Free Radical Research, Volume 38 Number 7 (July 2004), pp. 683–689

Taylor & Francis
healthsciences

The Influence of Extracorporeal Circulation on the Susceptibility of Erythrocytes to Oxidative Stress

JEAN-JACQUES LAHET[a], CAROL COURDEROT-MASUYER[a], FRANÇOIS LENFANT[b], ETIENNE TATOU[c], CATHERINE VERGELY[a], MICHEL DAVID[c] and LUC ROCHETTE[a,*]

[a]*Laboratoire de Physiopathologie et Pharmacologie Cardiovasculaires Expérimentales, Facultés de Médecine et de Pharmacie, IFR No 100, 7 Bd Jeanne d'arc, BP 87900, 21079 Dijon Cedex, France;* [b]*Département d'Anesthésie et de Réanimation, Hôpital général, CHU Dijon Cedex, France;* [c]*Service de Chirurgie Cardiovasculaire, Hôpital du Bocage, 1 Bd Jeanne d'arc, 21033 Dijon Cedex, France*

Accepted by Professor F.J. Kelly

(Received 10 December 2003; In revised form 10 March 2004)

Extracorporeal circulation (ECC), a necessary and integral part of cardiac surgery, can itself induce deleterious effects in patients. The pathogenesis of diffuse damage of several tissues is multifactorial. It is believed that circulation of blood extracorporeally through plastic tubes causes a whole body inflammatory response and a severe shear stress to blood cells. The aim of this study was to evaluate the level of oxidative stress and its deleterious effect on red blood cell (RBC) before (pre-ECC), immediately after (per-ECC) and 24 h after an ECC (24 h post-ECC). Several indicators of extracellular oxidative status were evaluated. The ascorbyl free radical (AFR) was directly measured in plasma using electron spin resonance (ESR) spectroscopy and expressed with respect to vitamin C levels in order to obtain a direct index of oxidative stress. Allophycocyanin assay was also used to investigate the plasma antioxidant status (PAS). Indirect parameters of antioxidant capacities of plasma such as vitamin E, thiol and uric acid levels were also quantified. RBC alterations were evaluated through potassium efflux and carbonyl levels after action of AAPH, a compound generating carbon centered free radicals. No changes in plasma uric acid and thiols levels were observed after ECC. However, vitamin E levels and PAS were decreased in per-ECC and 24 h post-ECC samples. Vitamin C levels were significantly lower in 24 h post-ECC and the AFR/ vitamin C ratio was increased. Differences in results had been noted when measurements took account of hemodilution. Increases of uric acid and thiols levels were observed after ECC. Vitamin E levels were not modified. However after hemodilution correction a significant decrease of vitamin C level was noted in 24 h post-ECC samples as compared to per-ECC sample. Whatever the way of measurement, vitamin C levels decreased suggesting the occurrence of ECC induced-oxidative stress. Concerning RBC, in the absence of AAPH, extracellular potassium remained unchanged between pre-, per- and 24 h post-ECC. AAPH induced a significant increase in extracellular potassium and carbonyls levels of RBC membranes, which was not modified by ECC. These results suggest the absence of alterations of RBC membrane during ECC despite the occurrence of disturbances in PAS. Such protection is of particular importance in a cell engaged in the transport of oxygen and suggests that RBC are equipped with mechanisms affording a protection against free radicals.

Keywords: Erythrocyte; Extracorporeal circulation; Antioxidant; Ascorbyl radicals

INTRODUCTION

Extracorporeal circulation (ECC), a necessary and integral part of cardiac surgery, can itself induce deleterious effects in patients. The pathogenesis of diffuse damage of several tissues is multifactorial. It is believed that circulation of blood extracorporeally through plastic tubing causes a severe stress to blood cells[1] and the whole body inflammatory response induced by ECC is responsible for post-operative organs dysfunctions and morbidity.[2–4] The pathogenesis of the development of organs dysfunctions is diverse. A systemic increase of various markers of oxidative stress has been demonstrated during ECC.[1,4] It is well recognized that oxygen free radicals are important triggers in the evolution of an inflammatory reaction.[5] Because a surgical operation generally enhances

*Corresponding author. E-mail: rochette@u-bourgogne.fr

ISSN 1071-5762 print/ISSN 1029-2470 online © 2004 Taylor & Francis Ltd
DOI: 10.1080/10715760410001702512

the generation of radical oxygen species and increases mechanical shear stress on erythrocytes, we sought to clarify the incidence of ECC on the plasma antioxidant status (PAS) and on the resistance of erythrocytes membranes to oxidative stress. Thus, in order to examine the influence of oxidative stress induced by ECC on red blood cells (RBC) membrane, we evaluated the carbonyl levels and potassium leak and the incidence of oxidative stress on RBC samples obtained after ECC. Indeed the incubation of RBC with a peroxyl radical generator is able to induce cell damages responsible for an early potassium efflux and a later hemolysis[6–11] probably due to the formation of hemolytic holes in the membrane.[11] This experimental approach was used in order to study erythrocytes membranes resistances towards oxidative stress.

MATERIALS AND METHODS

Patients

Eight patients (5 men and 3 women, aged mean 69 ± 7 years) were selected for the study. All these patients presented an angina rated at CCS III (4 cases) or CCS IV (4 cases). The mean pre-operative left ventricular ejection was 51 ± 18%. All patients gave written informed consent and the study protocol was approved by the local medical ethics committee (CCPRB–Dijon). To obtain a homogenous group, patients with the following characteristics were considered for the study: (1) left ventricular ejection fraction 40% or more; (2) age 76 years or less; (3) intraoperative aortic clamping time between 80 and 150 min. Exclusion criteria were known: corticosteroid use within the previous 15 days or a history of hematologic, hepatic or renal disease.

Anesthesia and Surgery

Patients were premedicated with flunitrazepam (0.1 mg/10 kg). Anesthesia was induced with midazolam or etonidate, and maintained with high dose of midazolam (50–100 μg/kg). Isoflurane was administred if necessary.

Extra-corporeal circulation was established with the use of roller pumps fitted with termino-capio SX membrane oxygenators. Three patients had 2 aorto-coronary bypasses, three patients 4 aorto-coronary bypasses, one patient had 3 and one patient had 5 aorto-coronary bypasses. Mean ECC duration was 121 ± 26 min.

Effects of ECC on Red Blood Cells

Erythrocytes Preparation

Blood samples were drawn from vein and put on dry tubes before, per-ECC (immediately) and 24 h after ECC. Erythrocytes were separated from blood plasma by centrifugation (5 min at 1600 rotations per minute (RPM), at 4°C and washed 5 times with an isotonic saline solution (NaCl 0.9%). The supernatant and buffy coat were carefully removed after each wash. After separation, 2 ml of packed erythrocytes were suspended in 16 ml of phosphate buffer at pH 7.4 in order to get a 10% suspension of erythrocytes.

Measurement of Carbonyl Groups of RBC Membranes

Different groups ($n = 8$) were studied:

a. erythrocytes in phosphate buffer alone taken from the patients: pre-, per- and 24 h post-ECC;
b. Effects of AAPH (50 mM) on erythrocytes separated from blood samples drawn at the different periods (pré-ECC, per-ECC and 24 h post-ECC).

Oxidation of proteins was carried out using the reaction between 2,4-dinitrophenylhydrazine (DNPH) and carbonyl groups.[8] A chromophore was obtained with an absorbance at 380 nm. A measure of 50 μl of the erythrocyte suspension were acidified with 500 μl of 20% trichloroacetic acid (TCA) and 500 μl of acidified acetone. After centrifugation at 11,000*g* (4°C) for 3 min, membranes of RBC were collected. These procedure was carried out 3 times. A measure of 500 μl of 2,4 DNPH were added to the pellet. Samples were incubated for 1 h at 25°C with mixing every 15 min. Proteins were precipated by adding 500 μl of 10% TCA and centrifugation. The pellet was washed 3 times using ethanol/ethylacetate mixture (1:1) and centrifuged. Proteins were suspended in 1 ml of 6 M guanidine (pH 2.3) and detected at 380 nm. In these experimental conditions, the possible traces of haemoglobin and residual reagent (DNPH) were removed. The carbonyl content was calculated using a molar extinction coefficient of 22,000. The total protein content of the erythrocyte suspension was determined by Lowry's method.[12]

Measurement of Extracellular Potassium of RBC

Different groups ($n = 8$) were studied:

a. Erythrocytes in phosphate buffer alone taken from the patients: pre-, per- and 24 h post-ECC;
b. Effects of AAPH on erythrocytes in presence of AAPH (50 mM final concentration) (pre-, per- and 24 h post-ECC).

Each sample was adjusted to a final volume of 5 ml by adding phosphate buffer as needed.

The samples were then incubated at 37°C. At T_0 and every 30 min during 2 h, 0.4 ml of the suspension was drawn, added to 0.75 ml of saline solution and spinned 5 min at 1500 rpm at 4°C. A measure of 0.25 ml of the supernatant was added to 4.75 ml of phosphate buffer and extra cellular potassium was measured using flame photometry method (Photomer 410, CIBA Corning, France). The concentration of potassium was expressed in nEq/mg of total proteins.

Determination of Plasma Antioxidant Status

Blood samples were collected before, immediately after ECC and also 24 h after ECC. Plasma was prepared immediately by centrifugation of blood at 3500 rpm for 10 min at 4°C using heparin as an anticoagulant.

Allophycocyanin Test

The oxygen radical absorbance capacity (ORAC) of plasma was measured according to the method of Cao *et al.*[13] The method is based on the property of allophycocyanin (APC) to loss its fluorescence when damaged by oxygen radicals. In the present work, peroxyl radicals were generated by AAPH and ORAC was assayed as the ability of plasma to delay the loss of APC fluorescence (Fluorimeter Kontron, SFM). The reaction mixture (2 ml) contained 37.5 nM APC, 3 mM AAPH in 0.075 M phosphate buffered saline, pH: 7.0 (Blank). A measure of 1 μl of plasma or 1 μM Trolox were added to the mixture in sample and reference standard, respectively. The fluorescence of APC at the emission of 651 nm and the excitation of 598 nm was measured every minute at 37°C until disappearance of APC fluorescence. The ORAC value of each plasma was calculated by measuring the net protection area under the quenching curve of APC. One ORAC unit was assigned the net protection provided by 1 μM final concentration Trolox. ORAC value of the samples was calculated as follows:

$$S = \text{Area under the quenching curve.}$$

$$\text{ORAC} = (S_{\text{sample}} - S_{\text{blank}})/(S_{1\,\mu\text{M Trolox}} - S_{\text{blank}})$$

Measurement of Plasma Vitamin C

Vitamin C was measured in plasma using the method described by Roe and Kuether.[14] Proteins were precipitated with 10% TCA and centrifuged. The protein-free supernatant (500 μl) was mixed with 100 μl of 30 mg/ml dinitrophenylhydrazine in 9 N sulfuric acid containing 4 mg/ml thiourea and 0.5 mg/ml copper sulfate and incubated at 37°C for 3 h. Following the addition of 750 μl of 65% (v/v) sulfuric acid, the absorbance was recorded at 520 nm.

Ascorbyle Radical Determination

The detection of ascorbyl radical was assayed by electron spin resonance (ESR) spectroscopy. ESR spectra were recorded on plasma samples with a BRUKER ESP 300 E spectrometer using a TM110 cavity and a flat-type quartz cell with the following instrument settings: modulation frequency = 100 Khz; modulation amplitude = 0.800 G, microwave power = 4 mW; time constant = 0.16 s; gain = 4×10^6; scan number = 5.

Determination of Plasma Vitamin E

For vitamin E measurements (α-tocopherol), proteins were precipitated with ethanol (v/v) and α-tocopherol was extracted into hexane (v/v) according to the method of Burton *et al.*[15] An external standard: α-tocopherol (Merck, France) was used. A measure of 20 μl of sample were analyzed by HPLC (Kontron system with a Kontrosil columm (150(4.6 mm, 5 μm particle sized). Mobile phase was a mixture of heptan/isopropanol (991). α-Tocopherol was quantified by a fluorescence method (excitation: 295 nm, emission: 325 nm, fluorimeter SMF 25 (Kontron Instruments, France) and Kontron autosampler 465. Vitamin E concentrations were expressed as mg/ml per mmol of total cholesterol. Plasma total cholesterol concentration was determined by an enzymatic method (Sigma diagnostics, France).

Uric Acid Measurement

Measurement of plasma uric acid was performed using test kits (Sigma diagnostics).

Thiols Plasma Levels

Thiol groups measurements were assayed according to the method of Tietze.[16] The plasma (200 μl) was reacted with 40 μl 10 mM of 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB or Ellman reagent) in 600 μl 0.25 mM of Tris buffer. The release of thionitrobenzoate was recorded spectrophotometrically at 412 nm 15 min later, after centrifugation (100 min, 300*g*). Results were expressed as μM.

Corrections for Plasma Dilution

The concentrations obtained in plasma were corrected for dilution by the following equation:

$$\text{Corrected value} = \text{measured value} \times \text{Hct 1} \times (1 - \text{Hct 2})/\text{Hct 2} \times (1 - \text{Hct 2})$$

where hematocrit Htc 1 is the initial hematocrit level in the first sample before ECC and Hct 2 is the sample hematocrit level.

TABLE I The mean pre-, per- and post-operative (24 h) biological status. Each data point represents the mean ± SEM for samples from 8 patients

	Hemoglobin g/100 ml	Hematocrit %	Proteinemia g/l
Pre-ECC	12.77 ± 1.25	40.84 ± 3.76	76.00 ± 4.39
Per-ECC	9.03 ± 1.68*	26.2 ± 4.63**	42.75 ± 1.82**
Post-ECC (24 h)	11.86 ± 1.29	34.42 ± 4.18*#	44.71 ± 2.24**

*$p < 0.05$ compared with pre-operative values with a one-way analysis of variance. **$p < 0.001$ compared with pre-operative values with a one-way analysis of variance. #$p < 0.001$ compared with per-operative values with a one-way analysis of variance.

Statistical Analysis

Results are expressed as mean ± SEM. Analysis of variance for repeated measures (ANOVA) and Tukey's test were used for statistical analyses. A value of $p < 0.05$ was considered to be significant.

RESULTS

The mean pre-, per- and 24 h post-operative hematological status is summarized in Table I. We observed a significant decrease of hematocrit and proteinemia during the per- and post-ECC periods.

Estimation of Membrane Carbonyls

The carbonyl levels of RBC membranes were not modified during pre-, per- and 24 h post-ECC periods. When RBC were submitted to AAPH-induced oxidative stress, carbonyl levels were significantly increased in pre- and post-ECC samples (Fig. 1).

Potassium Leak

In the absence of AAPH, RBC did not release potassium. AAPH induced a marked increase in the extra-cellular concentrations of potassium. This evolution did not differ in pre-ECC, per-ECC and 24 h post-ECC samples (Fig. 2).

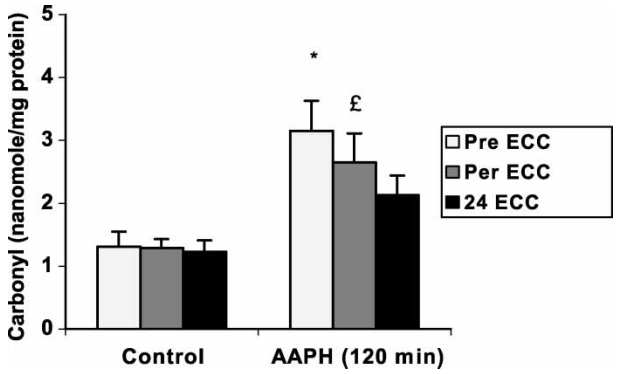

FIGURE 1 Evaluation of carbonyl levels of RBC membranes after addition of 50 mM AAPH. Each data point represents the mean ± SEM for samples from 8 patients. *$p < 0.05$ versus control pre-ECC. £ $p < 0.05$ versus control per-ECC.

Measurement of Plasma Antioxidant Capacity

The ORAC of plasma was lower in samples collected per-ECC (2.12 ± 0.21) as compared to values obtained before ECC (3.16 ± 0.21) ($p < 0.05$) (Fig. 3).

Estimation of Plasma Antioxidants

Vitamin C levels were significantly lower 24 h after ECC as compared to values pre-ECC values (4.78 ± 0.90 mg/l versus 11.45 ± 2.31 mg/l; $p < 0.05$). However, when values were corrected for hemodilution, a significant decrease in plasma vitamin C concentrations was observed 24 h after ECC (6.40 ± 1.40 mg/l versus 11.45 ± 2.31 mg/l; $p < 0.05$) in comparison with the values obtained immediately after ECC.

The ascorbyl radical/vitamin C ratio was significantly increased ($p < 0.05$) 24 h after ECC as compared to pre-ECC values (pre-ECC: 0.76 ± 0.13 AU/mg/l; 24 h post-ECC: 1.34 ± 0.20 AU/mg/l).

A significant decrease of vitamin E levels was observed ($p < 0.01$) in the course of ECC but when values were corrected for hemodilution, no significant difference was shown.

A significant increase in plasma uric acid was observed after correction by hemodilution (94.89 ± 13.91 per-ECC; 53.79 ± 11.29 pre-ECC; $p < 0.05$).

The global plasma concentration of thiols did not vary during ECC, but a significant increase of thiol levels were observed after ECC when values were corrected for hemodilution (827 ± 53 μM per-ECC versus 513 ± 20 μM pre-ECC; $p < 0.01$) (Table II).

DISCUSSION

During cardiopulmonary bypass surgery where the heart of the patient is transiently arrested the surgical procedure, the blood is removed from systemic venous circulation and pumped through an extra-corporeal oxygenator before going back to the systemic arterial circulation. Cardiopulmonary bypass is often associated with the development of myocardial dysfunctions and it is known that these patients are subjected to a high degree of surgical risk.[17] The present study was planned to study whether ECC was associated with an oxidative stress and with modifications of RBC functions. Our results indicate a significant decrease of plasma antioxidant capacities 24 h after ECC which may be attributed to the development of an oxidative stress. Our findings are in accordance with previous results obtained in our group demonstrating that a systemic

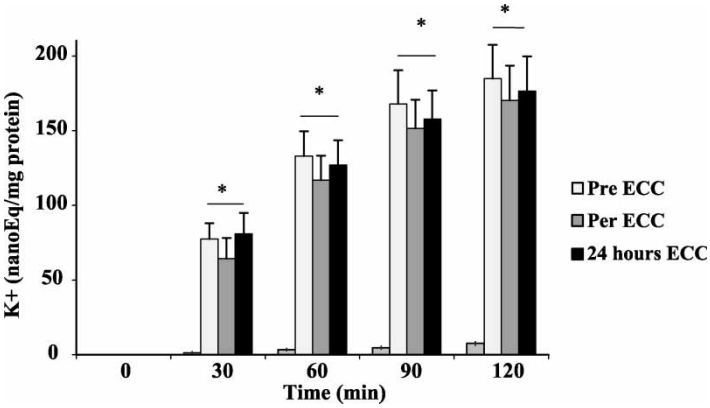

FIGURE 2 Potassium leak. RBC were exposed to 50 mM AAPH. Each data point represents the mean ± SEM for samples from 8 patients. *$p < 0.001$ versus control.

oxidation stress occurred in patients undergoing heart surgery, illustrated by the increase alkyl and alkoxyl radicals detected and quantified by ESR spectroscopy.[1] which may be attributed to the "persistant" activation of neutrophils.[6,16–19] However, it is important to note that in our experimental conditions, the assay procedure using AAPH reveals mainly the contribution of hydrosoluble antioxidants. The popularity in the use of the thermolabile azo initiator derives from the constant and easily quantitated rate of radical production.

Concerning the variations in plasma thiols, they reflect changes in tissue thiols resulting from oxidative stress induced injury. In the present study, we observed an increase of corrected plasma thiol levels after ECC as already been noticed by Castelli *et al.*[20] Plasma antioxidant compounds levels were corrected in order to account for the hemodilution that occurs during ECC. In this way, the increase of corrected plasma thiol levels after ECC may be due to a release of glutathione (GSH) from cells such as RBC. This hypothesis is in agreement with studies of Ferrari *et al.*[21] who showed in patients subjected to aortocoronary bypass, an increase of plasma total glutathione (GSH + GSSG) and a decrease of the GSH/GSSG ratio indicating the presence of an oxidative stress. Usually, the protection of human plasma against free radical injury is offered by a wide spectrum of antioxidants. We showed here that the PAS was decreased during ECC, reflecting a reduced capacity of plasma to protect its environment from free radical injuries.

In our work, the corrected plasma vitamin C concentration decreased 24 h after ECC while no changes in corrected plasma vitamin E levels was observed. The same results were reported by Ballmer *et al.*[22] It has been proposed that AFR can be used as a marker for the oxidation of vitamin C and as a non invasive indicator of oxidative stress.[23–24] The increase of ascorbyl radical/vitamin C ratio observed in our study seems to reflect the degree of oxidative stress associated to the ECC. Moreover, it is known that vitamin C regenerates vitamin E by reducing tocopherol radical which is produced when vitamin E scavenges a peroxyl radical; the result of this reaction being the formation of AFR. When plasma is exposed to a free radical generating

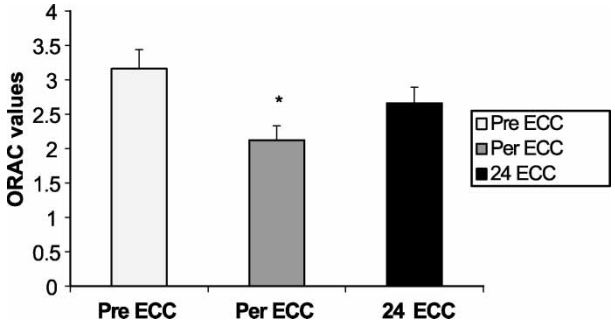

FIGURE 3 Total plasma antioxidant capacity pre, per and 24 h after ECC. Each data point represents the mean ± SEM of samples from 8 patients. *$p < 0.05$ versus pre-ECC.

TABLE II Plasma parameters during ECC. Each data point represents the mean ± SEM for samples from 8 patients

	Pre-ECC	Per-ECC	24 h Post-ECC
Vitamin C (uc) mg/l	11.45 ± 2.31	8.58 ± 1.68	4.78 ± 0.90*
Vitamin C (cor) mg/l	11.45 ± 2.31	18.29 ± 3.88	6.40 ± 1.40$
Ascorbyl radical/vit C AU/mg/l	0.76 ± 0.13	0.92 ± 0.18	1.34 ± 0.20*
Vitamin E (uc) mg/l	9.76 ± 1.07	6.13 ± 0.84*	4.96 ± 0.56*
Vitamin E (cor) mg/l	9.76 ± 1.07	12.42 ± 2.95	7.05 ± 1.19
Vitamin E/Cholesterol (mg/l/mmol)	2.06 ± 0.28	2.09 ± 0.35	3.14 ± 0.57
Uric acid (uc) mg/l	53.79 ± 11.29	48.89 ± 8.99	48.38 ± 11.67
Uric acid (cor) mg/l	53.79 ± 11.29	94.89 ± 13.91*	66.16 ± 18.26
Thiols (uc) μM	513 ± 20	422 ± 36	432 ± 35
Thiols (cor) μM	513 ± 20	827 ± 53**	554 ± 63

uc = crude values uncorrected for hemodilution. cor = corrected values using hematocrit. AU = Arbitrary Unit. * $p < 0.05$ versus Pre ECC, ** $p < 0.01$ versus Pre ECC, $ $p < 0.05$ versus Per ECC.

system, lipid peroxidation does not occur until all of the vitamin C has been oxidized.[25,26] In our present study, we also noticed an increase in corrected plasma uric acid levels after ECC, these results being consistent with others works.[20,27] It has been postulated that the antioxidant properties of uric acid were of biological importance *in vivo*.[1,20,27] Uric acid is derived from adenine and guanine-based purine compounds through the action of the xanthine oxidoreductase. Systemic uric acid could also derive from a cellular free radical attack on nucleic acid compounds or could be the result of restricted perfusion of peripheral tissues during ECC, leading to adenosine triphosphate and adenosine degradation.

Red cell membrane may be considered as a particularly interesting object for the study of oxidative attack during ECC.[28,29] These cells constitute a large stock of oxygen from which the potentially dangerous free radicals may be derived. The echinocytic shape transformation of RBC during cardiopulmonary bypass has already been reported.[30] Origins of these alterations remain uncertain but several evidences suggest that oxidative stress associated with the inflammatory reaction may be implicated. Lipid peroxidation mediated by free radicals is known to be one of the important causes of cell membrane damages such as increase of membrane fluidity and permeability. In our study, we did not notice major changes of RBC membrane functions during ECC since carbonyl formation and K^+ leak were not affected by ECC. However, after AAPH stimulation, carbonyl levels were significantly increased in RBC membranes taken before and after ECC. These observations could be explained by the release of cell outer membrane fragments following the action of pumps. AAPH generates carbon centered free radicals in a temperature and time dependent manner.[28] For concentrations lower than 25 mM, potassium efflux is a good indicator for early membrane damages significant hemolysis occurring after two hours.[29] No change in potassium efflux induced by AAPH was observed in our study after ECC as compared to pre-ECC samples. These observations are in accordance with previous studies reported by Reinhart *et al.*[30] studying the influence of ECC on RBC deformability and membrane lipids. An increase of lipid peroxidation was not demonstrated and the ratio saturated /unsaturated fatty acids remained constant during ECC. These authors noted that the deformability of RBC, as assessed by the resistance to filtration of washed RBCs through pores, was not affected by ECC.

In conclusion, the present results suggest that an oxidative stress is still present in plasma 24 h after ECC but has no effect on RBC functions. Such protection on these RBC is of particular importance suggesting that these cells are equipped with mechanisms affording protection against the formation and the propagation of free radicals.

Acknowledgements

The authors gratefully acknowledge the Conseil Régional de Bourgogne, the Faculty of Medicine and the Centre Hospitalier Universitaire (CHU) Dijon.

References

[1] Clermont, G., Vergely, C., Jazayeri, S., Lahet, J.J., Goudeau, J.J., Lecour, S., David, M., Rochette, L. and Girard, C. (2002) "Systemic free radical activation is a major event involved in myocardial oxidative stress related to cardiopulmonary bypass", *Anesthesiology* **96**(1), 80–87.

[2] Menashé, P. and Peynet, J. (1995) "Normothermic cardioplegia. Is aortic cross-clamping still synonymous with myocardial ischemia?", *Ann. Thorac. Surg.* **54**, 472–478.

[3] Moat, N.E., Evans, T.E., Quinlan, G.J. and Gutteridge, J.M.C. (1993) "Chelatable iron and copper can be release from extracorporally circulated blood during cardiopulmonary bypass", *Fed. Eur. Biochem. Soc.* **328**, 103–106.

[4] Peuchant, E., Carbonneau, M.A., Dubourg, L., Thomas, M.J., Perromat, A., Vallot, C. and Clerc, M. (1994) "Lipoperoxidation in plasma and red blood cells of patients undergoing haemodialysis: vitamins A, E and iron status", *Free Radic. Biol. Med.* **16**, 339–346.

[5] Proulx, M., Maupoil, V., Rochette, L. and Du Souich, P. (1995) "Increase in antilipoperoxidant activity of plasma as a consequence of an inflammatory reaction induced by subcutaneous turpentine in the rabbit", *Free Radic. Res.* **23**(3), 245–254.

[6] Caramelo, C., Riesco, A., Outeirino, J., Millas, I., Blum, G., Monzu, B., Casaco, V., Sanchez, L., Mosquera, J.R. and Casado, S. (1994) "Effects of nitric oxide on red blood cells: changes in erythrocytes resistance to hypotonic hemolysis and potassium efflux by experimental manoeuvres that decrease nitric oxide", *Biochem. Biopys. Res. Commun.* **199**, 447–457.

[7] Pieri, C., Moroni, F. and Marra, M. (1996) "Food restriction increases the protection of erythrocytes against the hemolysis induced by peroxyl radicals", *Mech. Ageing Dev.* **87**, 15–23.

[8] Sandhu, I.S., Ware, K. and Grisham, M.B. (1992) "Peroxyl radical mediated hemolysis: role of lipid. Protein and sulfhydryl oxidation", *Free Radic. Res. Comm.* **16**, 111–122.

[9] Tatum, V.L. and Chow, C.K. (1996) "Antioxidant status and susceptibility of sickle erythrocytes to oxidative and osmotic stress", *Free Radic. Res.* **25**, 133–139.

[10] Vissers, M.C., Stern, A., Kuypers, F., Van Den Berg, J. and Winterbourn, C.C. (1994) "Membrane changes associated with lysis of red blood cells by hypochlorous acid", *Free Radic. Biol. Med.* **16**, 704–712.

[11] Sato, Y., Kanazawa, S., Sato, K. and Suzuki, Y. (1998) "Mechanism of free radical-induced hemolysis of human erythrocytes. II. Hemolysis by lipid-soluble radical initiator", *Biol. Pharm. Bull.* **21**, 250–256.

[12] Lowry, O.H., Rosebrough, N.J., Fair, A.L. and Randall, R.J. (1951) "Protein measurement with the folin phenol reagent", *J. Biol. Chem.* **193**, 265–275.

[13] Cao, G., Alessio, H. and Cutler, R.G. (1993) "Oxygen-radical absorbance capacity assay for antioxidants", *Free Radic. Biol. Med.* **14**, 303–311.

[14] Roe, J.H. and Kuether, C.A. (1943) "The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivates of dehydroascorbic acid", *J. Biol. Chem.* **147**, 399–407.

[15] Burton, G.W., Webb, A. and Inglod, K.U. (1985) "A mild, rapid and efficient method of lipid extraction for use in determining vitamin E/lipid ratios", *Lipids* **20**, 29–39.

[16] Tietze, F. (1969) "Enzyme method for quantitative determination of nanogram amounts of total and oxidized glutathione:applications to mammalian blood and others tissues", *Anal. Biochem.* **27**, 502–522.
[17] Kirlin, J.W. and Theye, R.A. (1963) "Cardiac performance after intracardiac surgery", *Circulation* **28**, 1061–1071.
[18] Brody, W.R., Reitz, B.A., Andrews, M.J., Roberts, W.C. and Michaelis, L.L. (1975) "Long-term morphologic and hemodynamic evaluation of the left ventricle after cardiopulmonary bypass", *J. Thorac. Cardiovasc. Surg.* **70**, 1073–1087.
[19] Morse, D., Adams, D. and Magnani, B. (1998) "Platelet and neutrophil activation during cardiac surgical procedures: impact of cardiopulmonary bypass", *Soc. Thorac. Surg.* **65**, 691–695.
[20] Castelli, P., Condemi, A.M., Brambillasca, C., Fundaro, P., Botta, M., Lemn, M., Vanelli, P., Santoli, C., Gatti, S. and Riva, E. (1995) "Improvement of cardiac function by allopurinol in patients undergoing cardiac surgery", *J. Cardiovasc. Pharmacol.* **25**, 119–125.
[21] Ferrari, R., Alfieri, O., Curello, S., Ceconi, C., Cargnoni, A., Marzollo, P., Pardini, A., Caradonna, E. and Visioloi, O. (1990) "Occurrence of oxidative stress during reperfusion of the human heart", *Circulation* **81**, 201–211.
[22] Ballmer, P.E., Reinhart, W.H., Jordan, P., Buhler, E., Moser, U.K. and Gey, K.F. (1994) "Depletion of plasma vitamin C but not of vitamin E in response to cardiac operations", *J. Thorac. Cardiovasc. Surg.* **108**, 311–320.
[23] Frei, B., England, L. and Ames, B.N. (1989) "Ascorbate is an outstanding antioxidant in human blood plasma", *Proc. Natl Acad. Sci. USA* **86**, 6377–6381.
[24] Buettner, G.R. and Jurkiewicz, B.A. (1996) "Catalytic metals, ascorbate and free radicals: combinations to avoid", *Radiat. Res.* **145**, 532–541.
[25] Van Asbeck, B., Hoidal, J., Vercellotti, G., Schwartz, B., Moldow, C. and Jacob, H. (1985) "Protection against lethal hyperoxia by tracheal insufflation of erythrocytes: role of red cell glutathione", *Science* **227**, 756–759.
[26] Miki, M., Tamai, H., Mino, M., Yamamoto, Y. and Niki, E. (1987) "Free-radical chain oxidation of rat red blood cells by molecular oxygen and its inhibition by tocopherol", *Arch. Biochem. Biophys.* **258**, 373–380.
[27] Pesonen, E., Korpela, R., Leijala, M., Sairanen, H., Pitkanen, O.M., Raivio, K.O., Venge, P. and Anderson, S. (1996) "Prolonged granulocyte activation, as well as hypoxanthine and free radical production after open heart surgery in children", *Intensive Care Med.* **22**, 500–506.
[28] Beauseigneur, F., Goubern, M., Chapey, M.F., Gresti, J., Vegely, C., Tsoko, M., Demarquoy, J., Rochette, L. and Clouet, P. (1996) "F1F0-ATPase, early target of the radical initiator 2,2′-azobis-(2-amidinopropane) dihydrochloride in rat liver mitochondria *in vitro*", *Biochem. J.* **320**, 571–576.
[29] Lahet, J.J., Lenfant, F., Courderot-Masuyer, C., Ecarnot-Laubriet, E., Vergely, C., Durnet-Archeray, M.J., Freysz, M. and Rochette, L. (2003) "*In vivo* and *in vitro* antioxidant properties of furosemide", *Life Sci.* **73**, 1075–1082.
[30] Reinhart, W.H., Ballmer, P.E., Rohner, F., Ott, P. and Staub, P.N. (1990) "The influence of extracorporeal circulation on erythrocytes and flow properties of blood", *J. Thorac. Cardiovasc. Surg.* **100**, 538–545.