1987

# Protection from reperfusion injury in the isolated rat heart by postischaemic deferoxamine and osypurinol administration

Stephen F. Badylak

Abby Simmons

John Turek

Charles F. Babbs
*Purdue University*, babbs@purdue.edu

# Protection from reperfusion injury in the isolated rat heart by postischaemic deferoxamine and oxypurinol administration

STEPHEN F BADYLAK, ABBY SIMMONS, JOHN TUREK, CHARLES F BABBS

Biomedical Engineering Center, and School of Veterinary Medicine, Purdue University, West Lafayette, Indiana, USA.

## Abstract

The Langendorff isolated rat heart preparation was used to determine the effect of oxypurinol, a xanthine oxidase inhibitor, and deferoxamine, an iron binding agent, on the extent of myocardial reperfusion injury after 60 minutes of ischaemia. Thirty rats were divided into three groups of 10. and an isolated heart preparation made from each rat. The isolated hearts were perfused for 15 minutes with a modified Krebs-Henseleit perfusate solution to permit stabilisation of the preparation. Each heart was then subjected to 60 minutes of total ischaemia at 37°C followed by 60 minutes of reperfusion with either saline treated perfusate, oxypurinol treated perfusate (1.3 mmol/litre), or deferoxamine treated perfusate (0.61 mmol/litre). Reperfusion injury was assessed by the total amount of creatine phosphokinase released into the perfusate, by changes in myocardial vascular resistance, and by morphological examination. The saline treated group released significantly more creatine phosphokinase into the perfusate than either the oxypurinol treated group ($p<0.05$) or the deferoxamine treated group ($p < 0.05$). The mean vascular resistance increased for all groups during the 60 minutes of reperfusion compared with that just before ischaemia but was significantly greater in the saline treated group than in the drug treated groups ($p < 0.01$). Ultrastructural examination of a randomly selected heart from each group after 60 minutes of reperfusion showed pronounced attenuation of mitochondria1 and endoplasmic reticulum swelling, increased maintenance of membrane integrity, and diminished separation of myofilaments in the oxypurinol treated and deferoxamine treated hearts. The mean cross sectional area of mitochondria after 60 minutes of reperfusion was significantly greater in the saline treated group than in the drug treated groups. Thus both oxypurinol and deferoxamine, given after 60 minutes of ischaemia at the onset of reperfusion, can protect the isolated rat heart from reperfusion injury,

Key words: free radical, iron, ischemia, mitochondria, myocardial damage, myocardial stunning, oxidative stress, superoxide, toxicity, xanthine oxidase

Cardiovascular Research, 1987; 21:500-506

Supported by grant HL-29398 from the National Heart, Lung, and Blood Institute, Bethesda, Maryland, USA.

The prompt return of circulation to ischaemic myocardium is necessary to sustain tissue viability. Reperfusion supplies the tissue with molecular oxygen and other essential energy forming substrates. However, the life sustaining reperfusion may also cause progressive tissue damage by initiating deleterious chemical reactions involving partially reduced forms of oxygen--a phenomenon referred to as reperfusion injury or the oxygen paradox.[1-5] Cytotoxic oxygen metabolites such as superoxide, hydrogen peroxide, and the hydroxyl radical have been implicated as the mediators of such reperfusion injury.[1, 3, 6, 7] These partially reduced oxygen species may be generated from a series of reactions that begin with the accumulation of xanthine, hypoxanthine, NAD(P)H, and low molecular weight free iron during of hypoxia.[1, 8] Reperfusion of the affected tissue then supplies oxygen to drive these reactions.[1-3] Xanthine oxidase, an enzyme present in virtually every tissue of the body, may be very important in the generation of superoxide, and iron may catalyse the formation of hydroxyl radical during reperfusion of transiently ischaemic tissues.

Support for this mechanism of injury has accumulated in recent years through reports of protection against reperfusion injury with compounds that scavenge free radicals or inhibit various steps in their formation. Superoxide dismutase plus catalase protected the *in vivo* dog heart,[9, 10] and the isolated rabbit heart,[11] from reperfusion injury when administered before the period of reperfusion. Allopurinol, a xanthine oxidase inhibitor, plus superoxide dismutase protected the in situ dog heart from reperfusion injury after 90 minutes of regional ischaemia.[12] Unlike most clinical situations, however, these studies tested the effects of drugs administered before the period of ischaemia. Practical prevention of reperfusion injury in the setting of coronary thrombolysis, for example, would require that the protective drug be given after the onset of ischaemia, just before reperfusion. It is therefore important to determine if treatment administered after the ischaemic injury, at the onset of reperfusion, can provide the same protection that pretreatment provides.

Previous studies in our laboratory have provided support for the ability of drugs to reduce reperfusion injury when given after the period of ischaemia, at the onset of reperfusion. Deferoxamine, an iron chelating agent, or allopurinol, a xanthine oxidase inhibitor, increased survival in a rat model of cardiorespiratory arrest and resuscitation when administered after the return of spontaneous circulation.[13, 14] The present study was conducted to determine if chelation of free iron by deferoxamine, or inhibition of superoxide production by the xanthine oxidase inhibitor, oxypurinol, are effective means of protecting against reperfusion injury when administered after ischaemia, at the onset of reperfusion.

## Materials and methods

### ISOLATED HEART MODEL

A Langendorff isolated rat heart preparation was used in this study.[15] After anaesthesia with ketamine hydrochloride (90 mg/kg, ip), the heart and proximal aorta were excised from each rat, quickly weighed, and immersed in ice cold perfusion solution. Myocardial contractions ceased within 5 sec. The aortic root was then cannulated and perfusion begun. Coronary artery perfusate entered the aortic root and coronary arteries from a reservoir set at 100 cm above the cannula to provide a perfusion pressure of 76 mmHg. A cardiac electrogram was recorded by connecting a

cardiac preamplifier to a pin electrode at the apex of the heart and to the metal perfusion cannula and displayed on a Physiograph recorder. The perfusion medium was a modified Krebs-Henseleit solution consisting of (mmol/litre): KCl 4.0, NaCl 100, $CaC1_2$ 2.0, $NaH_2PO_4$ 2.0, and lactate 30, warmed to 37 °C. The perfusate was adjusted to a pH of 7.4 with NaOH, and bubbled with 100% $O_2$ after the heart had been mounted on the perfusion apparatus, a stab incision was made in the left ventricular apex and a 1.0 cm long polyethylene drain inserted to allow exit of accumulated Thebesian venous drainage.

**EXPERIMENTAL DESIGN**

Thirty male Wistar rats, each weighing between 350-450 g, were divided into three groups of 10 each: saline treated, deferoxamine treated, and oxypurinol treated. The isolated heart preparation from each rat was allowed a 15 min period of stabilisation, followed by 60 min of total ischaemia, during which time the heart was immersed in a nitrogen bubbled bath of Krebs-Henseleit solution at 37 °C to minimise oxygen reactions at the epicardial surface. The heart was then reperfused for 60 min with oxygenated perfusate. The deferoxamine treated hearts were reperfused with perfusate that contained 0.61 mmol/litre deferoxamine mesylate. The oxypurinol treated hearts were reperfused with perfusate that contained 1.3 mmol/litre oxypurinol. The saline treated hearts were reperfused with Krebs-Henseleit solution that contained a volume of isotonic saline equivalent to the volume of drug solution added in the other groups (2 ml).

ASSESSMENT OF INJURY

Myocardial injury was evaluated by three criteria: changes in coronary vascular resistance, changes in creatine phosphokinase release into the effluent, and changes in morphology. The rate of perfusate flow through the myocardium was measured by timed collection, and aliquots of effluent were reserved for creatine phosphokinase determination immediately after cannulation, at 5 min and 1 min before ischaemia, at 1 min and 5 min after the beginning of reperfusion, and at 10 min intervals thereafter.

The myocardial vascular resistance was calculated by dividing the pressure (kPa where 1 kPa = 7.5 mmHg) by the flow rate (litre/min) and was expressed as kPa-min/litre. The creatine phosphokinase assay was based on a modification of the method of Rosalkii[6] and was expressed as Sigma units (SU) released per minute per gram of tissue. One SU of creatine phosphokinase will phosphorylate one nanomole of creatine per minute at 25 °C under the conditions of the test. Triplicate control assays were performed with oxypurinol, deferoxamine, creatine phosphokinase standard, and experimental samples to ensure that these drugs, or the Krebs-Henseleit solution itself, did not interfere with creatine phosphokinase activity. There was less than 3% variation in measured creatine phosphokinase activity in any of these quality control tests. The creatine phosphokinase assays were all performed within 2 hours of collection.

The morphological evaluation of the myocardium used specimens collected at the end of reperfusion (t =+60 min). The heart was fixed by perfusion with Trump's solution (a mixture of 2% glutaraldehyde and 10% buffered formalin), followed by collection of standardised sections from the mid-left ventricular free wall for ultrastructural examination. These sections for electron microscopy were then stored in cold Trump's solution. Standard procedures were used for the

preparation of this tissue for transmission electron microscopy. Morphometric studies were done to measure the mitochondrial cross sectional area, and a qualitative assessment was made of other morphological features such as membrane integrity, intracellular oedema and myofibrillar architecture. The morphometric studies used the Video Image Analysis System (WAS; SciCom Computer Consultants Co, Irvine, CA, USA) and a 10 x 10 cm grid, which was placed over each of five randomly selected photomicrographs (X 8250 magnification) of tissue that was taken from the mid-left ventricular free wall of each heart. Systematic measurement of the cross sectional area of the mitochondria closest to 25 preselected points on each grid was done. Therefore, a total of 125 mitochondria from each heart were evaluated. A mean (± SEM) value was then calculated for each group for subsequent statistical comparison, Serial transverse sections of the remaining heart tissue from each preparation were fixed in 10% formalin then later embedded in paraffin, sectioned, and stained with haematoxylin and eosin for light microscopical examination.

Unlike the serial determinations of creatine phosphokinase release and vascular resistance, the morphological evaluation of the hearts could be done only at a single point in time. Therefore, four separate groups of three hearts each were evaluated as controls against which the three treated groups could be compared. Control group 1 was examined after the 15 min stabilisation period with no subsequent ischaemia or reperfusion. Control group 2 was allowed a 15 min period of stabilisation then subjected to 60 min of ischaemia without any reperfusion before fixation. Control group 3 was subjected to 60 min of ischaemia followed by 60 min of reperfusion without any treatment. Control group 4 was allowed to beat continuously for the 135 min of the study with no period of ischaemia.

### STATISTICAL ANALYSIS

The mean values for the change in myocardial vascular resistance, the creatine phosphokinase measurements, and the mitochondrial cross sectional area were determined for each group of rats at each collection time. Student's t test was used (two tailed analysis) to test the null hypothesis that there was no difference for these values between each drug treated group and the saline treated group. In addition, an analysis of variance (ANOVA)[17] was performed for the mean mitochondrial cross sectional area for the three treated groups and the four control groups. A p value of 0.05 was considered significant.

## Results

### VASCULAR RESISTANCE

The mean vascular resistance increased for all groups during the 60 min of reperfusion, compared with that just before ischaemia (t = -1 min), but the increase was significantly greater in the saline treated group than in the drug treated groups. The vascular resistance (kPa-min/litre) increased by 2870 (± 273) for the saline treated group, by 722 (± 282) for the deferoxamine treated group ($t = 3.50$, $df = 18$, $p < 0.01$) , compared with the saline treated group), and by 549 (± 141) for the oxypurinol treated group ($t = 3.94$, $df = 18$, $p < 0.01$, compared with the saline treated group). There was no significant difference between the deferoxamine treated and the

oxypurinol treated groups. The sequential vascular resistance values, determined at each collection time, for each group are shown in Figure l (a).

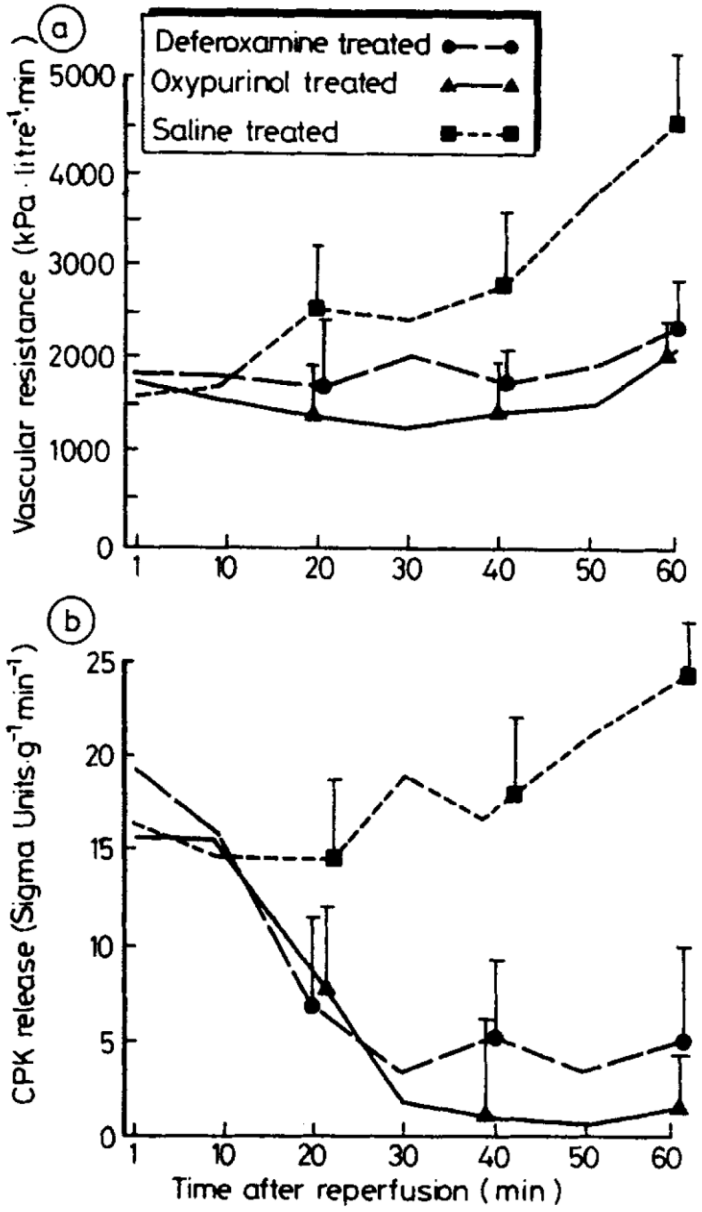


**FIG 1** ***(a) Sequential mean(±SEM) myocardial vascular resistance and (b) sequential mean (±SEM) values for creatine phosphokinase for the three groups during the period of reperfusion. For clarify, solid symbols for data points are omitted at times 1 and 10 min.***

CREATINE PHOSPHOKINASE RELEASE

The mean total creatine phosphokinase released during the entire reperfusion period was significantly greater for the saline treated group than for either of the drug treated groups. The total creatine phosphokinase released (SU per gram per 60 min of reperfusion) was 1137 (± 311) for the saline treated group, 452 (± 209) for the deferoxamine treated group ($t = 4.10$, $df = 18$, $p < 0.01$, compared with the saline treated group), and 231 (± 200) for the oxypurinol treated group ($t = 4.21$, $df = 18$, $p < 0.01$, compared with the saline treated group). There was no significant difference between the drug treated groups. The sequential creatine phosphokinase release (namely, creatine phosphokinase released at each collection time, as opposed to total creatine phosphokinase released) is shown in Figure 1 (b).

MORPHOLOGICAL FINDINGS

There were no observable differences in the light microscopical appearance of the three treatment groups or the four control groups. All hearts showed an occasional indistinct loss of cross striations but no definitive evidence of myodegeneration. However, the ultrastructural features showed pronounced alterations. The mitochondrial cross sectional area of all groups is given in the table. The values for control group 1 (pre-ischaemia), control group 2 (ischaemia and no reperfusion), and control group 4 (no ischaemia and continuous perfusion) were significantly less than all other groups. However, both the deferoxamine treated and the oxypurinol treated groups had significantly smaller mitochondrial cross sectional area than either control group 3 (ischaemia and reperfusion) or the saline treated group. All groups that were subjected to ischaemia showed increased interfibrillar space suggestive of intracellular oedema. Control group 3 (ischaemia and reperfusion) and the saline treated group also showed pronounced distortion of mitochondria1 cristae, myofibrillar disruption, and sarcoplasmic reticulum membrane damage (Figure 2). Both of the drug treated groups showed moderate amounts of intracellular oedema but no evidence of ruptured membranes.

**TABLE** ***Mitochondrial cross sectional area. Values are mean (±SEM)***

| *Group* | *Cross sectional area ($\mu m^2$)* |
|---|---|
| Control 1 | 1.19(0.04)† |
| Control 2 | 1.41(0.14)† |
| Control 3 | 2.50(0.10) |
| Control 4 | 1.39(0.13)† |
| Saline treated | 2.50(0.13) |
| Deferoxamine treated | 2.14(0.09)*† |
| Oxypurinol treated | 1.85(0.12)*† |

Control groups: 1— preischaemia; 2 — ischaemia/no reperfusion; 3 — ischaemia/reperfusion/no treatment; 4 — no ischaemia/continuous perfusion.
†$p<0.01$, significantly different from the value for control group 3 and the saline treated group.
*$p<0.01$, significantly different from the value for control group 1.

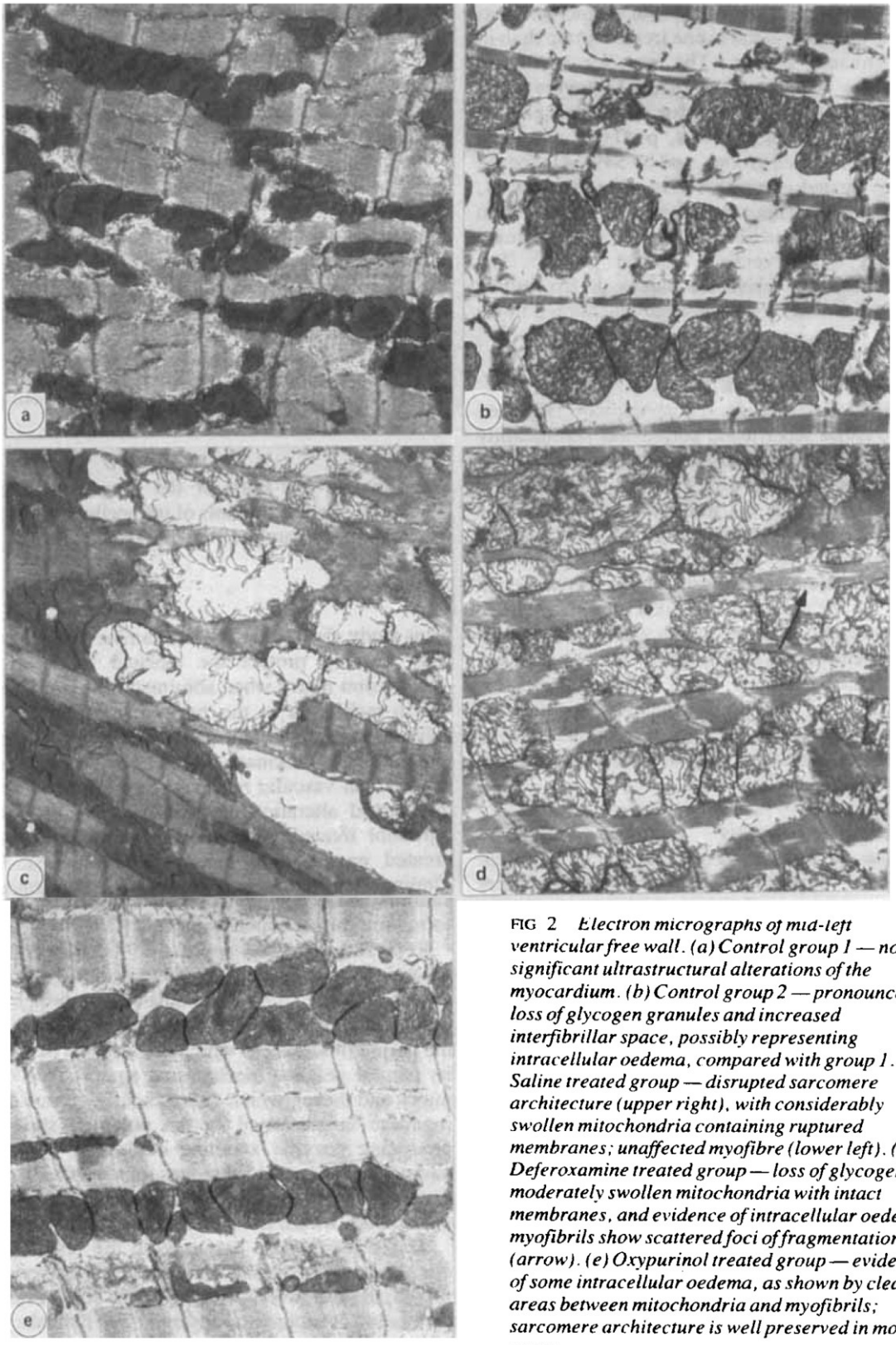


FIG 2 *Electron micrographs of mid-left ventricular free wall. (a) Control group 1 — no significant ultrastructural alterations of the myocardium. (b) Control group 2 — pronounced loss of glycogen granules and increased interfibrillar space, possibly representing intracellular oedema, compared with group 1. (c) Saline treated group — disrupted sarcomere architecture (upper right), with considerably swollen mitochondria containing ruptured membranes; unaffected myofibre (lower left). (d) Deferoxamine treated group — loss of glycogen, moderately swollen mitochondria with intact membranes, and evidence of intracellular oedema myofibrils show scattered foci of fragmentation (arrow). (e) Oxypurinol treated group — evidence of some intracellular oedema, as shown by clear areas between mitochondria and myofibrils; sarcomere architecture is well preserved in most areas.*

## Discussion

This study showed that deferoxamine and oxypurinol can partially protect the isolated rat heart from reperfusion injury when administered after 60 min of warm ischaemia at the beginning of recirculation. Myocardial protection was evident by diminished creatine phosphokinase release, smaller increases in myocardial vascular resistance, and less severe morphological alterations in the deferoxamine and oxypurinol treated groups compared with the saline treated or the non-treated control groups. These findings lend additional support for the concept of xanthine oxidase mediated, iron catalysed free radical formation during reperfusion injury.

Reperfusion injury appears to be dependent on several events, some of which begin during the period of ischaemia.[18] These events can be thought of as occurring in three phases. The first phase consists of the period of anoxia in which the tissue is depleted of its natural defence mechanisms against free radical injury and during which endogenous xanthine dehydrogenase is converted to the potential superoxide producing enzyme xanthine oxidase.[1, 19] Reducing equivalents and other substrates for superoxide radical generation such as hypoxanthine and xanthine accumulate within the cells during this phase.[1]

The second phase begins with the restoration of circulation to ischaemic tissue and is characterised by the rapid and overwhelming, but still reversible, generation of superoxide and other free radicals. These free radicals accumulate in an environment now depleted of its natural defence mechanism[4, 6] Iron may play a key role at this stage of injury by converting the relatively innocuous superoxide radicals into the strongly oxidising hydroxyl radical via the superoxide driven, iron catalysed Haber-Weiss reaction.[20, 21]

The third phase is irreversible and begins with the initiation and propagation of cell protein and membrane lipid peroxidation by the free radical species (especially the hydroxyl radical) generated during phase two. This phase of injury is characterised by pronounced morphological and functional changes of the tissue. The present study was based on this stepwise hypothesis for the pathogenesis of reperfusion injury and the known action of oxypurinol (inhibition of the xanthine oxidase produced in phase one) and deferoxamine (inhibition of iron catalysed reactions in phase two).

Allopurinol, a less water soluble inhibitor of xanthine oxidase than oxypurinol, has been shown to provide protection in animal models of cardiorespiratory arrest,[14] intestinal ischaemia,[22] myocardial infarction,[11, 12] haemorrhagic shock,[23, 24] skin grafts,[25] and kidney transplantation.[26, 27] However, except for the cardiorespiratory arrest studies, the allopurinol was administered before the ischaemic insult and was not analogous to most clinical situations of ischaemia and reperfusion.

Deferoxamine has been shown to increase the survival of rats after cardiorespiratory arrest and resuscitaton when given after the return of spontaneous circulation . In addition, Myers and colleagues have shown that deferoxamine, given during the hypoxic period, could decrease creatine phosphokinase release from isolated rabbit hearts after 60 min of warm ischaemia.[28]

The results of the present study add additional support for the therapeutic effectiveness of a xanthine oxidase inhibitor and an iron chelator in reperfusion injury and support the theory of xanthine oxidase mediated, iron catalysed free radical production in reperfusion injury. It is noteworthy that this protection is achieved with the drug administered at a clinically relevant time (namely, after the ischaemic insult and during reperfusion).

Alternative explanations for the effectiveness of oxypurinol and deferoxamine should be considered. Xanthine oxidase inhibitors such as oxypurinol and deferoxamine may act by conserving purine nucleotide pools in ischaemic and reperfused tissue, thus providing a potential source of intracellular energy substrates. One may also speculate that these drugs have membrane stabilising effects. The lack of an assay directly to measure free radicals within tissues has forced investigators to make conclusions based on associations with suspected free radical events, rather than by directly measured cause and effect relations.

The importance and extent of myocardial reperfusion injury in clinical practice are unknown. However, the increased use of coronary artery thrombolysis and coronary angioplasty suggests that this type of injury may become more common. The delayed and progressive nature of this injury indicates that there is an opportunity for therapeutic intervention in the acute postischaemic period. An effective treatment for reperfusion injury would have the potential to reduce the significant morbidity and mortality that occurs in the days and weeks that follow such conditions as coronary artery occlusion followed by thrombolysis or cardiac arrest with resuscitation.

## References

1 McCord JM. Oxygen-derived free radicals in postischemic tissue injury. N Engl J Med 1985;312:159-63.

2 Hearse DJ, Humphrey , Nayler WG, et al. Ultrastructural damage associated with reoxygenation of the anoxic myocardium. J Mol Cell Cardiol 1975;7:315-24.

3 Meerson FZ, Kagan VE, Kozlov YP, et al. The role of lipid peroxidation in pathogenesis of ischemic damage and the antioxidant protection of the heart. Basic Res Cardiol 1982;77:465-85.

4 Hearse DJ, Humphrey SM, Bullock GR. The oxygen paradox and the calcium paradox: two facets of the same problem? J Mol Cell Cardiol 1978;106:41-68.

5 Hess ML, Manson NH. Molecular oxygen: friend and foe. The role of the oxygen free radical system in the calcium paradox, the oxygen paradox, and ischemia reperfusion injury. J Mol Cell Cardiol 1984;16:969-85.

6 Guarnieri C, Flamigni F, Caldarera CM. Role of oxygen in the cellular damage induced by re-oxygenation of hypoxic heart. J Mol Cell Cardiol 1980;12:797-808.

7 Hammond B, Hess ML. The oxygen free radical system: potential mediator of myocardial injury. J Am Coll Cardiol 1985;6:2 15-20.

8 Krause GS, Joyce KM, Nayini NR, et al. Cardiac arrest and resuscitation: brain iron delocalization during reperfusion. Ann Emerg Med 1985;11:1037-43.

9 Przyklenk K, Kloner RA. Superoxide dismutase plus catalase improve contractile function in the canine model of the "stunned myocardium". Circ Res 1986;58:148-56.

10 Jolly SR, Kane WJ, Bailie GD, er al. Canine myocardial reperfusion injury. Its reduction by the combined administration of superoxide dismutase and catalase. Circ Res 1984;54:277-85.

11 Shlafer M, Kane PF, Kirsh MM. Superoxide dismutase plus catalase enhances the efficacy of hypothermic cardioplegia to protect the globally ischemic, reperfused heart. J Thorac Cardiovasc Surg 1982;83:830-39.

12 Werns SW, Shea MJ, Mitsos SE, et al. Reduction of the size of infarction by allopurinol in the ischemic-reperfused canine heart. Circulation 1986;3:518-24.

13 Kompala SD. Babbs CF. Effect of deferoxamine on late deaths following CPR in rats. Ann Emerg Med 1986;15:405-7.

14 Badylak SF, Babbs CF, Kougias C, et al. The effect of allopurinol and dimethylsulfoxide upon long term survival in rats following cardiorespiratory arrest and resuscitation. Am J Emerg Med 1986;4:313-8.

15 Langendorff O. Untersuchungen am uberlebender Saugertierherzen. Pfliigers Archiv fur die gesamte Phvsiologiedes Menschen undder Tiere. 1895;61:291-332.

16 Rosalki SB. An improved procedure for serum creatine phosphokinase determination. J Lab Clin Med 1967;69:696-705.

17 Dunn OJ. ed. Basic statistics: a primer for the biomedical sciences. New York: John Wiley 1977;122-35.

18 Babbs CF. Role of iron ions in the genesis of reperfusion injury following successful cardiopulmonary resuscitation: preliminary data and a biochemical hypothesis. Ann Emerg Med 1985;14:777-83.

19 Roy RS, McCord JM. Superoxide and ischemia: conversion of xanthine dehydrogenase to xanthine oxidase. In: Greenwald R. Cohen G. eds. $O_2$ radicals and their scavenger systems. vol 2, cellular and molecular aspects. New York: Elsevier Science, 1983:145-53.

20 Aust SD, Svingen BA. The role of iron in enzymatic lipid peroxidation. In: Free radicals in biology. New York: Academic Press. 1982.

21 Morehouse LA, Thomas CE. Aust SD. Superoxide generation by NADPH-cytochrome P-450 reductase, the effect of iron chelators and the role of superoxide in microsomal lipid peroxidation. Arch Biochem Biophys 1984;232:336-77.

22 Granger DN, Rutili G, McCord JM. Superoxide radicals in feline intestinal ischemia. Gastroenterology 1981;81:22-9.

23 Crowell JW, Jones CE, Smith EE. Effect of allopurinol on hemorrhagic shock. Am J Physiol 1969;216:744-48.

24 Cunningham SK, Keaveny TV. Effect of a xanthine oxidase inhibitor on adenine nucleotide degradation in hemorrhagic shock. Eur Surg Res 1978;10:305-13.

25 Manson PN, Anthenelli RM, Im MJ, et al. The role of oxygen-free radicals in ischemic tissue injury in island skin flaps. Ann Surg 1983;198:87-90.

26 Owens ML. Lazarus HM, Wolcott MW, et al. Allopurinol and hypoxanthine pretreatment of canine kidney donors. Transplantation 1974;17:424-27.

27 Toledo-Pereya LH, Simmons RL. Najarian JS. Effect of allopurinol on the preservation of ischemic kidneys perfused with plasma or plasma substitutes. Ann Surg 1974;180:780-82.

28 Myers CL, Weiss S J . Kirsh MM, et al. Involvement of hydrogen peroxide and hydroxyl radical in the oxygen paradox: reduction of creatine kinase release by catalase, allopurinol or deferoxamine, but not by superoxide dismutase. J Mol Cell Cardiol 1985;17:675-84.

29 Earnest MP. Yamell PR, Memll SL, et al. Long term survival and neurological status after resuscitation from out-of-hospital cardiac arrest. Neurology 1980;30:1298-305.