

Plasma antioxidants: evidence for a protective role against reactive oxygen species following cardiac surgery

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SUMMARY. Total plasma antioxidant status (TPAS), lipid peroxide concentration (LPX) and cardiac troponin T (cTnT) were measured in 24 patients undergoing coronary artery bypass grafting (CABG) with cardiopulmonary bypass (CPB). Samples were obtained preoperatively and at 1.5 h, 6 h, 24 h and 72 h after CPB. The absolute TPAS values were significantly lower at 1.5 h, 6 h, 24 h and 72 h after CPB than were preoperative values ($P < 0.05$). The LPX concentration was significantly elevated at 1.5 h after CPB ($P < 0.05$). Cardiac troponin T concentrations were significantly elevated at all time points postoperatively ($P < 0.05$). Preoperative TPAS values were significantly correlated with the magnitude of fall in TPAS at 1.5 h ($P < 0.05$). The greater the fall in TPAS between 0 and 1.5 h, the less LPX was formed between 0 and 1.5 h. The LPX at 1.5 h displayed a significant correlation with cTnT release from myocardial myocytes ($P < 0.05$). These data provide evidence for the first time that the consumption of antioxidants during CABG surgery with CPB protects against the production of reactive oxygen species and subsequent myocyte necrosis. Furthermore, the availability of protective antioxidants is dependent upon preoperative TPAS.

Additional key phrases: total antioxidant status, lipid peroxides, cardiac troponin T

There is increasing evidence to suggest that coronary artery bypass grafting (CABG) with cardiopulmonary bypass (CPB) can result in postoperative complications in the form of reperfusion injury causing myocyte death. Cells reversibly injured at the end of a period of ischaemia become irreversibly damaged at the time of reperfusion. It has been previously demonstrated by the indirect measurement of lipid peroxidation products¹⁻⁴ that the production of reactive oxygen species (ROS) occurs during CPB. During the same time period, the plasma concentration of cardiac troponin T is also significantly increased, indicating myocyte necrosis.⁵

Reactive oxygen species are directly cytotoxic to the myocardial myocytes^{6,7} and it is currently thought that myocardial reperfusion injury centres around both the intracellular and

extracellular production of ROS during cardiac surgery.⁸⁻¹¹ ROS can be generated by activated neutrophils,¹²⁻¹⁵ the electron transport chain of mitochondria, the xanthine oxidase system, high oxygen tension¹⁶ and other, as yet unknown, mechanisms.

There are several ways in which ROS are capable of damaging the myocardium and other organs.¹⁷ In consequence, all organisms possess complex intracellular and extracellular antioxidant defence and repair mechanisms. Oxidative stress¹⁸ occurs when these mechanisms are overwhelmed by the generation of ROS. Oxidative stress can cause damage to human cells by a number of different mechanisms.^{19,20} Polyunsaturated fatty acids and other membrane esters are the most susceptible classes of biomolecule to free radical attack. The resulting lipid hydroperoxide (LPX) is found at a concentration proportional to the amount of ROS.

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The aim of the present study was to assess whether, in patients undergoing CABG with CPB, total plasma antioxidant status (TPAS) could be protective against oxidative stress (as measured by LPX concentration) and against consequent myocyte necrosis [using cardiac troponin T (cTnT) as an indicator].

METHODS

Subjects

Twenty-four patients undergoing CABG with CPB were studied. The mean age for the group was 63 years (range 44-83 years) and comprised 17 men and 7 women. Patients in cardiogenic shock were excluded from the study. Informed consent was obtained from all patients, with the approval of the St Mary's Local Research Ethics Committee.

Operative technique

After median sternotomy and harvesting the internal mammary artery, 300 units/kg body weight of heparin sulphate were intravenously administered. When the activated clotting time was 480 s or more, cardiopulmonary bypass was established between a two-stage venous cannula in the right atrium and the arterial return to the ascending aorta. Additional heparin doses were given every hour to keep the activated clotting time longer than 480 s. A membrane oxygenator (CML membrane fibre oxygenator, Cobe Inc., Denver, Colorado, USA) was used for extracorporeal circulation and the system was primed with 1 L of Ringer's lactate. Myocardial protection was achieved with the use of intermittent fibrillation. Nasopharyngeal temperature was maintained at approximately 33°C during the CPB period. After the completion of each distal anastomosis, the aortic cross-clamp was released and the proximal anastomoses were performed with the heart beating. Systemic rewarming was initiated shortly before the completion of the last distal anastomosis. No banked blood was used during the operation. Up to 5 grafts were performed in each patient, with a mean ischaemia time of 47 ± 3.2 min (range 24-92) and mean CPB time of 99 ± 5.4 min (range 67-174).

Anaesthesia

None of the patients received any drugs known to influence measures of TPAS or LPX activity pre- or postoperatively or during the operation and CPB.

Blood sampling

Peripheral venous blood samples for TPAS, LPX and cTnT were obtained before the operation and 1.5, 6, 24 and 72 h after CPB.

The venous samples were drawn into vacuum tubes containing dry lithium heparin and immediately placed on ice. The plasma was then separated within 30 min of collection by centrifugation (4°C 3000 rpm for 10 min). All samples were collected in the fasting state. The plasma was then frozen at -70°C until analysis. The samples were stored for up to 1 year.

Measurement of plasma lipid hydroperoxide

Lipid hydroperoxide concentrations were measured using the Peroxoquant kit (lipid compatible formulation) purchased from Pierce & Warriner (UK) Ltd, Chester, UK. This assay is based on the Fox 2 assay.²¹ The LPX in the plasma converts ferrous iron to ferric iron at an acidic pH. The ferric iron then complexes with xylenol orange dye to give a chromagen with absorption maximum at 595 nm. For each sample to be tested, a blank was prepared in which the plasma was pretreated with TRIS (2 carboxyethyl)-phosphine hydrochloride [TCEP; Pierce & Warriner (UK) Ltd.] to reduce all the hydroperoxides in the sample. All samples were centrifuged at 12 000 g for 10 min. The absorbance of the supernatant was measured at 595 nm on a Labsystem Mark 2 Plus ELISA plate reader [Life Science International (UK) Ltd, Basingstoke, Hampshire, UK]. The absorbance difference between the 'blank' and 'test' plasma was read from a standard curve generated with 0-15 μ M H₂O₂ (Sigma Chemical Company, Poole, Dorset, UK).

Measurement of total antioxidant status

Total plasma antioxidant status was measured using an assay kit purchased from RANDOX Laboratories Ltd, Ardmore, Crumlin, Co Antrim, UK. The assay was based on a method developed by Miller and coworkers.²² This is a two reagent assay performed on serum or plasma. ABTS [2,2-Azinobis (3-ethyl benzothiazoline 6-sulphonate)] is incubated with a peroxidase (metmyoglobin) and H₂O₂ to produce the radical cation ABTS^{•+}. This has a stable blue-green colour which is measured at 600 nm on the Cobas Bio Version 8326 analyser (Roche Diagnostica, Roche Products Limited, Welwyn Garden City, UK). Antioxidants in the sample cause a suppression of this colour

production to a degree that is proportional to their concentration.

Measurement of cardiac troponin T concentrations

Cardiac troponin T was measured using the Troponin T enzymun test (Cat. no. 1556-428) purchased from Boehringer Mannheim (Lewes, UK), on the Boehringer ES 300 autoanalyser. This is a colorimetric immunoassay using a biotinylated anti-troponin T antibody and anti-troponin T peroxidase conjugate added to a streptavidin-coated vial. After washing, the substrate chromogen solution containing ABTS⁺ and hydrogen peroxide were added and the resulting colour change was measured.

Statistical analysis

Both TPAS and LPX concentrations were normally distributed and two-way analysis of variance was performed to detect significant differences between time points. cTnT values were not normally distributed, therefore changes in cTnT concentration between time points and preoperative values were analysed using the Mann-Whitney U test, a non-parametric analysis. Differences were considered significant at a probability level of *P* less than or equal to 0.05.

RESULTS

Correction for haemodilution

During surgery, the circulating blood volume was diluted with 1 L of Ringer's lactate solution used to prime the CPB pump. The LPX and cTnT were therefore corrected for haemodilution using the formula below.²³ It was necessary to correct the concentrations of these biochemical markers in order to accurately assess their production. However, TPAS was analysed both uncorrected (TPAS) and corrected [TPAS(C)], the former to reflect the absolute concentration of antioxidants to which the tissue is exposed and the latter to assess comparative consumption or production of antioxidant substances within plasma.

$$\text{Corrected concentration} = M \times \frac{(1 - \text{Ht}(T) \text{ after operation})}{(1 - \text{Ht}(0) \text{ before operation})}$$

where M is the measured concentration of either TPAS or LPX or cTnT, Ht(0) is the haematocrit value before the operation, and Ht(T) is the haematocrit value at a particular time point.

Total plasma antioxidant status

During CPB, the absolute concentration of TPAS showed a significant decrease ($P=0.0002$) 1.5 h postoperatively which was sustained for 24 h, with a gradual increase towards preoperative values (see Table 1, Fig. 1). By 72 h, values were significantly higher than at 24 h ($P=0.001$).

When TPAS values were corrected for haemodilution (see Table 1, Fig. 1) the initial fall in concentration was not significant and values increased significantly beyond preoperative values 72 h postoperatively ($P=0.001$), indicating the synthesis or secretion of antioxidants.

Compared with preoperative values there was a marked fall in TPAS by 1.5 h with a slow recovery from 24 h onwards. At 72 h TPAS was still significantly reduced (see Table 1, Fig. 1).

Lipid peroxide concentration and cardiac troponin T

The LPX concentration was significantly elevated 1.5 h after CPB ($P=0.0121$). By 6 h after CPB the concentration had fallen to preoperative values (see Table 1, Fig. 2).

Cardiac troponin T concentrations were significantly elevated at all time points postoperatively, with a peak at 6 h (see Table 1, Fig. 3; $P=0.0001$).

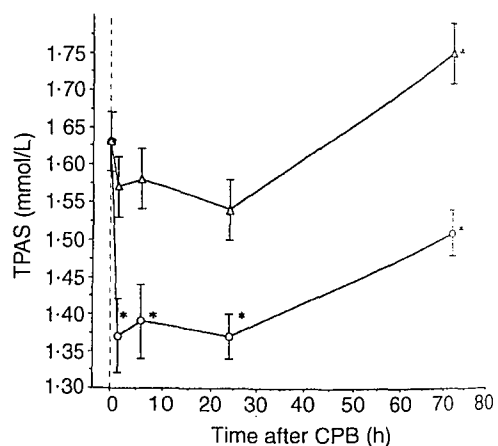


FIGURE 1. Changes in absolute total plasma antioxidant status (TPAS; ○) and volume-corrected TPAS [TPAS(C); △] values (mean ± standard error, *n* = 24) at 1.5, 6, 24 and 72 h after cardiopulmonary bypass (CPB). * = significant difference from preoperative values ($P \leq 0.001$).

TABLE 1. Total plasma antioxidant status (TPAS), corrected TPAS [TPAS(C)], lipid peroxide concentration (LPX), corrected cardiac troponin T [cTnT(C)] and haemoglobin values preoperatively and at 1.5 h and 6 h during cardiopulmonary bypass surgery and at 24 h and 72 h post-operatively in 24 patients

	Preoperative	1.5 h	6 h	24 h	72 h
TPAS (mmol/L)	1.63 ± 0.04	1.37* ± 0.05	1.39* ± 0.05	1.37* ± 0.03	1.51* ± 0.03
TPAS(C) (mmol/L)	1.63 ± 0.04	1.57 ± 0.04	1.58 ± 0.04	1.54 ± 0.04	1.75* ± 0.04
LPX(C) (μmol/L)	4.7 ± 0.54	7.19* ± 0.78	5.56 ± 0.85	5.66 ± 0.63	5.64 ± 0.47
cTnT(C) (ng/ml)	0.015	0.72*	1.39*	1.01*	0.64*
	(0.01–0.04)	(0.36–1.6)	(0.55–3.09)	(0.53–2.16)	(0.33–1.33)
Haemoglobin (g/dL)	13.4 ± 0.5	10.3* ± 0.4	10.7* ± 0.4	11.1* ± 0.3	10.6* ± 0.8

* = significant difference from preoperative values ($P < 0.05$).

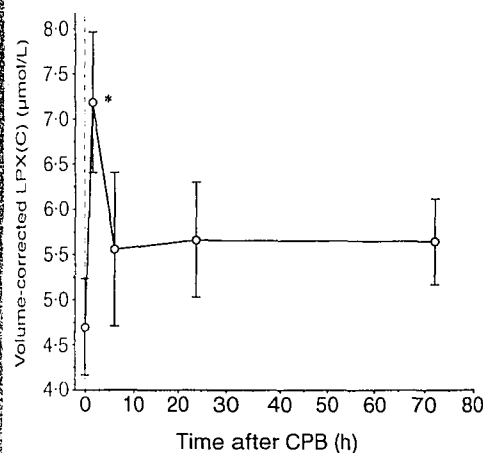


FIGURE 2. Volume-corrected lipid peroxide [LPX(C)] concentration (mean ± standard error, *n* = 24) at 1.5, 6, 24 and 72 h after cardiopulmonary bypass (CPB). * = significant difference from preoperative values ($P < 0.01$).

Correlations between TPAS, LPX and cTnT

There was a significant correlation ($P=0.0018$) between preoperative TPAS and the decrease in TPAS(C) from preoperative values to those measured 1.5 h after CPB (Fig. 4). Therefore, the higher the preoperative TPAS value, the greater the fall in TPAS after 1.5 h (Fig. 4).

The decrease in TPAS(C) from preoperative values (between TPAS(C) time 0 and time 1.5 h) showed a significant correlation with the increase in LPX between time 0 and time 1.5 h (Fig. 5; $P=0.0221$). So, the greater the fall in TPAS(C) between 0 and 1.5 h, the less LPX formed over the same period.

The LPX at 1.5 h displayed a significant correlation ($P=0.0057$) with cTnT release from myocardial myocytes at this time point (Fig. 6).

This correlation was also apparent at 6, 24 and 72 h (data not shown) as well as with the area under the cTnT curve (Fig. 7; $P=0.0005$).

Ischaemia time (CPB time) and TPAS, LPX and cTnT

There was no significant correlation between the fall in TPAS from time 0 to 1.5 h and CPB time or the number of grafts performed. Also, there was no significant correlation between the rise in LPX and cTnT from time 0 to 1.5 h and the CPB time or the number of grafts. There was also no significant correlation between the area under the curve of LPX and cTnT and CPB time or the number of grafts performed.

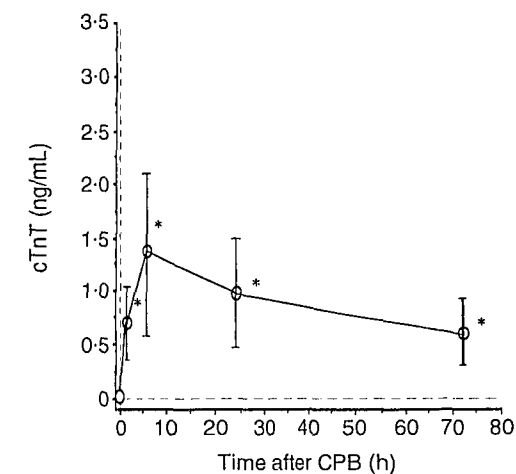


FIGURE 3. Volume-corrected cardiac troponin T (cTnT) concentration (mean ± standard error, *n* = 24) at 1.5, 6, 24 and 72 h after cardiopulmonary bypass (CPB). * = significant difference from preoperative values ($P < 0.0001$).

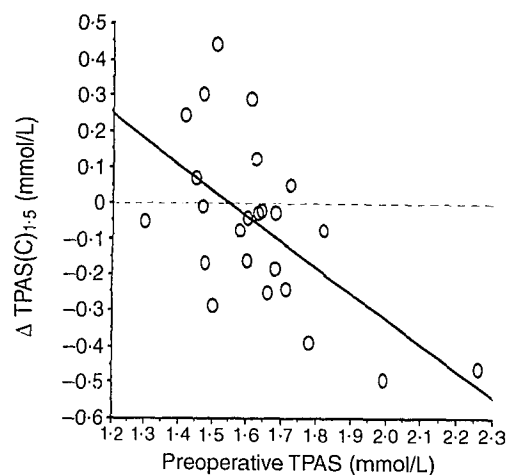


FIGURE 4. Scatterplot and regression analysis showing a significant correlation ($P=0.0018$) between the total plasma antioxidant status (TPAS) and the decrease in volume-corrected TPAS [TPAS(C)] from preoperative to 1.5 h after cardiopulmonary bypass ($n=24$, $r=0.6$).

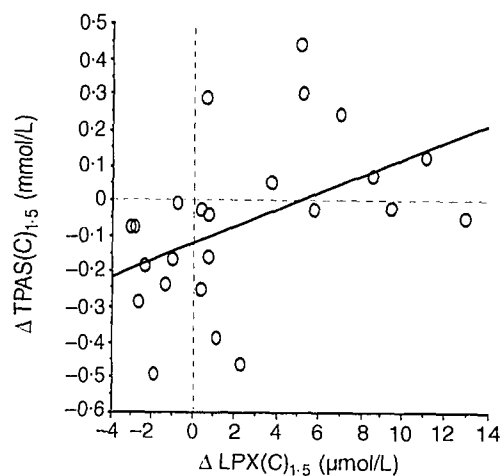


FIGURE 5. Scatterplot and regression analysis showing a significant correlation ($P=0.0221$) between the decrease in volume-corrected total plasma antioxidant status [TPAS(C)] from preoperative values to 1.5 h after cardiopulmonary bypass, and the increase in lipid peroxide (LPX) concentration over the same period ($n=24$, $r=0.46$).

DISCUSSION

During cardiac surgery, exposure of the patient's blood to an extra-corporeal circuit increases the concentration of complement factor C5a^{14,15}

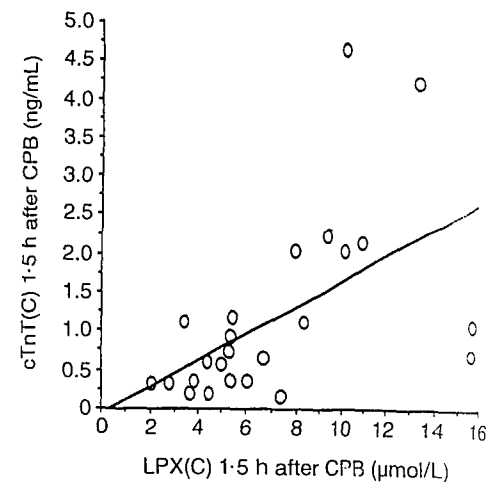


FIGURE 6. Scatterplot and regression analysis showing the correlation between volume-corrected lipid peroxide [LPX(C)] concentration at 1.5 h after cardiopulmonary bypass (CPB) and volume-corrected cardiac troponin T [cTnT(C)] release from myocardial myocytes at that time point ($n=24$, $r=0.55$, $P=0.005$).

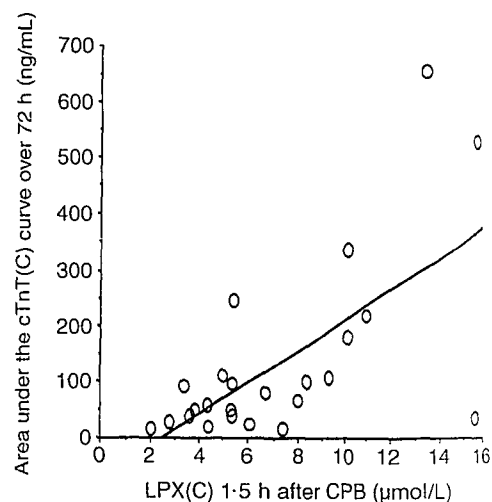


FIGURE 7. Scatterplot and regression analysis showing the correlation between volume-corrected lipid peroxide [LPX(C)] concentration at 1.5 h after cardiopulmonary bypass (CPB) and total volume-corrected cardiac troponin T [cTnT(C)] over 72 h (area under the curve) ($n=24$, $r=0.65$, $P=0.0005$).

which activates neutrophils leading to the liberation of oxygen free radicals (OFR). Neutrophil activation and cytotoxic secretion is thought to be responsible for the majority of

OFR released into the extracellular compartment.²⁴ Also, reperfusion of the myocardium after a period of aortic cross-clamping ischaemia may lead to the release of free radicals from a variety of pathways in monocytes, endothelial cells or both.²⁴

There has been a very limited number of studies demonstrating oxidative stress following the reperfusion of the human heart. One of the original studies was by Ferrari *et al.*²⁵ who reported changes in glutathione in the coronary sinuses plasma during post-ischaemic reperfusion. More recent studies have given an indirect measurement of an increase in OFR activity during CPB in human studies.^{1-6,26,27} However, there have not been any studies giving direct measurement of an increase in oxidative stress during CPB in humans.

In this study, we measured TPAS, LPX and cTnT during CPB. TPAS gives a measure of the combined activity of antioxidant species in plasma. LPX is an indicator of oxidative stress and results from the oxidation of plasma and membrane lipids by ROS. cTnT is an indicator of myocardial myocyte necrosis.

We found the TPAS to be significantly reduced for 72 h following CPB. The reason for this fall in antioxidant status is unclear. When we corrected TPAS values for haemodilution in our study we found that TPAS(C) was depressed for 24 h, although not to a statistically significant level. TPAS(C) was then significantly increased at 72 h compared with preoperative values, implying that plasma antioxidants were regenerated between 1 and 3 days postoperatively.

In contrast to our study, Tovoinen and coworkers² showed that TPAS values measured by the total radical-trapping antioxidant parameter (TRAP) method²⁸ during CPB increased significantly when values were corrected for haemodilution. TPAS in that study was measured at 13 time points: before, during and 1 h after CPB in 6 patients undergoing routine coronary artery surgery. However, when the uncorrected values were taken from the study they show a significant decrease in TPAS from preoperative values until 5 min after CPB. By 1 h after CPB, the TPAS in the Tovoinen study returned to preoperative values, contrary to our findings.

The major difference between our study and the Tovoinen study was the method of TPAS analysis used. We used the ABTS assay, based on a method by Miller *et al.*²² with an intra-

assay coefficient of variation (CV) of 1.69% and an interassay CV of 3.49%. The TRAP assay used by Tovoinen and coworkers has a high degree of inherent imprecision caused by the instability of the oxygen electrode over the sample analysis time which can be up to 2 h.²¹ This may have contributed to the discrepancy in the results between the two studies, particularly as the Tovoinen study used a relatively small number of patients.

The findings of the present study are in agreement with research conducted by Pyles and coworkers²⁹ investigating TPAS over time in children undergoing operations (with CPB) for congenital heart disease. They described a significant depression of TPAS peri- and post-operatively. They also showed that age and bypass time were significantly correlated with TPAS value after bypass. Such relationships were not present in our study, possibly implying that adults respond to CPB in different ways than children. It has been suggested that infants tolerate CPB less well than adults and that differences between adults and children may be related to the maturity of the plasma proteins and their resistance to denaturation in response to CPB.^{30,31}

Plasma LPX concentrations give a simple and reliable index of oxidative stress.²¹ The LPX measured would have included oxidized plasma and membrane lipids attacked by both extra- and intra-cellular sources of ROS.

The preoperative concentrations of LPX in the 24 patients averaged $4.7 \mu\text{mol/L} \pm 0.54$ (mean \pm standard deviation), which compares favourably with studies of healthy control individuals using an identical assay technique.³² In the present study, there was a significant rise in LPX after 1.5 h to a mean value of $7.19 \mu\text{mol/L} \pm 0.78$ (mean \pm standard deviation). Concentrations at all other time points were elevated but not significantly above preoperative values.

Therefore, despite absolute TPAS being significantly depleted for over 72 h, the LPX concentration remained significantly elevated for less than 6 h. It would seem to indicate that during the surgical procedures OFR species are formed rapidly within the first 1.5 h after CPB, reaching a peak before 1.5 h and then falling back to preoperative values by 6 h. The rapid production of ROS in this study is consistent with ROS production following ischaemia and reperfusion radical formation, as well as neutrophil activation and ROS secretion.

The greater the TPAS preoperatively, the greater the depletion of TPAS after 1.5 h. The patients with the greatest fall in TPAS had a lower increase in LPX formation as shown by the significant inverse correlation between the amount of depletion of TPAS preoperatively to 1.5 h after CPB and the increase in LPX concentrations over the same time period. This is the first direct demonstration of a rise in a free radical marker (LPX) with a simultaneous fall in antioxidant activity during cardiac surgery.

Cardiac troponin T values as a specific marker of myocardial myocyte necrosis³³ were significantly increased at all time points when compared with preoperative values.

There was a significant correlation between LPX at 1.5 h and cTnT at 1.5, 6, 24 and 72 h (Figs 6 and 7), giving direct evidence that the degree of oxidative stress is related to the extent of myocyte necrosis.

It seems that a higher TPAS value may be protective against the formation of LPX. The plasma antioxidants may act as a 'free radical sink'.³⁴ These were depleted perhaps because of sacrificial oxidation, leading to the observed fall in TPAS.

Plasma contains many antioxidants including vitamin E, C, beta carotene, transferrin, uric acid, protein thiols and caeruloplasmin.²⁰ Our study indicates that the comparative composition of the plasma antioxidant system may vary in individuals, with an unknown component of the TPAS being highly protective against oxidative stress and consequent cellular necrosis.

Rice-Evans and coworkers used the term 'antioxidant gap' to describe the difference between serum TPAS and the sum of the serum albumin and uric acid activity and found that a decline in 'antioxidant gap' after myocardial infarction was associated with a significantly higher mortality rate.³⁵ The details of the morbidity and mortality of the patients after discharge from hospital are not available; however, the events we have studied during CPB and the work by Rice-Evans show that antioxidant status may have an important role in determining the long-term outcome of the operation.

With future studies, it would be interesting to elucidate the exact changes in differing components of the plasma antioxidant defence system.

Conclusions

This study strongly suggests that a high preoperative TPAS and the consumption of

plasma antioxidants confers some protection against OFR production, LPX generation and subsequent myocardial myocyte necrosis. Our study gives the first direct evidence of an increase in oxidative stress during CABG with CPB, as evidenced by the rise in the free radical marker LPX with a concomitant fall in antioxidant activity during cardiac surgery.

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