective agents in the cardioplegic solution (Hearse et al 1981, pp.151).

Chemical induction of cardiac arrest is most commonly produced by mild hyperkalemia (Melrose et al 1955). Within its optimal concentration range (between 10 and 30 meq/L), potassium very effectively arrests the heart in diastole with few side effects on tissue (Tyers et al 1977, Buckberg 1979). Substantial depletion of calcium and sodium can also induce cardiac arrest (Gebhard et al 1983). However, they
also produce harmful effects on the cells (Gordon et al 1978, Sachror et al 1979, Jynge 1980b) and it would appear simpler to produce cardioplegia with solutions like the one proposed by Hearse (1976).

The second component is hypothermia caused by local cooling. Numerous experimental studies have shown that hypothermia and chemical cardioplegia complement each other and together ensure additive tissue protection (Hearse et al 1980, Jynge 1980a, Preusse et al 1979).

When the optimally formulated CPS is used during cardioplegia, myocardial deterioration is primarily due to MI/R injury. With greater understanding about the mechanisms involved in the MI/R damage, it should be possible to prevent or at least delay some of these critical changes using carefully designed interventions. Several protective interventions have been incorporated experimentally and/or clinically in cardioplegic procedures. These possibilities include the following: (a) Adding glucose and insulin or intermediates plus alternate substrates of oxidative metabolism to stimulate the anaerobic energy production (Lolley et al 1974, Teoh et al 1990, Rousou et al 1986). (b) Supplying of ATP and creatine phosphate (Hearse et al 1976). (c) Buffering with bicarbonate to combat acidosis of anaerobic glycolysis during ischemia (Jynge et al 1981, Buckberg 1979).

Experimental evidence suggests that the optimum pH during
cardioplegia is alkaline (7.9-8.1). (d) Providing mannitol or other agents to counter cell swelling (Goto et al 1991). (e) Including ion channel blockers such as the calcium channel blockers, verapamil and nifedipine (Rodd-Nicholson et al 1978, Clark et al 1977), and beta blockers (Magee et al 1979). (f) Adding several anaesthetic agents such as procaine (Kay et al 1978). In addition to inducing electromechanical arrest, procaine is thought to stabilize cell membranes. (g) Inclusion of free radical scavengers in CPS (Bando et al 1988). (h) Inclusion of oxygen to maintain aerobic energy production. At the present time both oxygenated crystalloid CPS and blood have been used clinically to minimize the oxygen debt arising during ischemic arrest (Bleese et al 1978, Buckberg 1979). The blood cardioplegic technique uses the blood as a vehicle of cardioplegic agents and oxygen. In 1978, Follette et al reported their findings on blood cardioplegia in animals and humans. They showed that mixing cool cardioplegic solution with autologous blood allowed prolonged aortic clamping to be accomplished in the animal experiments and resulted in postoperative recovery in patients compared with unmodified blood. Other groups compared the blood cardioplegia with crystalloidal cardioplegia in laboratory experiments and clinical trials (Engelman et al 1980, Feindel et al 1984, Fremes 1984). Blood cardioplegia provided better myocardial protection than did crystalloidal cardioplegia. As a kind of
oxygen carrying vehicle, perfluorocarbons have been employed as cardioplegic adjuvants and have improved recovery of cardiac function (Flaherty et al. 1984, Rousou et al. 1982, Hicks et al. 1983, Kanter 1981). PFCs have increased oxygen solubility at low temperature (Naito and Yokoyama 1978). After Magovern et al. (1982) indicated that blood failed to deliver significant amounts of oxygen to protect myocardium at lower temperatures, perfluorocompounds as a means of oxygenating crystalloid cardioplegic solutions became more appealing. Novick et al. (1985) compared the protective effect of crystalloid, blood and Fluosol-DA cardioplegia on hypertrophied pig myocardium. They concluded that Fluosol-DA cardioplegia was the most effective.

4. Perfluorocarbons

Perfluorocarbons (PFCs) are predominantly comprised of carbon and fluorine. They may contain other atoms such as nitrogen, oxygen and bromide (Yokoyama et al. 1983). PFCs are chemically inert, insoluble in other materials and resistant to thermal and radiation damage. They are good solvents for gases. Approximately 40 ml of oxygen can dissolve in 100 ml of most PFCs and CO$_2$ is at least twice as soluble as oxygen (Sargent and Seffle 1970). These properties of PFCs make them potentially useful for a variety of biological purposes, such as gas transport. One well-known application is as a blood substitute. PFCs may
be gases, liquids or solids. For use in blood substitute preparations, liquid PFCs are preferred. Because they are hydrophobic, it is necessary to emulsify them for intravenous use and organ perfusion. Presently, the most extensively used emulsifying agents are Pluronic F68 and various phospholipids (Geyer 1988). Emulsions which are used as blood substitutes must have a suitable vapor pressure and short retention time in tissue. Only a small number of PFCs are currently viable candidates for biological use and most have been proven unsatisfactory (Geyer 1988). PFCs exert no osmotic or oncotic pressure. Electrolytes or plasma volume expanders must be added to circumvent this deficiency (Alexander et al 1975).

The solubility of \( O_2 \) and \( CO_2 \) in liquid PFCs makes them well suited to carry oxygen from the lungs to other tissues and to transport \( CO_2 \) from tissues to the lungs (Sloviter et al 1969). Animal research with PFCs as blood substitutes has produced good results (Geyer 1975) and their initial clinical application has been successful (Mitsuno 1988, Chen and Yang 1988). Bloodless animals perfused with PFC emulsion survive and rapidly produce new plasma proteins and red cells (McCoy 1988). Geyer (1975) also demonstrated that rats, perfused with PFC, tolerated toxic levels of carbon monoxide. In contrast to red blood cell, PFCs can simultaneously carry oxygen, carbon monoxide and carbon dioxide. The oxygen solubility of PFCs is not affected by
carbon monoxide.

Although PFCs are inert compounds and usually nontoxic, they can produce some side effects when administered intravenously for a fairly long period of time. The major adverse effects relate to excessive deposition in the liver and spleen. Anaphylactoid reactions, which include complement activation, hypotension, leukopenia, and other changes in the immune system, have also been observed (Clark et al 1973, Ciazza et al 1988, Zhang et al 1988, Lowe and Bollands 1988). Some perfluorinated compounds are peroxisome proliferators and act as a positive modulator of hepatocarcinogenesis. Abdellatif et al (1991) determined that perfluorooctanoic acid can significantly increase the incidence of hepatocellular carcinoma in response to carcinogenic agents.

PFC emulsions have been successfully used to preserve organs such as kidney, liver and heart for transplantation (Fuchinoue et al 1986, Baba et al 1988, Biro et al 1989, Tomera and Geyer 1982).

Another important application of PFCs is as an oxygenating agent. Not only do the PFC containing products have excellent \( O_2 \)-carrying capacity, they also release \( O_2 \) at a higher partial pressure than would occur with a plasma/red blood cell mixture. Since PFC particles are approximately \( 1/70 \) the size of red blood cells, they can penetrate past partial vascular obstructions into areas that are
inaccessible to red blood cells. PFC particles load and unload $O_2$ faster than do erythrocytes. The lower viscosity of PFC emulsions facilitates a higher flow into tissues. (Fischer et al 1986, Geyer, 1975).

Hypoxia limits tumor radiotherapeutic efficacy because oxygen is a potent radiosensitizer. At usual radiotherapeutic levels, only a small amount of damage can be produced by direct interactions between radiations such as X-ray or gamma rays and biological molecules. Most critical damage is produced indirectly by a variety of radicals and other chemically reactive species which are generated through chemical reactions in the tissue initiated by radiation. Oxygen participates in these chemical reactions leading to radiation damage. Solid tumors have a poor blood supply and consequently contain insufficient oxygen tension to allow efficient radiotherapy (Rockwell 1988). The beneficial rheological characteristics of PFC emulsions (i.e. small particle size and low viscosity) allow them to increase tumor oxygenation and improve radiotherapy (Teicher and Rose 1984).

PFC emulsions have also been used to treat brain ischemia. Because the brain is so rapidly affected by hypoxia, it is crucial to replenish the supply of $O_2$ as rapidly as possible. Both experimental and clinical studies have demonstrated the efficacy of these blood substitutes in brain ischemic tissue (Clark et al 1989, Chen et al 1988).
MI/R injury is initiated by insufficient oxygen supply. PFCs which possess effective oxygen carrying ability and therefore can delay hypoxia have been obvious candidates to rescue ischemic hearts. Glogar et al (1981) demonstrated that PFC preparations markedly reduced the infarcted area in a regionally ischemic canine heart model when they partially replaced the blood and the animals breathed 100% oxygen. Rude et al (1982) indicated that PFC emulsions improved oxygen supply to the myocardium in a similar regional ischemic model. Based on the isolated rabbit heart global ischemic model, Parrish et al (1984) argued that the benefits shown by PFCs when used during reperfusion could be attributed to their superior rheological properties. The special rheological characteristics of PFCs may also be one of the mechanisms by which they limit ischemic damage when used during occlusion episodes (Glogar 1981). Bajaj et al (1989) demonstrated that myocardial preservation by PFCs was associated with their ability to suppress the enhanced neutrophil margination and infiltration into myocardium caused by ischemia. Oxygenated perfluorochemical cardioplegic solutions can support aerobic metabolism and therefore delay depletion of high-energy phosphate during cardiac arrest (Rousou et al 1982, Flaherty et al 1984, Bando et al 1988). Inclusion of PFCs in cardioplegic solution prevented intracellular acidosis and the accumulation of lactate, creatine phosphokinase and
thiobarbituric acid reactive substances (products of lipid peroxidation) in the coronary effluent.

One of the most serious deficiencies of PFC blood substitutes is their limited oxygen carrying capacity, related to their low PFC content (Arlen et al 1988). For instance, the first commercial preparation, Fluosol-DA, contains only 20% (w/v). One of the brominated fluorochemicals, perfluorooctylbromide (PFOB), was first synthesized as a nontoxic contrast agent for fluoroscopy, NMR imaging and ultrasonography. PFOB has the capability to form reasonably stable emulsions at higher concentrations than most other PFCs (Long et al 1988). A 100% (w/v) emulsion, which is easy to achieve, has more than four times the oxygen-carrying capacity of Fluosol-DA (a 20% PFC emulsion) (Riess 1988). Biro et al (1989) tested the cardioprotective effects of this novel PFC emulsion (50% w/w) on a modified Langendorff rat heart preparation. They found that after switching the perfusate from aqueous perfusate to PFOB emulsion, contractile performance of hearts was significantly higher. However, protective effects of this PFC emulsion have not been determined in MI/R injury models.

5. Tationale for These Experiments

A 100% PFOB emulsion has a very high oxygen-carrying capacity. MI/R injury occurs during cardioplegic processes.
This damage is initiated by hypoxia. The hypothesis that oxygenated PFOB-containing cardioplegic solutions can prevent the decline in cardiac function associated with cardioplegia was tested in the modified Langendorff rat heart preparation.

Several mechanisms of successful interventions in MI/R injury exist. They include prevention of the depletion of ATP and endogenous free radical scavengers such as glutathione (GSH). Also, endogenous antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPD) can be maintained or induced. The ability of PFOB to preserve endogenous antioxidant defenses and ATP levels during crystalloid cardioplegia was also determined in these experiments.

Although cold cardioplegia is commonly performed in clinical surgery, hypothermia is still controversial (Lessana et al 1992). Hypothermia is an alternative way to protect the myocardium by slowing metabolism and degenerative processes. The aim of the current study was to define the cardioprotective effects of PFOB emulsions. Application of hypothermia in this research would make the result more difficult to interpret since there would be another protective factor to consider. In order to simplify this research, all investigation was undertaken at normal temperature (37°C).
MATERIALS AND METHODS

ANIMALS AND HOUSING:

Male Sprague-Dawley rats, weighing 400-500g, were used. Two rats were housed together in a wire-mesh cage over wood-chip bedding. Purina lab chow and water were available ad lib during 12 hour light-dark cycles. All animals were allowed a minimum of 2 days acclimatization before use in the experiments.

REAGENTS AND DRUGS:

Drugs and reagents were obtained from the indicated sources. Sodium pyruvate, Tetrabutylammonium hydrogen sulphate (TBAHS), Ethylenediaminetetraacetic acid (EDTA), Glutathione, reduced form, Glutathione reductase, B-Nicotinamide adenine dinucleotide, reduced form (NADH), B-Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), Sulfosalicylic acid, Triethanolamine, Diethylenetriamine-pentacetic acid (DTPA), 2,4-Dinitrophenylhydrazine, Pyrogallol, Triton X-100 (Sigma Chemical Co. St Louis, Mo). Potassium chloride (KCl), Sodium bicarbonate (NaHCO₃), Calcium chloride (CaCl), Sodium phosphate monobasic (KH₂PO₄), Potassium phosphate dibasic (K₂HPO₄), Sodium hydroxide (NaOH), Tris (buffer), Acetonitrile, (Baker Chemical Co. Phillisburg, NJ). Potassium phosphate monobasic (KH₂PO₄), Perchloric Acid,

EXPERIMENTAL PROTOCOL:

1. Cardioplegic reperfusion injury:

   Male Sprague-Dawley rats, weighing 400-500g, were anesthetized with sodium pentobarbital (100 mg/Kg i.p.), intubated to maintain ventilation during cardiac procedures, and heparinized. The aorta was isolated and a cannula inserted. Hearts were perfused with modified Krebs-Henseleit (KH) solution (NaCl 112 mM; KCl 5 mM; MgSO₄·7H₂O 1.2 mM; KH₂PO₄ 1 mM; NaHCO₃ 25 mM; Dextrose 11.5 mM; Na Pyruvate 2 mM; CaCl·2H₂O 1.25 mM), aerated with 95% O₂-5% CO₂ gas mixture, at a pressure of 80 mmHg and a temperature of 37°C. Hearts were suspended in a heated muscle bath filled with KH solution. The left
atrium was removed to allow the insertion of a balloon cannula into the left ventricle. The balloon cannula was connected to a pressure transducer to monitor heart rate and left ventricular developed pressure (LVDP).

Hearts were allowed to equilibrate for 15 minutes before the balloon was inflated to 5 mmHg LVDP. After 20 minutes, coronary flow (CF), heart rate (HR), and LVDP were recorded. A one-ml sample of perfusate was obtained for determination of lactate dehydrogenase (LDH) activity. LDH was determined with a kit supplied by Sigma (Procedure No.500) as described in Appendix A. LDH release is expressed as Berger-Broida (B-B) units/ml. Cardiac function estimated as double product (DP) was calculated as (HR x LVDP /1000). Hearts were switched to another reservoir which contained the St. Thomas' Hospital Cardioplegic Solution No.2 (CPS) (NaCl 110mM; KCl 16mM; MgCl$_2$·6H$_2$O 16mM; CaCl$_2$·2H$_2$O 1.2mM; NaHCO$_3$ 10mM), aerated with 95% O$_2$-5% CO$_2$ gas mixture. After perfusion for 3 minutes, the flow of CPS was shut off and the KH in the bath was replaced by the same cardioplegic solution. After a 30 minute global ischemia, the hearts were reperfused by KH and the CPS in the bath was replaced with KH. Following a 30-minute period of reperfusion, values for HR, LVDP, CF, DP and LDH activity were determined again.
2. PFOB emulsions:

Emulsification of PFOB was accomplished by adding the appropriate weight of the compound to the appropriate volume of CPS containing 2.7% Pluronic F-68, a surfactant. The mixtures were sonicated with a Branson Sonifier resulting in a cloudy, but stable, emulsion. The emulsions were aerated with 95% O₂-5% CO₂ for 15 minutes before use. Hearts exposed to PFOB were treated the same as control hearts, except that they were subjected to a single infusion of PFOB emulsion to fill the coronary circulation and then immersed in the PFOB emulsion. Values for HR, LVDP, CF, DP and LDH activity were again determined prior to cardioplegia and after 30 minutes of reperfusion.

3. Tissue preparation:

At the end of the 30 minute reperfusion period, left ventricular tissue was rapidly frozen and stored in liquid nitrogen. Less than 1 week later ATP and intracellular antioxidant assays were performed with the wet tissue. A control group of hearts were frozen just prior to ischemia for determination of preischemic values. Contents of the substances and activity of enzymes were expressed per gram of wet tissue weight.

4. Assays:

The detailed procedures for intracellular substance assays are described in the Appendix A.

a. ATP was measured by counter ion HPLC as modified from the
method of Sellevold et al (1986). ATP was extracted from tissue with 0.42M perchloric acid. The perchloric acid was then neutralized with 1.0 M KOH in the presence of an indicator, 0.1% chlorphenol red. A C-18 reverse phase column was used. The mobile phase was a degassed 10% acetonitrile : 90% KH₂PO₄ mixture. ATP standard solutions were used to establish the standard curve for quantifying the ATP level in tissue.

b. Glutathione (GSH) and glutathione disulfide (GSSG) were determined enzymatically by the DTNB-GSSG reductase recycling procedure (Anderson, 1985). The samples were deproteinized by treatment with 5% 5-sulfosalicylic acid. GSH was oxidized by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to GSSG with stoichiometric formation of 5-thio-2-nitrobenzoic acid (TNB). GSSG was rapidly reduced by GSSG reductase and NADPH which were added in the reaction mixture. The rate of TNB formation was followed at 412 nm and was proportional to the sum of GSH and GSSG present. GSSG was measured by masking GSH with 2-vinylpyridine before running the assay. GSH was then calculated by subtracting the GSSG from the total glutathione.

c. Glutathione peroxidase (GPD) was determined by continuous monitoring of GSSG formation in the reaction mixture (Flohe and Gunzler 1984). GSH is oxidized by GPD to GSSG. The GSSG is then reduced by an excess of glutathione reductase to
maintain a constant level of GSH. The rate of concomitant oxidation of NADPH is proportional to GPD activity and can be monitored spectrophotometrically.

d. The total amount of superoxide dismutase (SOD) was assayed by the pyrogallol auto-oxidation method (Maestro and McDonald 1985). This assay is based on the ability of SOD to scavenge superoxide anion radicals. Removal of these molecules decreases the overall rate of pyrogallol autoxidation. The tissue samples were homogenized in 10 mM potassium phosphate buffer supplemented with 30 mM KCL.

e. Catalase (CAT) samples were prepared in the same buffer used for SOD. Prior to the determination, ethanol and Triton X-100 were added into the sample to increase the observable catalase level by decomposing the inactive complex of catalase with H₂O₂ or directly activating the enzyme. Samples were added to a standardized amount of H₂O₂ solution. The H₂O₂ which remained in the reaction mixture after 3 minutes was titrated with potassium permanganate. The catalase activity was inversely proportional to the amount of H₂O₂ left in the reaction mixture (Cohen et al 1970).

5. Statistics:

ANOVA and Newman-Keuls procedures were employed to determine significant differences among multiple treatment means (Steel and Torrie 1960). (p < 0.05 = significant difference)
RESULTS

Preliminary studies showed that a three minute coronary perfusion with CPS stopped all hearts. The following 35 minutes of ischemia and an additional 30 minutes of reperfusion with KH produced significant decreases in left ventricular contractile function (LVDP and Double Product) and coronary flow. A significant increase in extracellular LDH accumulation also occurred. No significant changes in heart rate were noted. The surfactant control (2.7% Pluronic F-68 in CPS) produced similar results (Figs. 1-5). We also observed that the ischemic time was very critical since 30 and 38 minutes of ischemia produced minimal and maximal injury, respectively.

Statistical analysis indicated no significant between-group differences for preischemic ventricular contractile, coronary flow values and extracellular LDH levels. Both 100% and 50% PFOB emulsions prevented the decline in left ventricular contractile function (LVDP, Double product) seen in control treatments. The 25% PFOB did not demonstrate this protection (Figs. 2 and 3). However, the decrease in coronary flow associated with reperfusion injury was not affected by any of the PFOB emulsions (Fig. 4). Heart rate was not altered by the PFOB emulsions (Fig. 1). The elevation in extracellular LDH was not significantly reduced by the 100% PFOB emulsion even though contractile function was sustained by this high concentration of the
emulsion (Fig. 5).

ATP, glutathione and endogenous antioxidant enzymes were only investigated in control and 100% PFOB treated hearts. Cardioplegia-induced ischemia and reperfusion reduced the tissue ATP concentration. The 100% PFOB was able to maintain a normal ATP level. There is no significant difference in ATP content between preischemic tissue and postischemic tissue treated with 100% PFOB (Fig. 6).

Table 1 shows that cardioplegia followed by reperfusion depleted the total glutathione and GSH levels in the myocardium, while the GSSG was not significantly decreased. The 100% PFOB prevented the loss of the total glutathione pool inside the cells. However, while there was no significant difference in GSH values between the preischemia control and the 100% PFOB treated group, the latter had less total glutathione content than the preischemic hearts.

The myocardial antioxidant enzymes GPD, CAT, and SOD were not significantly altered by the cardioplegia-induced MI/R injury. The 100% PFOB failed to modify any of these intracellular antioxidant defenses (Table 2).
Fig. 1. Pre-Ischemia = preischemic value. Post-Ischemia = value after 35 minutes of ischemia and following 30 minutes of reperfusion. PFOB = perfluorooctylbromide emulsions in cardioplegic solution administered during time of ischemia. Pluronic F-68 represents a control containing 2.7% of this surfactant. Bars represent mean ± SEM. Number of rats used is given above the bars.
EFFECT OF PFOB ON LEFT VENTRICULAR DEVELOPED PRESSURE


Fig. 2. Terms are the same as for Fig. 1.

* = significantly different from its respective preischemia value. ** = significantly different from its respective preischemia value and from the control value. (p < .05 = significant difference).
EFFECT OF PFOB ON DOUBLE PRODUCT

Fig. 3. Terms and marks are the same as for Fig.1 and Fig.2.
Double product = (heart rate x left ventricular developed pressure)/1000.
EFFECT OF PFOB ON CORONARY FLOW

Fig. 4. Terms and marks are the same as for Fig.1 and Fig.2.
EFFECT OF PFOB ON LDH ACTIVITY

Fig. 5. Terms and marks are the same as for Fig.1 and Fig.2.
**EFFECT OF PFOB ON ATP LEVELS IN HEART TISSUE**

- **Pre-isc**
- **Post-isc control**
- **Post-isc, 100% PFOB**

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**Fig. 6.** Pre-isc = preischemic value. Post-isc control = control value after 35 minutes of ischemia and following 30 minutes of reperfusion. Post-isc 100% PFOB = value after ischemia and reperfusion with administration of 100% PFOB emulsion in cardioplegic solution during ischemia. Bars and sample size are given in the same way as for Fig. 1. * = significantly different from all other values (p < .05).
Table 1. Effects of PFOB on Glutathione Levels in Heart Tissue

<table>
<thead>
<tr>
<th></th>
<th>Pre-Ischemia Control</th>
<th>Post-Ischemia Control</th>
<th>Post-Ischemia 100% PFOB</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GSH (umol/g tissue)</strong></td>
<td>1.44 ± 0.060 (n = 11)</td>
<td>0.79 ± 0.080&lt;sup&gt;a&lt;/sup&gt; (n = 6)</td>
<td>1.22 ± 0.07&lt;sup&gt;b&lt;/sup&gt; (n = 6)</td>
</tr>
<tr>
<td><strong>GSSG (umol/g tissue)</strong></td>
<td>0.23 ± 0.012 (n = 11)</td>
<td>0.19 ± 0.009 (n = 6)</td>
<td>0.19 ± 0.016 (n = 6)</td>
</tr>
<tr>
<td><strong>GSSG + GSH (umol/g tissue)</strong></td>
<td>1.67 ± 0.050 (n = 11)</td>
<td>0.95 ± 0.072&lt;sup&gt;a&lt;/sup&gt; (n = 6)</td>
<td>1.41 ± 0.057&lt;sup&gt;a,b&lt;/sup&gt; (n = 6)</td>
</tr>
</tbody>
</table>

All values are Mean ± SEM.

<sup>a</sup> Significantly different from preischemic value.

<sup>b</sup> Significantly different from postischemia control value.
Table 2. Effects of PFOB on the Activity of Myocardial Antioxidant Enzymes

<table>
<thead>
<tr>
<th></th>
<th>Pre-Ischemia Control</th>
<th>Post-Ischemia Control</th>
<th>Post-Ischemia 100% PFOB</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GPD</strong></td>
<td></td>
<td></td>
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<tr>
<td>(umol NADPH oxid/min/g tissue)</td>
<td>20.38 ± 0.65 (n = 8)</td>
<td>17.73 ± 0.87 (n = 6)</td>
<td>18.84 ± 1.52 (n = 6)</td>
</tr>
<tr>
<td><strong>CAT</strong></td>
<td></td>
<td></td>
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<tr>
<td>(k x 10⁻²)</td>
<td>2.95 ± 0.15 (n = 6)</td>
<td>2.37 ± 0.24 (n = 6)</td>
<td>2.83 ± 0.09 (n = 6)</td>
</tr>
<tr>
<td><strong>SOD</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(units/g tissue)</td>
<td>257.9 ± 25.1 (n = 6)</td>
<td>252.8 ± 17.4 (n = 6)</td>
<td>241.7 ± 26.0 (n = 6)</td>
</tr>
</tbody>
</table>

gpd = Glutathione peroxidase, cat = Catalase, sod = Superoxide dismutase.

All values are Mean ± SEM.
DISCUSSION

The control data indicated that this cardioplegic model caused reproducible and measurable MI/R injury simulating that induced by hypothermic cardioplegia. Shlafer et al (1982) tested cardioprotective effects of several solutions administered during ischemia in an isolated rabbit heart preparation. They demonstrated that following a 2 hour hypothermic cardioplegia (27°C) and a 1 hour reperfusion with oxygenated physiological saline solution, hearts incurred significant decreases in LVDP, left ventricular compliance, the maximum rate of left ventricular pressure development (dP/dt) and coronary flow. No differences in heart rates were noted. LDH released during reperfusion was also significantly increased. Similar changes in these important indicators of cardiac function were also found by Ledingham et al (1990) in their hypothermic cardioplegic model with isolated rat hearts, in which the same CPS as used in this laboratory was employed to induce ischemia (4 hours at 4°C).

After MI/R, myocardial tissue lost 72.8% of its preischemic ATP content (postischemia 154 ± 16.2 vs preischemia 546 ± 62 nmol/g wet tissue). These data are in agreement with the findings of other investigators who employed long hypothermic cardioplegia to induce MI/R damage (Rousou et al 1986, Wyatt et al 1989). The decline of ATP content has been noted in almost every ischemic and
reperfused model. There is good correlation between myocardial ATP level and left ventricular function (Flaherty et al 1984, Ledingham et al 1990, Ferrari et al 1985). All this evidence indicates that this normothermic cardioplegia-induced model of MI/R injury is similar to that produced by hypothermic cardioplegia in terms of changes in myocardial functional and biochemical parameters. However, it is superior to the others in terms of simplifying procedures and shortening experimental time. Additionally, a variety of levels of hypothermia (4°C--30°C) has been applied by different authors and this variability makes comparisons difficult. It is well established that homogeneous distribution of cooling during cardioplegia is difficult to achieve in patients with coronary artery stenoses or occlusions (Menash et al 1988). Under these situations, the myocardium is not entirely cooled during cold cardioplegia. Warm cardioplegia is currently used in some medical facilities for myocardial protection during open heart procedures (Lessana et al 1992). This model does mimic some clinical practices. The protective effect of PFOB emulsions indicates that this model may be used to evaluate the efficacy of various treatments which might significantly enhance the cardioprotection provided by cardioplegia.

The PFOB emulsions produced a dose-dependent protection against MI/R injury as measured by ventricular contractile function. Both 100% and 50% PFOB significantly increased
the extent of recovery of cardiac function while the effect of 25% PFOB could not be distinguished from the control. These results support the hypothesis that enhanced delivery of oxygen to the heart during ischemic arrest can improve myocardial preservation.

ATP levels and DPs were not significantly decreased after hearts were arrested with CPS containing 100% PFOB and reperfused with KH. Therefore, the high correlation between the preservation of cardiac function and maintenance of ATP observed by others (Godin et al, 1989, Sellevold et al, 1986) was verified in the current study. There is good experimental evidence that oxygenated PFC emulsions can enhance the intramyocardial oxygen tension (Rude et al, 1982, Novick et al, 1985). This maintains aerobic metabolism which is otherwise lost during ischemia. PFOB emulsion appeared to be capable of maintaining sufficient aerobic metabolism to prevent ischemia-induced loss of ATP. A large data base supports the conclusion that other PFC emulsions can retard high-energy phosphate decline during ischemia (Rousou et al, 1982, Flaherty et al, 1984, Bando et al, 1988). When Rude et al (1982) and Kanter et al (1981) compared PFC and blood cardioplegia, they proposed that the beneficial effects are not only due to PFC emulsion's high oxygen transporting ability, but also due to the augmented myocardial O₂ consumption in the ischemic hearts receiving oxygenated PFC emulsions (i.e. the amount of oxygen which can be actually...
taken up by the myocardium is high). This may occur as a consequence of faster unloading rate of oxygen and significant rheological features such as smaller particle size and lower viscosity (Fisher et al 1986). Our study did not address these possible mechanisms.

Carbon dioxide has about 2.5 times higher solubility than oxygen in PFCs (Geyer 1988). During ischemia the relatively high pressure of CO$_2$ in the myocardium partially accounts for the tissue damage (Rude et al 1982). PFOB could absorb this byproduct of ischemia and therefore diminish the tissue damage.

PFOB emulsions failed to significantly alter the decrease in coronary flow that followed reperfusion. This evidence does not seem to correspond to the protection of contractile function by highly concentrated emulsions. It has been reported previously that PFC emulsion-perfused hearts had decreased coronary flow compared with those receiving normal physiological solution (Rahamathulla et al 1985). The increased viscosity of PFC emulsions may account for this event (O'Neill et al 1981). Menash et al (1988) indicated that applying a PFC emulsion during cardioplegia diminished postischemic coronary flow even though crystalloid cardioplegic solution did not exhibit decreased coronary flow after reperfusion in their isolated rat heart model. The mechanism of this phenomenon has not been determined. In our case, one possibility would be that PFOB
emulsions failed to modify the vascular damage caused by MI/R.

GSH, a cofactor of GPD, has a critical role in the protection of myocardium against oxidative damage either by inactivation of lipid peroxides or by eliminating hydrogen peroxide. The depletion of tissue GSH has been referred to as a direct manifestation of myocardial oxidative injury (Guarnieri et al 1980). Thirty five minutes of cardioplegia and reperfusion induced a reduction of the tissue GSH/GSSG ratio. This reduction results from a significant decline in GSH with GSSG unchanged. This does not agree with other reports and is difficult to interpret. Increased oxidative stress leads to the depletion of GSH, which should in turn cause accumulation of GSSG in cell. However, there was a marked release of LDH from the damaged tissue. This implies that the cell membrane was disrupted and GSSG may have been lost from the cells into the coronary effluent upon reperfusion. Ferrari et al (1985) have shown that reperfusion of severely injured ischemic hearts causes a marked and sustained release of GSH and GSSG as result of peroxidation of cellular membranes. The percentage of GSSG release is higher than that of GSH.

PFOB retarded the loss of intracellular GSH that normally occurs in MI/R. This indicates that the maintenance of oxygenation during cardioplegic arrest prevents oxidative stress upon readministration of
oxygenated physiological perfusate. Formation of free radicals is initiated during myocardial ischemic phase (Rao et al 1983) and GSH is depleted before reperfusion (Ceconi et al 1988). PFOB may also reduce the free radical reactions caused by lower oxygen tension when the hearts were ischemic. However, the present experiments have not confirmed this mechanism. Another contribution to this event could be preservation of the cellular membrane. Decreased lipid peroxidation in PFOB treated hearts could help maintain the cellular membrane integrity and prevent the leakage of GSH from the cell. ATP plays a critical role in membrane function and integrity (Burton et al 1980, Jennings et al 1978). The high ATP level in PFOB treated hearts may also contribute to this protection.

The activities of the antioxidant enzymes (SOD, GPD, CAT), which some investigators repeatedly demonstrated to be lessened after MI/R injury (Guarnieri et al 1980, Meerson et al 1982, Julicher et al 1984), were not affected by MI/R in this model. However, there are numerous papers that present unchanged values or even increased enzyme activity after the damage (Arduini et al 1988, Godin et al 1989, Shlafer et al 1987). These discrepancies may relate to differences in the duration of ischemia and reperfusion and the use of different animal models. In this case, the cardioplegic solution applied during the ischemic episode may contribute to the inability to detect any variation. Although
protective, PFOB was not associated with any significant effect on these enzymes and did not induce these enzymes in the tissue. This implies that the mechanism of the protection by PFOB in this model may be due to its ability to transport gases.

LDH release was not significantly diminished by the 100% PFOB although this concentration provided cardioprotection in terms of mechanical and biochemical parameters. LDH release did not correlate well with cardiac function. This lack of correlation has been found in previous studies in our laboratory and by other investigators (Grover and Sleph 1989, Irem et al. 1991).

In conclusion, the results have shown that this normothermic cardioplegia-induced MI/R injury model is similar to the other MI/R models and can be used to study the mechanisms and treatment of MI/R damage. Enhanced oxygen delivery during cardioplegia can diminish MI/R injury. The data suggest that PFOB enhances oxygen delivery and might be used to reduce damage that occurs during cardiac surgery and organ preservation.
BIBLIOGRAPHY


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APPENDIX A

ASSAY PROCEDURES
LACTATE DEHYDROGENASE ACTIVITY

Reagents:

Na pyruvate solution: 100 mg/L
NADH solution (prepared immediately before use): 100 mg/ml
Color reagent: 200 mg/L of 2,4-Dinitrophenylhydrazine in 1 N HCl
NaOH solution: 0.4 N.

Instruments:

37°C water bath
Bausch & Lomb Spectronic 21

Procedure:

1). 1 ml of Na pyruvate solution and 10 ul of NADH solution were pipetted into each of two labelled test tubes (blank and sample), the tubes were placed in the 37°C water bath for 5 minutes.
2). 0.5 ml of sample or blank (Krebs-Henseleit solution) were added and mixed gently. Timer was started and the tubes were kept in the 37°C water bath.
3). After exactly 30 minutes of incubation, the tubes were removed from water bath and 1 ml of color reagent was added to each tube. Tubes were mixed thoroughly and allowed to remain at room temperature for 20 minutes.
4). 10 ml of NaOH solution were added to each tube and mixed well.
5). Between five and thirty minutes after adding the NaOH solution, absorbance was read and recorded with water
as reference at a wavelength of 525 nm.

6). The LDH activity in the sample was expressed by as B-B units/ml. B-B units/ml = (O.D.\text{sample} - O.D.\text{blank}) \times 1200.

ADENOSINE TRIPHOSPHATE

Reagents:

Perchloric acid: 0.42 mM

KOH solution: 1 M

pH indicator: 0.1% chlorphenol red

Phosphate buffer: 215 mM KH$_2$PO$_4$ and 2.3 - 8 mM tetrabutylammonium hydrogen sulphate (TBAHS), pH 6.3

Mobile phase: 90% phosphate buffer: 10% acetonitrile

Instruments:

IEC B-20A Centrifuge

Milton Roy HPLC system

Procedure:

1). Frozen tissue was quickly removed from liquid nitrogen and homogenized in perchloric acid solution (10\% W/V) on ice.

2). The homogenate was spun for 10 minutes at 10,000 rpm in the chilled (1-3°C) centrifuge.

3). The supernatant was transferred to a clean tube and KOH solution was added to precipitate and neutralize perchloric acid in the presence of the indicator.
4). The mixture was filtered through a microfilter and kept on ice until 20 ul of sample was injected into the HPLC system.

5). The mobile phase was degassed before use. The flow rate was maintained at 0.7 ml/min and the spectrophotometer was set at 259 nm.

6). Different ATP standard solutions were prepared with the same procedure and used to establish the standard curve for determination of the ATP in the tissue samples.

GLUTATHIONE (GSH) AND GLUTATHIONE DISULFIDE (GSSG)

Reagents:

5-Sulfosalicylic acid solution: 5%

Stock buffer: 143 mM KH₂PO₄ and 6.3 mM EDTA, pH 7.5

NADPH solution: 0.248 mg/ml in stock buffer. Stored at ice cold and prepared daily

5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB): 6 mM DTNB was prepared in the stock buffer and stored frozen. The stork buffer was thawed before use and kept on ice.

GSSG reductase: The stock enzyme was diluted to 266 U/ml with stock buffer and stored on ice during the experiment.

GSH standards: Standards were diluted daily in 5-sulfosalicylic acid from a frozen 16 mM stock solution which was prepared weekly in
5-sulfosalicylic acid
2-Vinylpyridine
Triethanolamine

Instruments:
IEC B-20A Centrifuge
Bausch and Lomb Spectronic 600 with constant temperature cuvette compartment

Procedure:
1). Tissue samples were homogenized on ice with 5-sulfosalicylic acid solution (10% W/V). The homogenate was centrifuged for 10 minutes at 10,000 rpm in the refrigerated (1-3°C) centrifuge.

2). For the total amount of glutathione assay, the supernatant was ready to use in the following procedure. For GSSG measurement, 0.5 ml of supernatant was mixed with 10 ul 2-vinylpyridine and 1 drop (approximate 30 ul) of triethanolamine. The final pH of the mixture was 6-7. The mixture were allowed to stay at room temperature for 60 minutes.

3). 700 ul of NADPH, 100 ul of DTNB and 175 ul of water were pipetted into a cuvette. The mixture was prewarmed at 30°C for 10 minutes in the constant temperature cuvette compartment of the spectrometer.

4). 25 ul of sample or standard solution was added.

5). 20 ul of glutathione reductase was added with mixing to initiate the reaction. Times were recorded when the
absorbance was 0.5, 1.0, 1.5, and 2.0 at 412 nm.

6). The reaction rate (ΔO.D./min) was estimated from the slope of the regressed line in which absorbance (O.D.) was plotted against time. The amount of GSH equivalents was determined from a standard curve in which the GSH equivalents present in the standard solutions were plotted against the reaction rate.

GLUTATHIONE PEROXIDASE (GPD) ACTIVITY

Reagents:

Phosphate buffer: 0.1 M KH$_2$PO$_4$, 1 mM EDTA, pH 7.0
GSSG reductase: 2.66 U/ml dilution in phosphate buffer from the stock enzyme
GSH standard: 10 mM GSH in water
NADPH solution: 1.5 mM NADPH in 0.1% NaHCO$_3$
H$_2$O$_2$ solution: 1.5 mM H$_2$O$_2$ in water

Instruments:

IEC B-20A Centrifuge
Bausch and Lomb Spectronic 600 with a constant temperature cuvette compartment

Procedure:

1). Tissue samples were homogenized in phosphate buffer (1% W/V) on ice and then spun for 10 minutes at 10,000 rpm in the refrigerated (1-3°C) centrifuge.

2). 500 ul of buffer, 100 ul of GSSG reductase, 100 ul of GSH standard and 100 ul of sample or phosphate buffer
as blank were pipetted into a cuvette. The mixture was preincubated at 37°C for 10 minutes in the constant temperature cuvette compartment of the spectrophotometer.

3). 100 ul of NADPH was added into the cuvette and mixed by inversion. The mixture was incubated for another 3 minutes.

4). 100 ul of \( \text{H}_2\text{O}_2 \) was pipetted into the mixture and quickly mixed to initiate the reaction. The absorbance at 365 nm was recorded for 5 minutes.

5). The rate of change of absorbance (\( \Delta \text{O.D.}/\text{min} \)) was estimated from the slope of the regressed line in which absorbance (O.D.) was plotted against time. The change of absorbance was caused by the oxidation of NADPH in the reaction mixture and therefore the rate of NADPH oxidation (mol NADPH oxidized/min) was calculated from the \( \Delta \text{O.D.}/\text{min} \) using the appropriate extinction coefficient (\( E=A/L\cdot C \)). The coefficient was measured by reading the absorbance of NADPH standard solution with the same cuvette and spectrophotometer. The GPD activity in samples was calculated from the difference of the mol NADPH oxidized/min between the sample and blank.
SUPEROXIDE DISMUTASE (SOD) ACTIVITY

Reagents:

Phosphate buffer: 10 mM K₂HPO₄ and 30 mM KCl pH 7.4

Tris buffer: 50 mM Tris (buffer), 1 mM diethylenetriamine pentacetic acid (DTPA), pH 8.2 (The solution is aerated by stirring vigorously at room temperature for 20 minutes before use)

Pyrogallol: 24 mM in 10 mM HCl.

Instruments:

Branson Sonifier 450

IEC B-20A Centrifuge

Bausch & Lomb Spectronic 21

Procedure:

1). The tissue sample was sonicated for 1 minute at an output setting of 5 in ice-cold phosphate buffer. The homogenate was then centrifuged at 10,000 rpm for 10 minutes in the refrigerated (1-3°C) centrifuge. The supernatant was used for determination of enzyme activity.

2). 3 ml of Tris buffer were added to 30 ul of supernatant in a cuvette. The reaction was initiated by adding 25 ul of pyrogallol solution. The change of the absorbance at 420 nm, caused by the formation of the oxidized pyrogallol, was recorded for 2-3 minutes.

3). A blank was run using the Tris buffer plus pyrogallol.

4). The autoxidation rate (ΔO.D./min) was estimated from

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the slope of the regressed line in which absorbance was plotted against time. The percentage of the autoxidation rate inhibited by the enzyme sample was calculated as: 
\[ \% \text{inhibition} = \frac{\Delta O.D._{\text{blank}} - \Delta O.D._{\text{sample}}}{\Delta O.D._{\text{blank}}} \times 100\% \]
One unit of SOD activity was defined as the amount which inhibits the rate of autoxidation by 50%.

CATALASE (CAT) ACTIVITY

Reagents:

- Phosphate buffer No. 1: 10 mM K_2HPO_4, 30 mM KCl pH 7.4
- Ethanol solution: 10% in the phosphate buffer No. 1
- Triton X-100 solution: 10% in phosphate buffer No. 1
- Phosphate buffer No. 2: 5 mM KH_2PO_4, 5 mM K_2HPO_4, pH 7.0
- \( \text{H}_2\text{O}_2 \) solution: 6 mM in phosphate buffer No. 2
- \( \text{H}_2\text{SO}_4 \) solution: 6 N in water
- \( \text{KMnO}_4 \) solution: 0.01 N in water

Instrument:

- Branson Sonifier 450
- IEC B-20A Centrifuge
- Bausch & Lomb Spectronic 21

Procedure:

1) Tissue samples were sonicated for 1 minute at the output setting at 5 in ice-cold phosphate buffer No. 1. The homogenate was then centrifuged at 10,000 rpm for 10 minutes in the refrigerated (1-3°C) centrifuge.
2). 0.1 ml of the supernatant was transferred to another tube and 10 ul of ethanol solution was added. The mixture was kept on ice for 30 minutes.

3). 10 ul of Triton X-100 solution was added into the mixture.

4). The mixture was diluted with 5 ml of phosphate buffer No.1 to prepare the final sample for assay.

5). 5 ml of cold \( \text{H}_2\text{O}_2 \) solution was added to each of two reaction vials labelled sample and blank. The reaction was initiated by adding 0.5 ml of sample or blank (0.5 ml of phosphate buffer No.1) to the vials.

6). After exactly 3 minutes, the reaction was stopped by rapidly adding 1 ml of \( \text{H}_2\text{SO}_4 \) solution to each tube.

7). A spectrophotometric standard was prepared by adding 5.5 ml of phosphate buffer No.2, 1 ml of \( \text{H}_2\text{SO}_4 \) solution and 7 ml of KMnO4 solution to the vial. The readout of the spectrophotometer was adjusted to read 1.0 absorbance unit for the standard.

8). 7 ml of KMnO4 reagent was added rapidly to each of the remaining reaction mixtures from step (6), mixed thoroughly, and read at 480 nm within 30-60 seconds.

9). Since the decomposition of \( \text{H}_2\text{O}_2 \) by catalase followed first-order kinetics, the catalase activity was expressed by the first-order reaction rate constant, \( k = \log(S_0/S_3) \times 2.3/t \). Here, \( S_0 = 1-\)the absorbance of blank, \( S_3 = 1-\)the absorbance of sample and \( t = 3 \text{ min} \).