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 cells ($P < 0.05$). 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.

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 refractory injury causing myocyte death. Cells reversibly injured at the end of a period of ischaemia become irreversibly damaged at the time of reperfusion. There has been demonstrated by the indirect measurement of lipid peroxidation products3,11 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 myocytes5 and it is currently thought that myocardial reperfusion injury centres around both the intracellular and extracellular production of ROS during cardiac surgery.3 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.

The aim of the present study was to assess whether, in patients undergoing CABG with CPB, total plasma antioxidant status (TPAS) would 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 patient’s aorta. Additional heparin doses were then 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 heparin was used during the operation.

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 stored at -70°C until analysis. 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 Peroxoxquant kit (lipid compatible formulation) purchased from Pierce & Warriner (UK) Ltd, Chester, UK. This assay is based on the Fox 2 assay and 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 an absorbance maximum at 595 nm. For each sample to be tested, a blank was prepared in which the plasma was replaced with distilled water and 0.12 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 μM 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 reagent assay performed on serum or plasma. ABTS [2,2 Azinobis (3 ethyl benzthiazoline-6-sulphonate) is incubated with horseradish 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 8260 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 colorometric 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. 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.

Corrected concentration = $M = \frac{(1 - Ht(0))}{(1 - Ht)}$

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

Total plasma antioxidant status
During CPB, the absolute concentration of TPAS showed a significant decline ($P=0.0002$) 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
Cardiac troponin T concentrations were significantly elevated at all time points postoperatively, with a peak at 6 h (see Table 1, Fig. 3; $P<0.005$).

The LPX concentration was significantly elevated 1.5 h after CPB ($P=0.0012$). By 6 h after CPB the concentration had fallen to preoperative values (see Table 1, Fig. 2).

Correlations between TPAS, LPX and cTnT
There was a significant correlation between preoperative TPAS and the decrease in LPX from time 0 to 1.5 h and the CPB time or the number of grafts. 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.

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.
DISCUSSION

During cardiac surgery, exposure of the patient’s blood to an extra-corporeal circuit increases the concentration of complement factor C5a14,15 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.16 Also, reperfusion of the myocardium after a period of aortic cross-clamping ischemia may lead to the release of free radicals from a variety of pathways in monocytes, endothelial cells or both.17

There has been a very limited number of studies demonstrating oxidative stress following reperfusion of the human heart. One of the original studies was by Ferrici et al.18 who reported changes in glutathione in the coronary artery during reperfusion. More recent studies have given an indirect measurement of an increase in OFR activity during CPB in human studies19,20,21. 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 depressed for 24 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, Tovoineen and coworkers10 showed that TPAS values measured by the TRAP method 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 Tovoineen study returned to preoperative values, contrary to our findings.

There was a significant difference between our study and the Tovoineen study. The method of TPAS analysis used was 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 3.69%. The TRAP assay used by Tovoineen 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.23 This may have contributed to the discrepancy in the results between the two studies, particularly as the Tovoineen study used a relatively small number of patients.

The findings of the present study are in agreement with research conducted by Pyles and coworkers24 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.25,26 Plasma LPX concentrations give a simple and reliable index of oxidative stress.27 The LPX measured would have included oxidized plasma and membrane lipids attacked by extracellular and intra-cellular sources of ROS.

The preoperative concentrations of LPX in the 24 patients averaged 4.7 ± 0.54 (mean ± standard deviation), which compares favourably with studies of healthy control individuals using an identical assay technique.28 In the present study, there was a significant rise in LPX after 1 h to a mean value of 7.9 ± 0.54 (mean ± standard deviation). Concentrations at all other time points were elevated but not significantly above preoperative values.

Although, despite absolute TPAS being significantly depleted for over 24 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 1h after CPB, reaching a peak before 1h and then falling back to preoperative values by 6h. The rapid production of ROS in this study is consistent with ROS production following ischemia and reperfusion radical formation, as well as neutrophil activation and ROS secretion.
The greater the TPAS preoperatively, the greater the depletion of TPAS after 1.5h. 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 preoperative depletion of TPAS preoperatively to 1.5h 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 necrosis were significantly increased at all time points when compared with preoperative values.

There was a significant correlation between LPX at 1.5h 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 myocardial 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’. These were depleted perhaps because of surgical 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. 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 ischemia was associated with a significantly higher mortality rate. 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.

REFERENCES


Plasma antioxidants in cardiac surgery 623


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