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FREE RADICALS IN HYPOTHERMIC RAT HEART PRESERVATION – PREVENTION OF DAMAGE BY MANNITOL AND DESFERRIOXAMINE

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The free radical mediated oxidation of proteins in stored hypothermic rat hearts has been investigated by the estimation of fluorescence (Ex 360, Em 440–460 nm) believed to result from the oxidation of tryptophan residues. Damage occurring during ischaemia results in the release of oxidised proteins. This damage is significantly attenuated by mannitol and desferrioxamine although the mechanism of action of the two compounds may be different.

KEY WORDS: heart, ischaemia, fluorescence, protein oxidation, mannitol, desferrioxamine.

INTRODUCTION

Considerable indirect evidence suggests that increased oxygen free-radical activity may be partly responsible for tissue damage in ischaemic anoxia.[1–3] Among the possible clinical implications of such a mechanism is the potential effect of free-radical scavangers on organ preservation for transplant surgery. It has been shown in recent years that oxygen free radical activity (though probably not superoxide or hydrogen peroxide) leads to changes in the fluorescence properties of proteins mainly as a result of the stepwise oxidation of tryptophan and, to a lesser extent, of other aromatic amino-acid residues.[4,5] The fluorescence generated (Ex 360, Em 440–460 nm) is readily distinguishable from the native fluorescence of proteins (Ex 280–290, Em 330–360 nm). This free-radical marker was used in the present investigation designed to explore the possible effect of mannitol and of desferrioxamine on the survival of hypothermic rat hearts.

METHODS

Heart perfusion

Isolated rat hearts were perfused on a modified Langendorff column using methods previously described[6] in three experimental groups. Following excision all hearts were

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immediately perfused (all hearts immediately resumed spontaneous contractility) at 37°C with Krebs-Henseleit solution (equilibrated with 95% O_2: 5% CO_2) for 20 min via the aortic cannula. The initial 50 ml of perfusate was discarded to remove blood contamination. The hearts were subjected to cardioplegia by the introduction of chilled cardioplegic solution via the aortic cannula and stored for 4 hr in St Thomas's cardioplegic solution at 0–2°C in ice. Subsequent reperfusion (the first 50 ml of perfusate was again discarded) was performed at 37°C for 20 min with: (1) Krebs–Henseleit solution; (2) Krebs–Henseleit solution + 100 mmol/l D-mannitol; or (3) Krebs–Henseleit solution + 3 mmol/l desferrioxamine mesylate. The perfusates were sampled serially at 5 min intervals for 20 min both before and after storage. In all three groups, spontaneous contractility resumed as the organs were perfused with Krebs solution at 37°C. No attempt at functional assessment of the hearts was made during Langendorff perfusion. The perfusates were stored at −20°C before analysis.

Fluorescence analysis

Perfusate specimens were thawed at room temperature and centrifuged at 2000 g for 10 min. Fluorescence spectra were recorded on a Perkin-Elmer MPF − 3 L scanning spectrofluorimeter and are reported uncorrected relative to a Perkin-Elmer block standard (block no 5, arbitrarily defined as 100 fluorescence units on sensitivity scale XI at Ex 400, Em 475 nm with excitation and emission slits of 12 and 14 nm respectively). All fluorescence intensities were recorded at 20°C. Two fluorescence measurements were made:

Ex 286, Em 340 nm – protein native fluorescence due primarily to tryptophan and tyrosine residues. Perfusate protein concentrations were estimated by comparing the fluorescence intensities of the perfusate specimens with those of standard protein solutions (0.5, 1.0, 2.0, 5.0 and 10.0 mg/l)

Ex 360, Em 440–460 nm – fluorescence thought to be generated by the free-radical or singlet-oxygen mediated oxidation of tryptophan residues. Fluorescence was expressed in arbitrary units and as arbitrary units/mg protein.

In both cases the fluorescence of the appropriate blanks (cardioplegic or Krebs–Henseleit solution) with and without the addition of desferrioxamine and mannitol were subtracted from the specimen values. All subsequent references to 'fluorescence' (unless otherwise indicated) are to fluorescence with the characteristics of Ex 360, Em 440–460 nm.

TABLE I

Effect of dialysis on the fluorescence (Ex 360, Em 440–460 nm) intensity of rat-heart reperfusion (t = 20 min) fluids

Specimen	Fluorescence Intensity (Ex 360, Em 440–460 nm)	
	Pre-Dialysis	Post-Dialysis
1	8.0	9.5
2	10.5	9.5
3	14.5	15.0
4	4.5	6.0
5	20.5	18.5

Dialysis

5 ml aliquots of reperfusion fluid (taken after 20 min reperfusion) from 10 experiments, were exhaustively displayed (Visking Tubing, Gallenkamp, London, UK., exclusion limit 13 × 10^3 daltons) at 4°C for 48 hrs with 2 changes of dialysis fluid (Krebs–Henseleit solution). Full fluorescence spectra were recorded before and after this procedure.

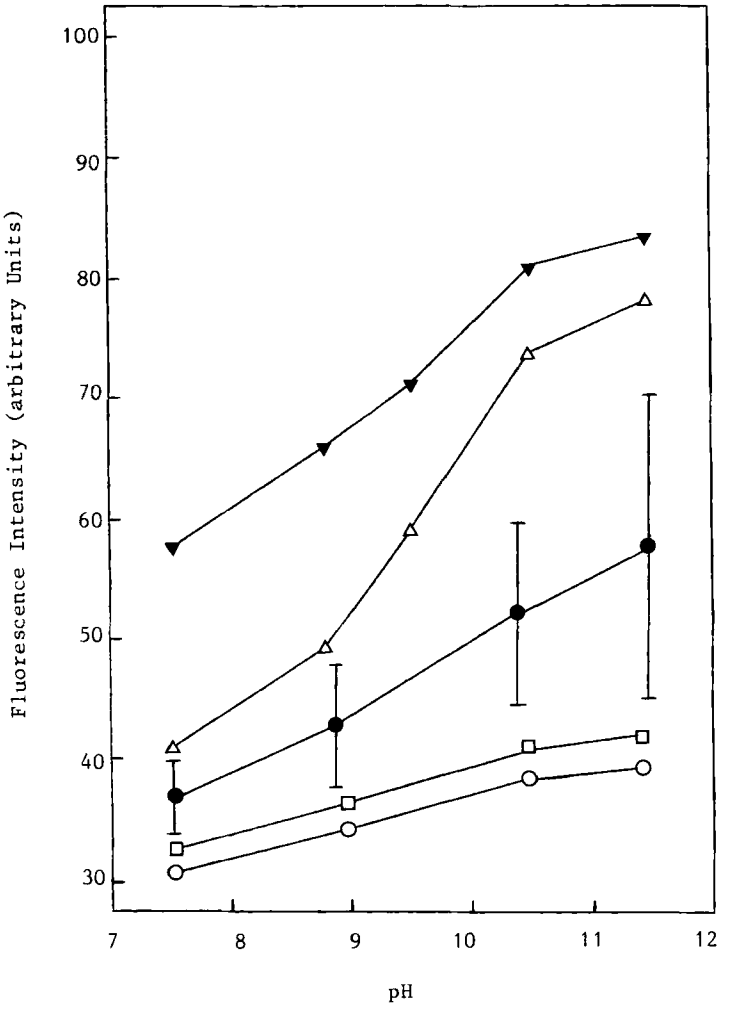
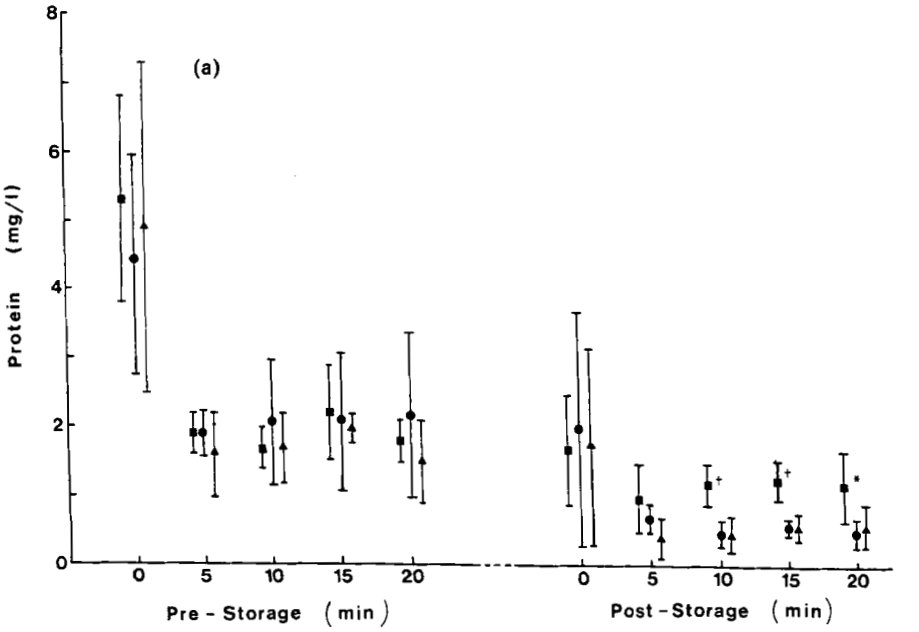
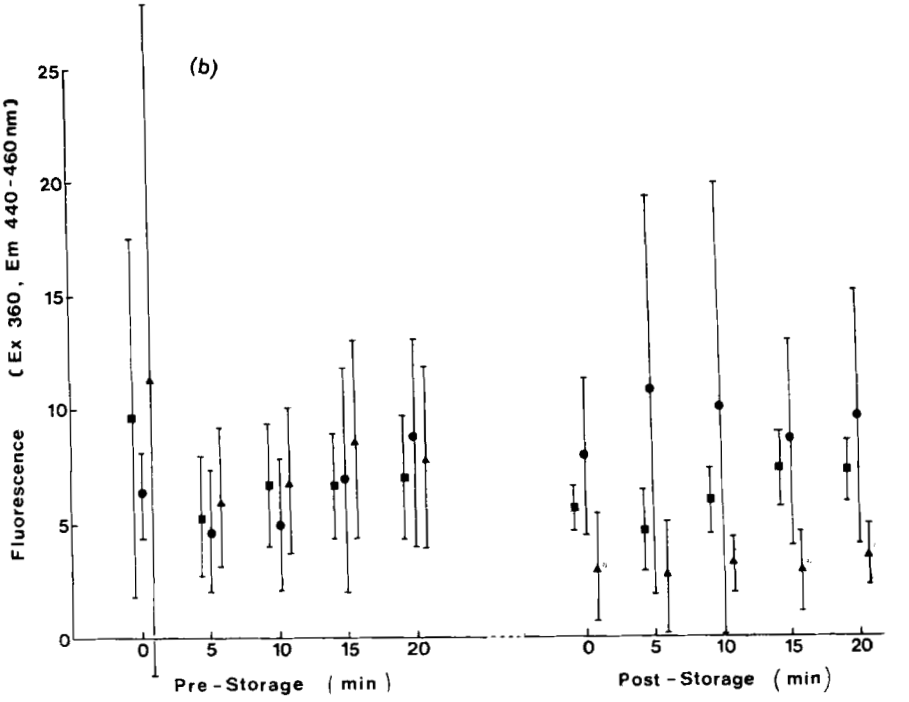


FIGURE 1 Effect of alkaline pH on the fluorescence intensity (Ex 360, Em 440–460 nm) of concentrated reperfusion fluid (●, n = 10, mean ± SD). Also shown is the effect of alkaline pH on the fluorescence (Ex 360, Em 440–460 nm) generated in pure tryptophan (1 mmol/l) and human gamma-globulin (2.5 g/l) solutions by UV-irradiation (▼, □ respectively) and a Cu^{2+}/H_2O_2 system (△, ○ respectively). pH changes were achieved by 10 μl additions of 1 mol/l NaOH to 3 ml of solution.





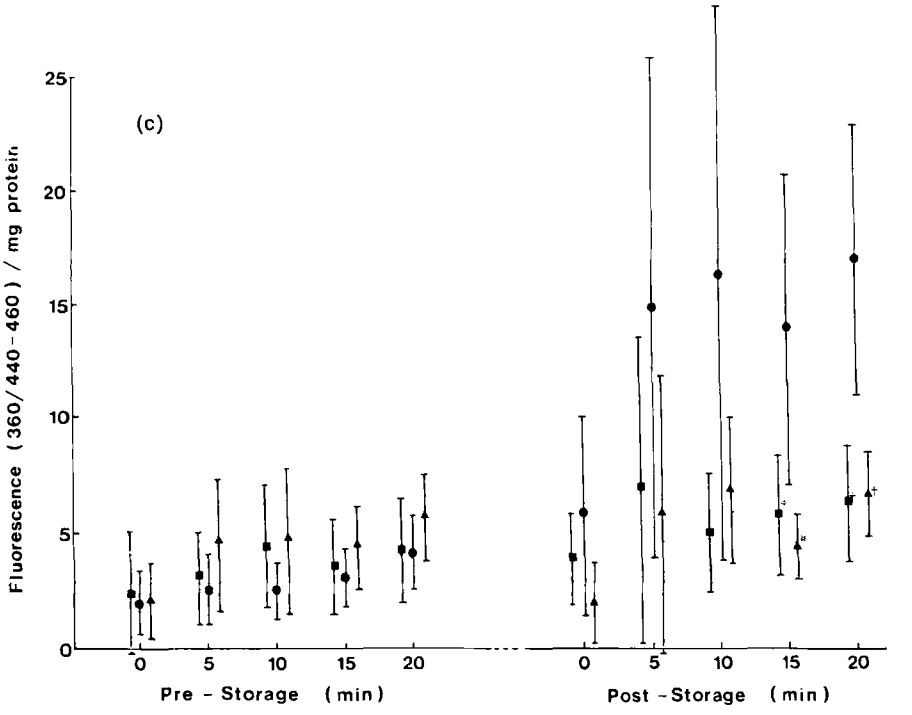


FIGURE 2 Levels of protein (a), fluorescence (Ex 360, Em 440–460 nm) (b) and fluorescence per mg protein (c) in rat-heart perfusate fluids pre (Krebs-Henseleit solution) and post 4 hr storage at 4°C. Reperfusion was performed in Krebs–Henseleit solution (n = 5, ●) and with the addition of 100 mmol/l mannitol (n = 5, ■) or 3 mmol/l desferrioxamine mesylate (n = 6, ▲). Results are expressed as mean ± SD. Significant differences compared to control, *0.05 > p > 0.01, +0.01 > p > 0.001.

Incubation with europium tri(2,2,6,6-tetramethylheptane-3-5-dionate) (europium (thd)$_3$)

Europium (thd)$_3$ (in methanol) was added to 3 ml aliquots of reperfusion fluid (taken after 20 min reperfusion) to give a final concentration of 50 μmol/l. Methanol was added to other aliquots as a control. Full fluorescence spectra were recorded. This was performed on reperfusion fluid from 10 experiments. Human gamma-globulin (2.5 g/l) and tryptophan (1 mmol/l) that had been exposed to UV-irradiation or incubation with Cu^{2+}/H_2O_2 as described[4] were similarly treated.

Incubation with alkali

The effect of alkaline pH on fluorescence intensity was investigated by the addition of 10 μl volumes of 1 mmol/l NaOH to 3 ml of reperfusion fluid (taken after 20 min reperfusion) concentrated by a factor of 20 by ultrafiltration. pH was monitored with a Kent-EIL 7046 digital pH meter. This was performed on reperfusion fluid from 10 experiments. Human gamma-globulin (2.5 g/l) and tryptophan (1 mmol/l) that had been exposed to UV-irradiation or incubation with Cu^{2+}/H_2O_2 were similarly treated.

RESULTS

Fluorescence was demonstrable in over 95% of the pre- and post-storage perfusate fluids. It was non-dialysable (table I) and was unaffected by incubation with europium $(thd)_3$. The fluorescence was appreciably enhanced by alkaline conditions in a similar manner to the fluorescence of tryptophan oxidation products (Figure 1). There was no marked difference between pre and post-storage perfusate protein concentrations (Figure 2a). Mannitol significantly increased the post-storage protein concentrations compared to controls (Figure 2a). Post-storage perfusate contained significantly ($p < 0.001$) elevated levels of fluorescence compared to pre-storage (Figures 2b and 2c). Mannitol and desferrioxamine reduced the absolute levels of fluorescence in post-storage perfusates compared to controls, although this was significant only for desferrioxamine (Figure 2b). Mannitol and desferrioxamine both significantly reduced fluorescence when expressed per mg protein (Figure 2c).

DISCUSSION

Several possible explanations have been advanced to account for the apparent paradox of oxidative free-radical damage caused by anoxia. First, Roy and McCord showed that in certain ischaemic tissues Ca^{2+} dependent proteolysis converts xanthine dehydrogenase to xanthine oxidase.[7] At the same time xanthine and hypoxanthine accumulate as a result of the catabolism of ATP.[8] When, on reperfusion, oxygen is readmitted to the tissue superoxide and hydrogen peroxide are generated. In support of this hypothesis several groups of workers have demonstrated the protective action of infused superoxide dismutase (SOD), catalase and allopurinol (a xanthine-oxidase inhibitor).[9–12] Second, reduced ADP levels will prevent cytochrome oxidase from using oxygen efficiently.[13] This may result in the electron-transport system releasing oxygen free radicals. Third, free arachidonic acid may accumulate in ischaemic tissue.[14] This may lead to an increased susceptibility to oxygen free-radical attack. Fourth, myocardial ischaemia has been shown to trigger complement activation,[15] the generation of chemotactic factors[16] and neutrophil migration.[17] This could lead to the release of free radicals by activated neutrophils and macrophages. Fifth, structural damage due to ischaemia, especially the release of free iron from storage forms, may be expected to favour free-radical activity.[18] Whatever the mechanism, the present experiments provide some support for the hypothesis of increased free-radical activity on reperfusion. In particular fluorescence could be detected in the vast majority of pre- and post-storage perfusate fluids. Simple chemical tests indicated that the fluorophore or fluorophores responsible for this fluorescence are similar to those generated in tryptophan and tryptophan-containing proteins by free radical activity.[4] Schiff bases have similar fluorescence characteristics with excitation and emission maxima in the region of 340–370 and 430–470 nm respectively.[19] However, the fluorescence intensity of these compounds is severely quenched at alkaline pH and reduced by 8–15% by incubation with europium $(thd)_3$.[20] The fluorescence observed in the perfusion fluids (which is enhanced at basic pH and unaffected by incubation with europium $(thd)_3$) cannot therefore be attributed to Schiff bases. Without evidence to the contrary we have attributed the fluorescence observed in rat heart perfusate fluids to the oxidation of protein tryptophan residues.

The post-storage perfusate contained significantly ($p < 0.001$) elevated levels of

fluorescence compared to pre-storage. This increase was not due to increased protein concentrations. These results indicate that more tryptophan residues are oxidised in proteins in post-storage fluids compared to pre-storage. The nature of the proteins contributing to fluorescence is uncertain. It is unlikely that native blood proteins or hemoglobin from lysed red cells contributed as the initial 50 ml of Krebs perfusate passing through the hearts was discarded. It may reflect free-radical damaged cardiac enzymes such as creatine kinase and glycogen phosphorylase which are known to be released from hearts as a consequence of ischaemia.[21]

Mannitol and desferrioxamine both reduce the absolute levels of fluorescence in post-storage fluids compared to controls, the significance of which increases when the data is expressed per mg protein. The results suggest, however, that their modes of action may be quite different. Mannitol appears to stimulate the release of non-oxidised protein thereby producing a low amount of fluorescence when expressed per mg protein. This may be an osmotic effect.[22] Desferrioxamine, on the other hand, produces a 'genuine' decrease in fluorescence.

These results indicate that the monitoring of fluorescence is a sensitive means of assessing protein damage in this model system. Damage occurring during cold ischaemia results in the release of oxidised proteins. This damage is attenuated by mannitol and desferrioxamine although their mechanisms of action may be different.

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