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Oxidative Stress is Evident in Erythrocytes as well as Plasma in Patients Undergoing Heart Surgery Involving Cardiopulmonary Bypass

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Objective: The aim of this study was to analyse the level and progression of oxidative stress, in both plasma and erythrocytes, during heart surgery involving cardiopulmonary bypass.

Materials and Methods: Twenty two patients undergoing cardiac surgery and considered to present a high/severe level of surgical risk were selected. We took five blood samples at different times during the cardiac surgery and analysed TBARS, α-tocopherol, coenzyme Q and retinol in plasma and TBARS (baseline levels and induced by Fe^{2+}-ascorbate oxidation), α-tocopherol, coenzyme Q and catalase, superoxide dismutase and gluthatione peroxidase activity in erythrocytes.

Results: Plasma results shown a decrease in both α-tocopherol and retinol concentration after starting CPB with respect to the reference level ($13.6 \pm 1.5\,nmol\,ml^{-1}$ vs. $22.0 \pm 3.0\,nmol\,ml^{-1}$ and $1.2 \pm 0.1\,nmol\,ml^{-1}$ vs. $1.8 \pm 0.2\,nmol\,ml^{-1}$, respectively ($p < 0.05$)). In comparison, in erythrocytes, all antioxidants, both enzymatic and non-enzymatic, increased in activity or concentration after starting CPB. Erythrocyte TBARS, both baseline levels and induced levels, followed a similar pattern, with an increase after starting CPB with respect to the reference level ($3.9 \pm 0.6\,nmol\,mg^{-1}$ of protein vs. $2.3 \pm 0.2\,nmol\,mg^{-1}$ of protein and $10.6 \pm 0.8\,nmol\,mg^{-1}$ of protein vs. $6.7 \pm 0.6\,nmol\,mg^{-1}$ of protein, respectively ($p < 0.05$)).

Conclusion: These results reveal an increase in oxidative stress after CPB, both in plasma and erythrocytes, and although the organism is capable of attenuating this stress by means of various antioxidative defence mechanisms, there is an increased possibility of post-CPB complications and thus of mortality.

Keywords: Oxidative stress; Cardiopulmonary bypass; Antioxidants; Erythrocyte; Plasma

INTRODUCTION

An ever-increasing number of patients are undergoing cardiac surgery[1] and in most cases cardiopulmonary bypass (CPB) is required. In the USA, 250,000 heart operations involving CPB are performed every year.[1] This process involves extracting blood from the heart, adding oxygen to it, removing carbon dioxide and then returning the blood to a high-capacity artery, thus interrupting the blood flowing through the heart and most of that passing through the lungs.[1,2] When the bypass is complete, it functions in series with the circulation to the rest of the body, providing irrigation and mechanical ventilation.[1,2] Nevertheless, it must not be forgotten that this technique is an aggressive one, as both blood pressure and flow are lowered.[1,2] Although CPB produces many beneficial effects, it also has disadvantages, because patients are

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subjected to a high degree of surgical risk.[3–5] Post-CPB mortality is low, at 1–2%[3,6] but subsequent complications may increase this rate.

In addition to the unphysiological hemodynamic conditions, one significant complication arising from CPB is the ischaemia reperfusion sequence syndrome,[4,7,8] in which a large number of free radicals are released, due, among other causes, to the metabolism of xanthine oxidase, to the activation of neutrophils, and to the oxidation of catecholamines, endothelial cells and prostaglandins.[4,8,9] These radicals give rise, among other effects, to a considerable degree of cell injury, both structural and functional, involving damage to membrane lipids (lipid peroxidation), denaturation of proteins, inactivation of enzymes and alterations to DNA.[4,10] Free radicals are considered responsible for systemic inflammation, one of the most significant aspects of the harmful effects of CPB, and also, according to some authors, for causing reperfusion-derived arrhythmias and myocardial dysfunction.[11–15]

It was within the above context that we determined the objective of this study, that is, to analyse the time-course of oxidative stress in plasma and erythrocyte from patients undergoing heart surgery involving cardiopulmonary bypass.

MATERIALS AND METHODS

Patients

To carry out this study, we selected 22 patients under cardiac surgery and considered to present a high/severe level of surgical risk, according to the Pettigrew scale,[16] which analyses several different indicators of the risk of surgery. The mean age was 59.1 ± 3.1 years and the surgery time was 222.6 ± 8.6 min. The clinical characteristics of the patients are show in Table I. The following conditions were applied as general criteria for inclusion in the project: (1) Age 20 years and over; (2) No haematological illness that might provoke a variation in erythrocyte levels; (3) Not subjected to transfusion during the three months prior to surgery; (4) Not subjected to prior surgery for valvuloplasty; (5) Absence of endocrine illness and not receiving hormone treatment; (6) Absence of severe or chronic anaemia. All patients, in accordance with the Helsinki agreement, gave their informed consent to the study, which was approved by the Ethics Committee of the "Virgen de las Nieves" Hospital.

TABLE I Patient clinical characteristics

Variable		
No of patients		22
Age (years)		59.1 ± 3.1
Associated cardiac pathology	Yes	9
	No	13
Other pathologies	Yes	7
	No	15
Surgery time (min)		222.6 ± 8.6

Anaesthetic and Surgical Procedure

All the patients received scopolamine and midazolam before operation. Non-invasive monitoring was established and, under local anaesthesia, the arterial system was cannulated. This procedure enables the internal monitoring of arterial tension and the patient's metabolic and blood-gas status condition. It was also used to obtain the blood samples (total volume 20 ml) required for this study. Samples were obtained at each moment of the intervention described below. The reference level (T0) was determined immediately after cannulation of the arterial system, before surgical intervention. We then administered hypnotics, opiates and muscle-relaxing agents (etomidate, fentanyl and pancuronium bromide, respectively), performed endotracheal intubation and established mechanical ventilation. Anaesthesia was maintained with reperfusions of fentanyl, atracurio and propofol. Following this, the central vein was cannulated and surgery began. The second sample (T1) was taken 10 min after beginning sternotomy. The third sample (T2) was taken after 15 min of CPB, and the fourth sample (T3) after 45 min. The final sample (T4) was obtained 20 min after the removal of the coronary bypass.

Analytical Results

All the chemicals products and solvents, of highest grade available, were acquired from Sigma (St. Louis MO, USA) and Merck (Darmstadt, Germany).

The blood was collected in heparinised tubes and centrifuged at 1750*g* for 10 min at 4°C in a Beckman GS-6R refrigerated centrifuge (Beckman, Fullerton, CA, USA) to obtain the plasma. The isolation of membranes and cytoplasm from the erythrocyte was carried out according to the protocol of Hanahan and Ekholm.[17]

The concentration of proteins in the erythrocyte membrane and cytoplasm was determined by the method of Lowry *et al.*[18]

Coenzyme Q, α-tocopherol and retinol in plasma were determined by high-performance liquid chromatography (HPLC), using the method described by Litarru *et al.*[19] with slight modifications. Prior extraction was carried out a mixture of hexane, ethanol/isopropanol (95:5) and sodium lauryl sulphate (2%). The hexane phase was removed and dried under a stream of nitrogen. The dry extract

was resuspended in the HPLC mobile phase (ethanol/water, 97:3). The coenzyme Q and the membrane α-tocopherol were also determined by HPLC. These molecules were extracted by a mixture of ethanol/petroleum (60:40) using the Kroger method,[20] after centrifugation at 2500 g for 5 min, the upper layer was collected by aspiration and the residue was re-extracted twice with 1 ml of petroleum ether. The dry residue of combined extracts was diluted in the HPLC mobile phase (ethanol/water, 97:3). In both techniques the HPLC system consisted of an apparatus equipped with a Diode Array Detector, model 168 (Beckman Instruments, Inc. Fullerton, CA, USA) and the column was a reverse-phase C18 Spherisorb ODS 1 of 25 × 0.46 cm reverse phase C18 column with a guard column containing the same material as the main column.

Catalase activity was determined following the method described by Aebi,[21] based on monitoring the H_2O_2 decomposition, a consequence of the catalytic activity of catalase, by spectrophotometric measures at 240 nm. Superoxide dismutase (SOD) was determined by the method of Fridovich *et al.*,[22] based on the inhibition by SOD of cytochrome C reduction and spectrophotometric measurement at 550 nm. For glutathione peroxidase (GPX), we used the technique of Flohé *et al.*,[23] a method based on the instantaneous formation of glutathione oxidised during the reaction catalysed by glutathione peroxidase.

Thiobarbituric acid reactive substances (TBARS) were measured spectrophotometrically.[24] The sample was mixed with TBA and acetic acid (pH 3.0) and heated at 90°C for 30 min. To lower the metal-catalysed auto-oxidation of lipids, BHT (0.01%) was added to the TBA reagent. After cooling, the chromogen was extracted in *n*-butanol and the absorbance of the organic phase was determined at 535 nm.

In erythrocyte membrane, we performed two separate determinations of TBARS, the first one, referred as baseline levels of TBARS, was made using the erythrocyte with no further induction. The second determination was developed after the induction of lipoperoxidation of the sample with Fe^{2+}-ascorbate.[25]

Statistical Analysis

Results are presented as mean ± standard error of the mean. One-way analysis of variance was used to test the time-dependent change in oxidative stress markers. *Post-hoc* mean comparisons were performed using multiple comparison test adjusted by Bonferroni. A *P* value less than 0.05 was considered statistically significant. Statistical processing was carried out using the SPSS package (SPSS for Windows, 9.0.1,1999, SPSS Inc., IL, USA).

RESULTS

No surgery or anaesthesia-related complication arose during this study.

Figure 1 shows the values obtained for plasma antioxidants [coenzyme Q (Fig. 1A), α-tocopherol (Fig. 1B) and retinol (Fig. 1C)]. All cases reveal the same pattern, with a significant decrease in concentrations in samples T2, T3 and T4 (15 min after starting CPB, 45 min after starting CPB and 20 min after ending CPB, respectively). In the case of α-tocopherol and retinol the reduction was statistically significant with respect to samples T0 and T1 (immediately after canalisation of the artery and 10 min after starting sternotomy, respectively) ($22.0 \pm 3.0\,\text{nmol ml}^{-1}$ and $21.3 \pm \text{nmol ml}^{-1}$ for α-tocopherol and $1.8 \pm 0.2\,\text{nmol ml}^{-1}$ and $1.6 \pm 0.1\,\text{nmol ml}^{-1}$ for retinol).

Plasma TBARS (Fig. 2) did not show statistically significant differences between any of the sample times registered. However, the highest value was observed at T2 ($5.0 \pm 0.2\,\text{nmol ml}^{-1}$).

In erythrocytes, all the antioxidants, both enzymatic and non-enzymatic, presented a significant increase in activity or concentration after the obtainment of the T1 sample. Catalase (Fig. 3A) shows its maximum value at T2 ($12.4 \pm 2.8\,\text{s}^{-1}\,\text{mg}^{-1}$ of protein), with statistically significant difference with respect to the reference level (T0) ($3.5 \pm 0.6\,\text{s}^{-1}\,\text{mg}^{-1}$ of protein). Glutathione peroxiodase (Fig. 3B) and superoxide dismutase (Fig. 3C), shows their maximum values at T2 and T3 with statistically significant difference with respect to the reference level (T0).

The highest concentration of both α-tocopherol (Fig. 4A) and coenzyme Q (Fig. 4B) was observed at T2 ($6.1 \pm 1.4\,\text{nmol mg}^{-1}$ of protein and $17.2 \pm 3.1\,\text{pmol mg}^{-1}$ of protein, respectively). In the case of α-tocopherol, the values at T3 and T4 remain higher than T0 values, with statistically significant differences.

Baseline levels TBARS in erythrocyte (Fig. 5A) shows a statistically significant increase at T2 with respect to the reference level ($3.9 \pm 0.6\,\text{nmol mg}^{-1}$ of protein and $2.3 \pm 0.2\,\text{nmol mg}^{-1}$ of protein, respectively). After the induction of lipoperoxidation by adding Fe^{2+}-ascorbate, TBARS (induced levels) (Fig. 5B) followed a similar pattern. So, the higher values were observed at T2 ($10.14 \pm 0.84\,\text{nmol mg}^{-1}$ of protein) and T3 ($9.1 \pm 0.8\,\text{nmol mg}^{-1}$ of protein), with statistically significant differences with respect to the reference level (T0) ($6.7 \pm 0.5\,\text{nmol mg}^{-1}$ of protein). At T4, the induced levels TBARS, remain

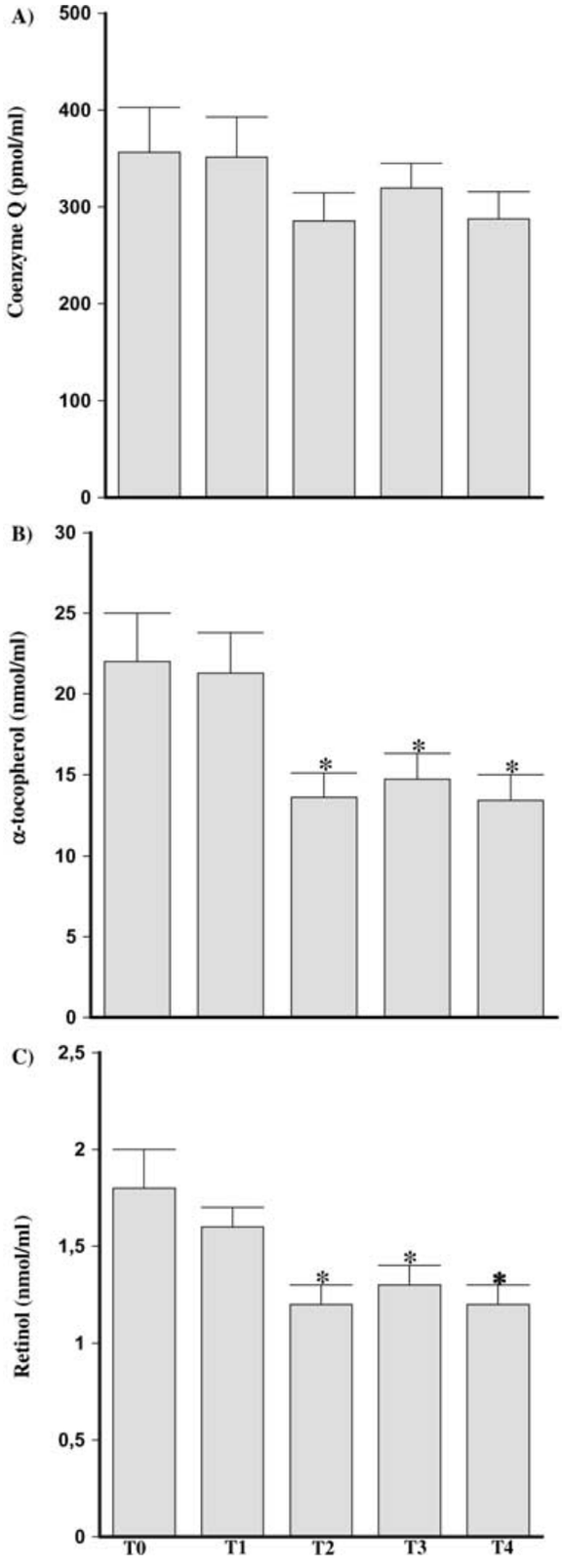

FIGURE 1 Concentration of Coenzyme Q (A), α-tocopherol (B) and retinol (C) in plasma. Values shown are mean ± SEM. *$p < 0.05$ compared with reference level (T0). T0: before surgical intervention; T1: 10 min after sternotomy; T2: 15 min after starting CPB; T3: 45 min after starting CPB; T4: 20 min after finalising CPB.

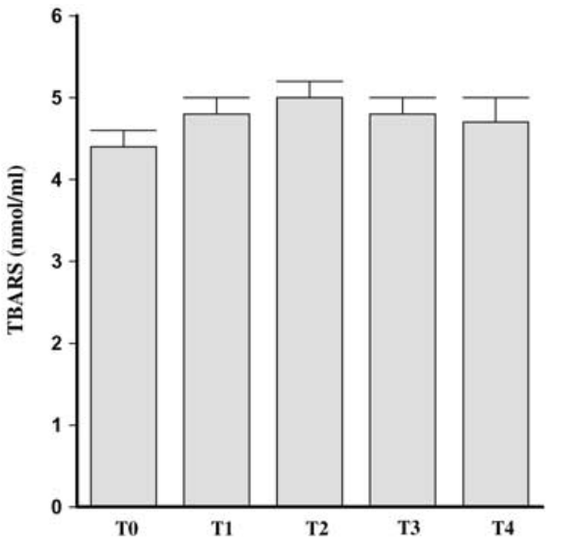

FIGURE 2 Concentration of TBARS in plasma. Values shown are mean ± SEM. *$p < 0.05$ compared with reference level (T0). T0: before surgical intervention; T1: 10 min after sternotomy; T2: 15 min after starting CPB; T3: 45 min after starting CPB; T4: 20 min after finalising CPB.

high, but without statistically significant difference with respect to T0.

DISCUSSION

Cardiac surgery normally requires the use of cardiopulmonary bypass (CPB). Evidences suggest that during cardiopulmonary bypass reactive oxygen species produced by activated neutrophils or by tissue reperfusion injury may be involved in pathogenesis of diffuse damage in patients undergoing cardiac surgery.[7,8,12,14]

In this study, we analysed the level and progression of oxidative stress, in both plasma and erythrocytes, produced during heart surgery involving cardiopulmonary bypass performed on patients presenting with cardiovascular risk. We consider it to be important to understand the behaviour of this oxidative stress in both compartments, because most of the studies give only information about these parameters in plasma.[5,26–28]

Our results show changes in TBARS and antioxidant capacity in both plasma and erythrocyte in response to CPB. These results support the hypothesis of increased oxidative stress in patients undergoing open heart surgery after to start CPB.[7,8,14,26–28]

We observed a slight increase in the content of TBARS in plasma at T2, although this was not statistically significant with respect to T0, the reference level in our study. Toivonen *et al.*[26] also observed a slight increase in TBARS in patients subjected to cardiac surgery with CPB, but likewise, the increase between different sample periods was

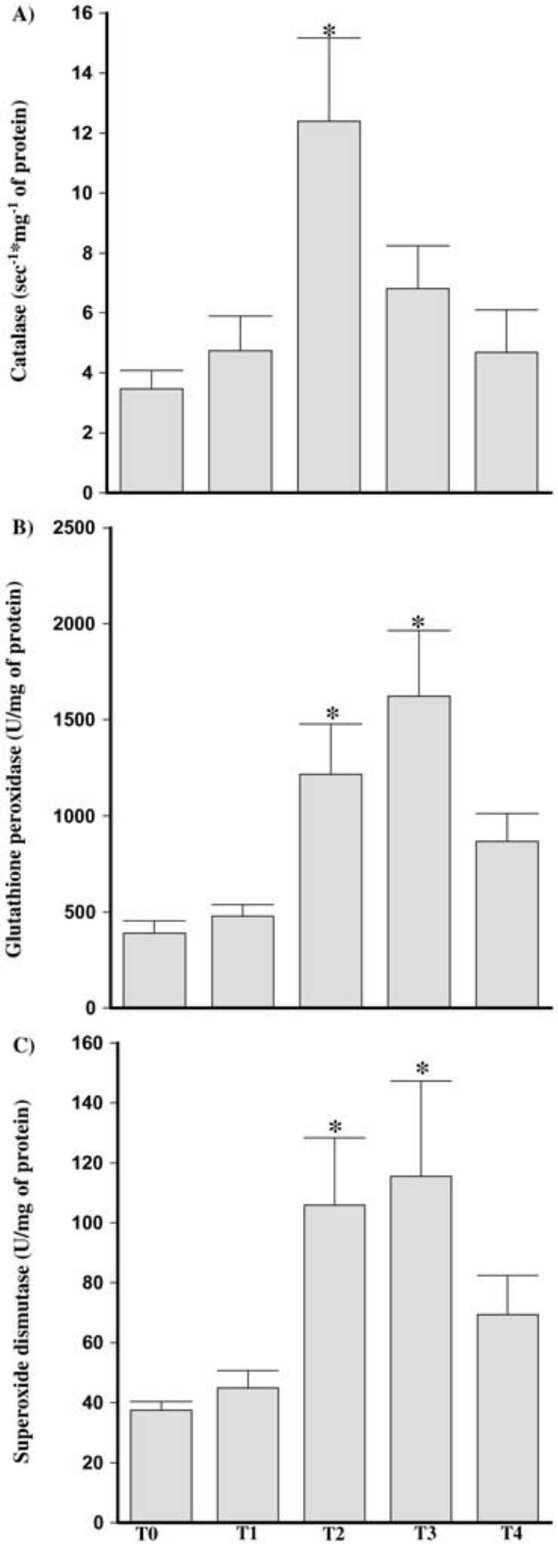

FIGURE 3 Activity of catalase (A), glutathione peroxidase (B) and superoxide dismutase (C) enzymes in erythrocytes. Values shown are mean ± SEM. *$p < 0.05$ compared with reference level (T0). T0: before surgical intervention; T1: 10 min after sternotomy; T2: 15 min after starting CPB; T3: 45 min after starting CPB; T4: 20 min after finalising CPB.

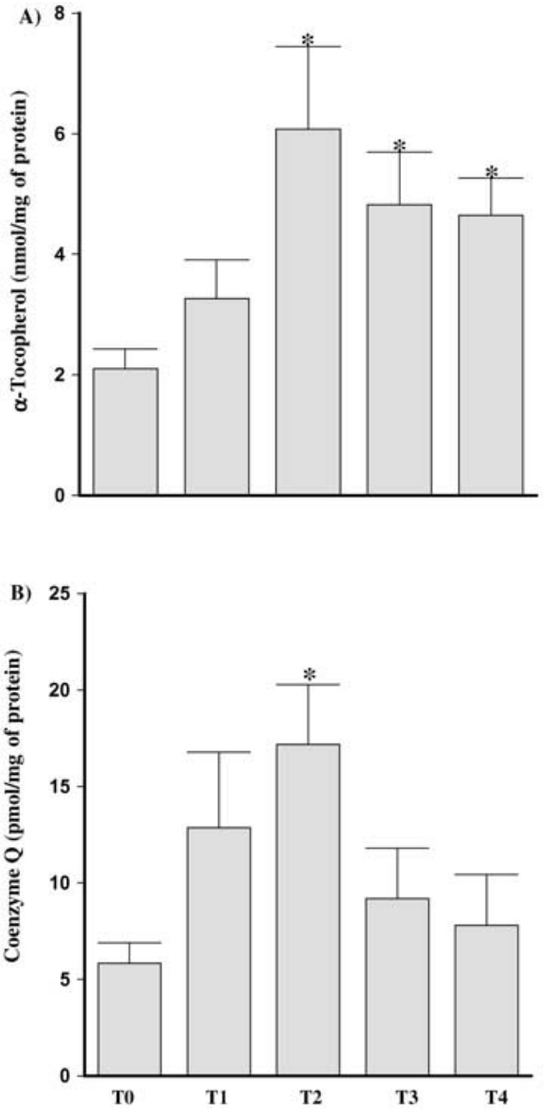

FIGURE 4 Concentration of α-tocopherol (A) and coenzyme Q (B) in the erythrocyte membrane. Values shown are mean ± SEM. *$p < 0.05$ compared with reference level (T0). T0: before surgical intervention; T1: 10 min after sternotomy; T2: 15 min after starting CPB; T3: 45 min after starting CPB; T4: 20 min after finalising CPB.

not statistically significant. In other studies, quantification of the content of malondialdehide in plasma has produced varied results.[26] We believe that these varied results in TBARS levels could be due to the use of different plasma dilution factors to correct the hemodilution during CPB, such as packed red cell,[26] g of protein,[27] and g of albumin.[29] In our study, we did not use any dilution factor because we considered that it is important to reflect an actual amount of free radical reaction products.

However, as evident in the results, despite this similarity of TBARS content in plasma, the marked fall in the levels of plasma antioxidants is indicative of the high degree of stress undergone by the patients during CPB[28,29] and it also constitutes compelling

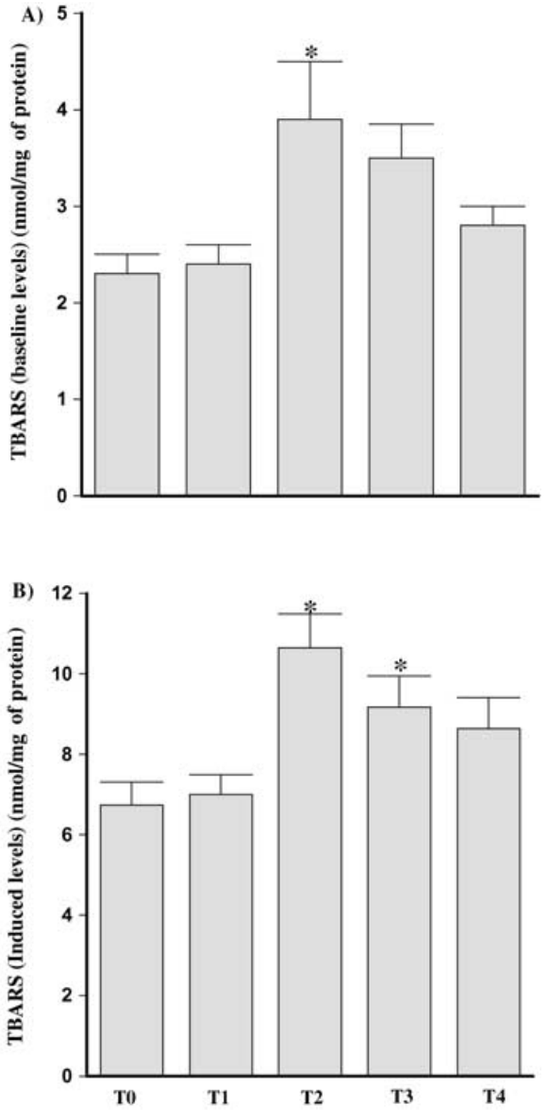

FIGURE 5 Concentration of TBARS (both baseline levels (A) and induced levels (B)) in the erythrocyte. Values shown are mean ± SEM. *$p < 0.05$ compared with reference level (T0). T0: before surgical intervention; T1: 10 min after sternotomy; T2: 15 min after starting CPB; T3: 45 min after starting CPB; T4: 20 min after finalising CPB.

evidence that CPB can be a contributory factor to post-surgery complications. The hemodilution accompanying CPB could be partly responsible for the remarkable reduction in plasma antioxidative capacity in cardiac surgery patients. Nonetheless, these results reflect an actual capacity of plasma antioxidative defence mechanism,[27] and, thus, increased susceptibility to oxidative stress during CPB is evident. The existence, therefore, of this reduction constitutes compelling evidence that CPB can be a contributory factor to post-surgery complications.

With respect to the erythrocytes we found a different behaviour. All the antioxidants, both enzymatic and non-enzymatic, increased in activity or concentration after starting the surgery. We believe these findings are novel as previous studies have only considered plasma responses in patients undergoing cardiac surgery.[5,26–28] This rapid antioxidant response in the erythrocyte is likely due to the increase in free radical production during CPB. Similar responses do exist in the literature,[30–32] although they are either in different cell types or involve different sources of oxidative stress. Nonetheless they indicate that after short-term oxidative stress there is an increase in the activity of some antioxidant enzymes and then this activity decreases to normal after a short period of time. This increase could be indicative of increased defence by the erythrocyte which helps decrease the oxidative stress. Likewise the rapid reduction in the level of coenzyme Q could be indicative of the high stress created.[33] This antioxidant seems to be used in an attempt to maintain the pool of α-tocopherol active in the erythrocyte membrane.

Erythrocyte (TBARS) baseline levels showed a statistically significant increase 15 min after starting CPB. After the induction of lipoperoxidation by adding Fe^{2+}-ascorbate, TBARS (induced levels) followed a similar pattern. The higher values were observed at T2 and T3. However, this level remained high until T4, but without statistically significant difference with respect to T0.

The results obtained, taking into account the circumstances of our experimental conditions, reveal a sharp increase in oxidative stress after cardiopulmonary bypass is established, both in plasma and in erythrocytes. Although the body is capable of attenuating this stress by means of various antioxidative defence mechanisms, this reaction leads to their depletion, increase the susceptibility against oxidative stress, as has been shown after induction of lipoperoxidation by adding Fe^{2+}-ascorbate, and therefore an increased possibility of post-CPB complications, and thus of mortality. With respect to the future of such interventions, this aspect should be taken into account; we believe it provides arguments in favour of creating wider-ranging intervention protocols and might justify either the use of a greater quantity of antioxidants or, alternatively, enzyme activity could be increased by pharmacological means, both before CPB is established and after it is removed.

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