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Induction of mitochondrial dysfunction in patients under cardiopulmonary by-pass: preliminary results

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Abstract. – OBJECTIVE: Cardiac surgery is often performed by cardiopulmonary by-pass (CPB), generally associated with organ dysfunction. The aim of this work was to determine if and how this phenomenon is related to mitochondrial damage. To this purpose, the effect of the addition of serum from CPB patients to human fibroblasts cultures on mitochondrial respiratory chain and oxidative phosphorylation (OXPHOS) activities was investigated.

PATIENTS AND METHODS: Serum samples of five patients were obtained before (pre-CPB) and after 6 h CPB weaning (CPB). Mitochondrial OXPHOS activities were examined by polarographic and spectrophotometric assays, and reactive oxygen species (ROS) production was measured by a spectrofluorimeter.

RESULTS: Addition of CPB serum to fibroblasts determined a decrease of mitochondrial oxygen consumption due to an inhibition of mitochondrial respiratory chain and some OXPHOS enzymes activities. This inhibition seems to be mainly related to a reduced activity of complex I.

CONCLUSIONS: Our data represent the first translational research evidence showing that CPB determines mitochondrial dysfunction which leads to impairment of OXPHOX activities and to an increase in ROS production, compromising tissue bioenergetic efficiency.

Key Words:
Translational research, Cardiopulmonary by-pass (CPB), Mitochondrial respiratory chain, Oxidative phosphorylation (OXPHOS), NADH-coenzyme Q oxydoreductase (complex I), Multiple organ dysfunction syndrome (MODS), Ischemia-reperfusion injury, Systemic inflammatory response syndrome (SIRS), Reactive oxygen species (ROS).

Introduction

Cardiac surgery is often performed with the help of cardiopulmonary by-pass (CPB), which maintains cardiopulmonary function by a pump and an oxygenator when the heart is stopped; CPB is not free of complications in the postoperative period.

Twenty percent of "low-risk" cardiac patients develop postoperative complications[1,2] and the incidence of multiple organ dysfunction syndrome (MODS) following CPB is 11% with a mortality rate of 41%[1,3]. Ischemia-reperfusion injury, endotoxemia, and contact of blood with the extracorporeal circuit release pro-inflammatory cytokines such as IL-1, IL-6, IL-8, TNF-α that may promote a systemic inflammatory response syndrome (SIRS)[1,4-8]. CPB

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may also promote apoptosis due to increased activity of Fas[9,10]. It has been demonstrated that serum of patients after weaning from CPB, when incubated with endothelial cells, showed apoptotic properties[11,12]. Apoptosis is related to mitochondrial dysfunction[13-15]. A dominant role of mitochondria is to produce energy of the cell Adenosine triphosphate (ATP) through respiration and oxidative phosphorylation (OXPHOS) system. OXPHOS consists of four multienzyme complexes (I-IV), forming the mitochondrial respiratory chain. The electron transfer along the respiratory chain creates a proton gradient across the inner mitochondrial membrane, which is used by Complex V (ATP synthase) to drive the ATP synthesis. Mitochondria are also the quantitative source of reactive oxygen species (ROS) in mammalian[16]. Abnormalities of the electron transport chain and OXPHOS are the most common causes of increased ROS production and mitochondrial diseases[17].

It has been shown that in several pathological conditions, serum levels of some mitochondria-related substances can be changed, included mesenchymal stem cells. Their levels have been associated to the pathology progression or have been considered as prognostic parameters. Plasma level of mitochondrial Coupling Factor 6 (CF6), an essential component of ATP synthase complex, increases in patients with coronary heart disease[18], acute myocardial infarction[19], stroke[20] or with type 2 diabetes mellitus[21]. Serum levels of mitochondrial Inhibitory Factor 1 (IF1), an inhibitor of ATPase activity, are independently associated with long-term prognosis in coronary artery diseases[22]. Mitochondrial dysfunction and oxidative stress in human fibroblasts cultures exposed to serum from septic patients have been reported[23]. In experimental models of sepsis, Complex I, Complex III, and Complex IV activities of the respiratory chain are reduced[24-27].

In this translational research work, we evaluate if CPB is able to affect mitochondrial function and determine "cytopathic hypoxia". For this purpose, we have measured the OXPHOS capacity in fibroblasts cell cultures incubated with serum samples obtained from patients before and after CPB.

Patients and Methods

Patients and Methods

All patients gave permission after they signed a written informed consent in accordance with the Helsinki Declaration, for the re-use of human bio-specimens in scientific research. The investigation was conducted in accordance with the current medical protocol as described by the Italian Government's NIH legislation. The procedure followed a precise individual medical anamnesis together with the required clinical evaluations performed at the Medical School-University of Bari "Aldo Moro" (Bari, Italy), in collaboration with the Elbasan University (School of Technical Medical Sciences, "A. Xhuvani"), Albania (Ethical Committee approval N. 496/1). Procedures were conducted according to Good Clinical Practice (GCP) and manufacture specifications.

In seven patients undergoing first elective heart surgery with CPB, blood samples were collected from the central venous catheter after induction of anaesthesia, and 6 h after weaning from CPB. After blood centrifugation at 500 g for 10 min at 25°C, the supernatant was preserved at –20°C until analysis. Anaesthesia management included premedication with morphine 10 mg s.c. 1 h before access to the operating room.

Anaesthesia was induced with propofol, 1-2 mg/kg, midazolam, 0.1-0.2 mg/kg, and fentanest 3-4 μg/kg. Neuromuscular blockade was obtained with rocuronium, 0.1 mg/kg for the intubation. Anaesthesia was maintained with continuous infusion of propofol 10 mg/kg/hr, fentanest, bolus of 50 μg every 30 min, and rocuronium 7 μg/kg/min. Extracorporeal circulation was conducted with aortic and atriumcaval cannulation. Cardioplegia was obtained with a cold solution. Pump flow was set from 4 to 4.5 L/min. Activated clotting time of 480 s was obtained with heparin bolus intravenous (i.v).

After surgery, clinical data and blood tests were collected to determine systemic inflammatory response syndrome (SIRS) occurrence. SIRS was defined according to The American College of Chest Physicians and the Society of Critical Care Medicine (ACCP/SCCM)[27].

Only patients with SIRS were enrolled in the study. Clinical conditions were determined by the APACHE II score (Acute Physiology and Chronic Health Evaluation II)[28] and SOFA (Sequential Organ Failure Assessment) score[29].

Cell Cultures and Mitoplasts Preparation

Neonatal normal human dermal fibroblasts (NHDF-neo; Cambrex, CC-2509, Paullo, Milan, Italy) were grown in the exponential phase in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Euroclone, Pero, MI, Italy) supplemented with 10% fetal bovine serum (FBS; Sig-

ma-Aldrich, MI, Italy), plus 2 mM L-glutamine (Euroclone, Pero, MI, Italy), 100 µg/ml penicillin (Euroclone, Pero, MI, Italy) and 100 µg/ml streptomycin (Euroclone, Pero, MI, Italy).

The cultures were incubated in 5% CO_2 humidified atmosphere at 37°C. FBS was replaced 12 h before the assay with 10% pre and post-CPB patient serum, while other components were not modified. For mitoplasts (i.e., mitochondria devoid of outer membrane) preparation, fibroblasts were harvested with 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA), and washed in phosphate-buffered saline (PBS) pH 7.4. Cells in PBS were exposed for 10 min on ice to 0.5 mg digitonin/mg cellular protein. Mitoplasts were prepared as in Papa et al[30], were pelleted at 10,000 g and resuspended in PBS[31].

Cellular Respiration

The cellular respiratory activity was measured polarographically[31] with a Clark-type oxygen electrode in a water-jacketed chamber, magnetically stirred at 37°C[8]. The cells were collected by trypsinization and centrifugation to 500 g per 3 min at 37°C and resuspended at 1-3×10^6 cells/ml in a buffer containing Sucrose 75 mM, Tris-HCl 30 mM, KCl 50 mM, EDTA 0.5 mM, Magnesium chloride ($MgCl_2$) 0.5 mM, KH_2PO_4 2mM. Cell suspensions were transferred to the polarographic chamber, and an aliquot was used for cell counting and protein determination with the Bradford method.

The substrates and inhibitors of mitochondrial OXPHOS were added at the following concentrations: oligomycin (1 µg/µl), carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (0.25 µM), Rotenone (1 µg/µl), succinate (1 M), digitonin (10%), antimycin A (1 µg/µl), ascorbate (1 M)+ N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD; 0.1 M) and potassium cyanide (KCN; 1.0 mM).

Respiratory control ratio (RCR) represents State III uncoupled/State IV oxygen consumption ratio (state IIIu/state IV). Respiration rates are expressed as femtomoles of molecular oxygen consumed per milligram of mitochondrial protein.

Assay of Mitochondrial Respiratory Complex Activities

The activity of NADH-coenzyme Q oxidoreductase (complex I, EC 1.6.5.3) was assayed essentially[32]. Mitoplasts were exposed to ultrasound energy for 15 s at 0°C, and Vmax and Km were determined using NADH as electrons donor to 360-374 nm with the Δε of 2.01 mM^{-1}. In 800 µl of Mix (K-Phosphate buffer 50 mM pH 7.4, $MgCl_2$ 2 mM, K-EDTA 2 mM, KCN 3 mM, antimycin A 1 µg/ml) 30 µg of mitochondrial proteins were added in presence of decylubiquinone 0.2 mM. The reaction was started with different concentrations of NADH (1.3-2.8 µM). The measurements were determined with and without rotenone 1µg/ml to discriminate the complex I activity. The Vmax and Kmof complex I were determined with Lineweaver-Burk equation following the oxidation of NADH. Cytochrome-C oxidase (complex IV, EC 2.3.3.1) activity was determined on mitoplasts in 700 µl of buffer (10 mM phosphate buffer, KCN, $MgCl_2$ 2 mM, pH 7.4) following the oxidation of ferrocytochrome c 10 µM.

Citrate synthase (CS) activity, used as enzymatic marker of the mitochondrial matrix, was assayed according to Taurino et al[33]. Briefly, mitochondrial proteins (30 µg/ml), 0.5 mM acetyl-coenzyme A, and 0.5 mM 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) were added to a Tris-HCl buffer (100 mM), pH 8.0. The reaction was started by the addition of 0.5 m oxalacetate, and the initial rate was measured following the reduction of DTNB at 419 nm ($\Delta\varepsilon$=163 $mM^{-1}\cdot cm^{-1}$).

The determination of ATPase (complex V) activity consisted of a coupled double enzymatic assay, as reported in Cavallo et al[34]. Since the mitoplasts were exposed to ultrasound energy, in 700 µl of Mix (sucrose 250 mM, Potassium chloride (KCl) 50 mM, $MgCl_2$ 5 mM and Tris-HCl 20mM pH 7.5) 30 µg of proteins were added in presence of pyruvate kinase 2 U, lactate dehydrogenase 2.5 U, phosphoenolpyruvate 1 mM, and NADH 200 µM. When ATP 0.5 mM is added, phosphoenolpyruvate is converted to pyruvate and pyruvate to lactate with the oxidation of NADH to 340 nm[31].

Membrane Potential Measurement

The mitochondrial membrane potential was measured following the fluorescence quenching of safranin at 525 nm (excitation) and at 575 nm (emission) with a spectrofluorimter (JASCO FP 6200). 1x10^6 cells were permeabilized with 20 µg/1x10^6 of digitonin and resuspended in 1 ml of buffer ICR (sucrose 75 mM, Tris-Cl 30 mM pH 7.4, KCl 50 mM, K-EDTA 0.5 mM, $MgCl_2$ 2 mM, Potassium phosphate monobasic ($KH_2PO_{4)}$ 2 mM, pH 7.4) with safranin (5 µM), and ciclosporyn A (33nM). The potential is generated by glutamate/malate (10/10 mM) and succinate (10 mM) addition.

ROS Measurements

Fibroblasts were treated with 10 μM dichlorofluorescein-diacetate DCF-DA (503-570 λ) for the detection of H_2O_2 with fluorimetric analysis. ROS production was measured using the cell permeant probe 2′-7′dichlorodihydrofluorescin diacetate (DCF-DA) which passively diffuses into cells where intracellular esterases cleave acetate groups to form the impermeable DCF-H2 which remains trapped within the cell. Cells were collected by trypsinization and resuspended in a small volume of PBS and were then incubated in 800 μl of PBS with 10 μM DCF-DA for 20 min in the dark at 37°C. The linear fluorescence increase (507 nm excitation and 530 nm emission wavelength), produced by the ROS-dependent oxidation of DCF-H2 to the fluorescent compound dichlorofluorescein (DCF), was measured with a Jasco FP6200 spectrofluorimeter.

Statistical Analysis

Clinical data are presented as absolute numbers, with standard deviation, or percentages. ROS generation, expressed as arbitrary fluorescence units, was calculated as percentage of those recorded before CPB cultured fibroblasts treatment. Results are analyzed with Student's *t*-test. $p<0.05$ was considered statistically significant.

Results

The pre-CPB and post-CPB serum were used to determine whether circulating factors can affect mitochondrial functions. Patients without clinical signs of SIRS in the post-operative time were excluded from the study. Clinical data are summarized in Table I.

Table I. Clinical data.

Patients data	(mean ± SD)
Sex (male/female)	4/1
Age (years)	65 ± 8.12
Ejection fraction %	53.6 ± 5.89
CPB (min)	88 ± 34.65
Minimum DaO_2 in CPB ($mlO_2/min/m^2$)	248.77 ± 42.35
Lactates in CPB (peak)	2.7 ±1.74
Glucose in CPB (peak)	142.2 ± 42.23
Minimum temperature in CPB	35 ± 1.08
SOFA Score	0.2 ± 0.45
APACHE II Score	7 ± 3.08
Predictive Death Rate %	8.14 ± 3.82

A significant difference between Complex I activity of cells incubated with serum pre-CPB and post-CPB can be observed. Results in Figure 1 demonstrate that, when compared to serum pre-CPB, the addition of serum post-CPB to human fibroblasts determines a significant decrease (i.e., nearly to 50%, $p= 0.017$) of complex I activity. Interestingly, a similar but less pronounced inhibition of ATPase activity was observed (Figure 1).

No statistically significant differences were found for Cytochrome c oxidase (COX) and citrate synthase activities in post-CPB *vs.*, pre-CPB serum-treated fibroblasts ($p =0.681$ and $p =0.614$, respectively) (data not shown). Figure 2 shows that the Vmax ratios of Complex I and Citrate Synthase (CI/CS) activities were significantly lower in post-CPB as compared to pre-CPB serum (CI/CS, $p =0.012$). The Vmax ratios of Complex I and Cytochrome c oxidase appears to be lowered in the post-CPB *vs.* pre-CPB samples, but the data is not statistically significant (CI/COX, $p =0.039$).

To prove the biological relevance of alteration in mitochondrial respiratory chain activity induced by serum addition to fibroblasts cultures, oxidative damage was then measured. ROS were significantly increased in cells incubated with post-CPB compared to pre-CPB serum samples ($p<0.001$) as reported in Figure 3. The membrane potential semi-quantitative analysis by fluorescence quenching, showed that after glutamate/ma-

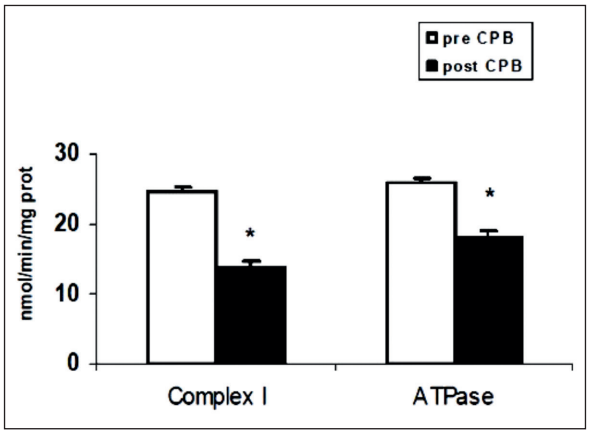

Figure 1. Effect of pre-CBP serum and post-CBP serum addition to human fibroblasts. The complex I activity in these cells showed a decrease of about 50% ($p= 0.017$) when post-CBP serum was added, with respect to the addition of pre-CBP serum. A similar but less pronounced inhibition was noted regarding the ATPase activity. Each bar is the mean ± standard error (SEM); the results are analyzed with Student's *t-test*. $p < 0.05$ was considered statistically significant. *Significantly different with respect to pre-CBP serum addition.

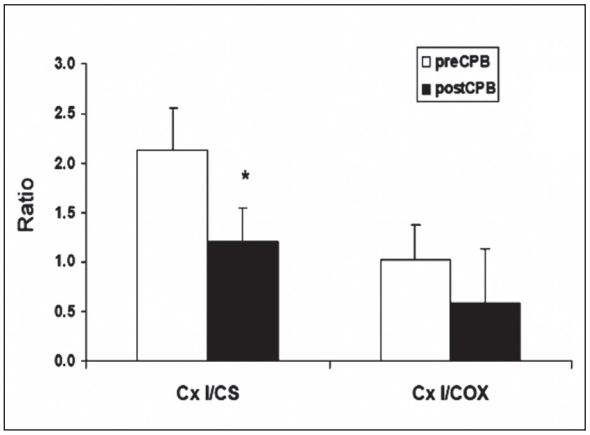

Figure 2. Ratios between Vmax of Complex I and Citrate Sintase (Cx I/CS) and between Vmax of Complex I and Cytochrome c oxidase (Cx I/COX) activities in pre-CBP and post-CBP serum-treated fibroblasts. Lower values were observed in post-CBP serum-treated cells (Cx I/CS, p= 0.012; Cx I/COX, p= 0.039, respectively). Each bar is the mean ± standard error (SEM); the results are analyzed with Student's *t-test*. p< 0.05 was considered statistically significant. *Significantly different with respect to pre-CBP serum addition.

late addition a plateau value was rapidly achieved in the post-CPB samples measurements, whilst in the pre-CPB samples no saturation of membrane potential no potential was observed in the time-scale of the experiments (Figure 4).

Rotenone addition reverses the potential in both conditions and successive succinate pulse returns to the pre-inhibition levels the fluorescence

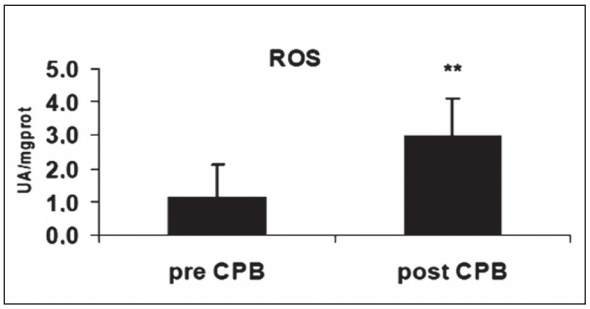

Figure 3. ROS measurements performed to prove the oxidative damage and the relevant alteration of mitochondrial respiratory chain activity induced by post-CBP serum addition to human fibroblasts. ROS levels in these cells were significantly higher after post-CBP serum incubation, compared to pre-CBP serum-treated fibroblasts. ROS generation was expressed as arbitrary fluorescence units and calculated as percentage of those recorded before CPB cultured fibroblasts treatment. Each bar is the mean ± SEM; the results are analyzed with Student's *t-test*. p < 0.05 was considered statistically significant. **Significantly different with respect to pre-CBP serum addition, with p < 0.001.

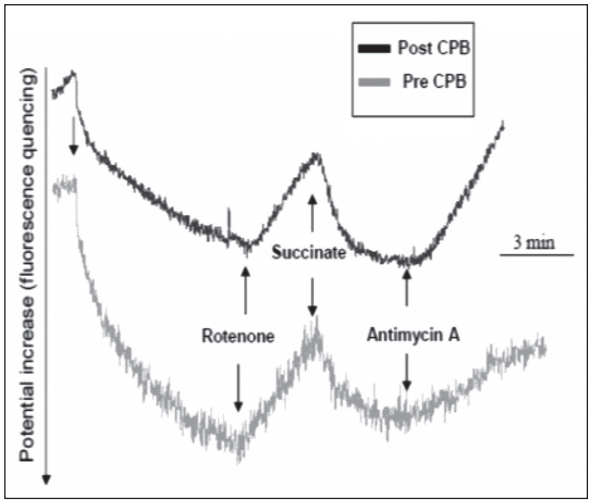

Figure 4. Membrane potential measurements by spectrofluorimetric analysis. It was observed a decrease of values after glutamate/malate but not succinate addition in pre-CBP serum-treated fibroblasts.

signals (Figure 4). Of note, successive inhibition by Antimycin A leads to membrane potential collapse with significantly different kinetics, as shown in Figure 4. The polarographic analysis underlined (Figure 5A) the reduction of endogenous respiration in cells incubated with serum post-CPB samples (p = 0.008).

A similar decrease was observed after incubation with oligomycin (state IV) and addition of the uncoupler CCCP (state IIIu; Figure 5A). The state IV and IIIu of respiratory chain decreased after post-CPB addition, (p=0.018 and p=0.001), while the succinate respiration was unaffected (Figure 5A). The ascorbate/TMPD respiration, a measure of isolated COX respiratory activity, was reduced (p=0.01; Figure 5A). Figure 5B shows that whilst the ratios between endogenous respiration, and succinate or ascorbate/TMPD respiration are not statistically different in the two different experimental conditions, the respiratory control ratio (RCR) shows a marked decrease in the post-CPB condition (p=0.007; Figure 5B).

Discussion

The data of this translational study show that addition to human fibroblasts cultures of serum from patients submitted to cardiac surgery with CPB induces abnormalities of mitochondrial functions. In particular, a decrease in cellular endogen respiration, associated with inhibition of some activ-

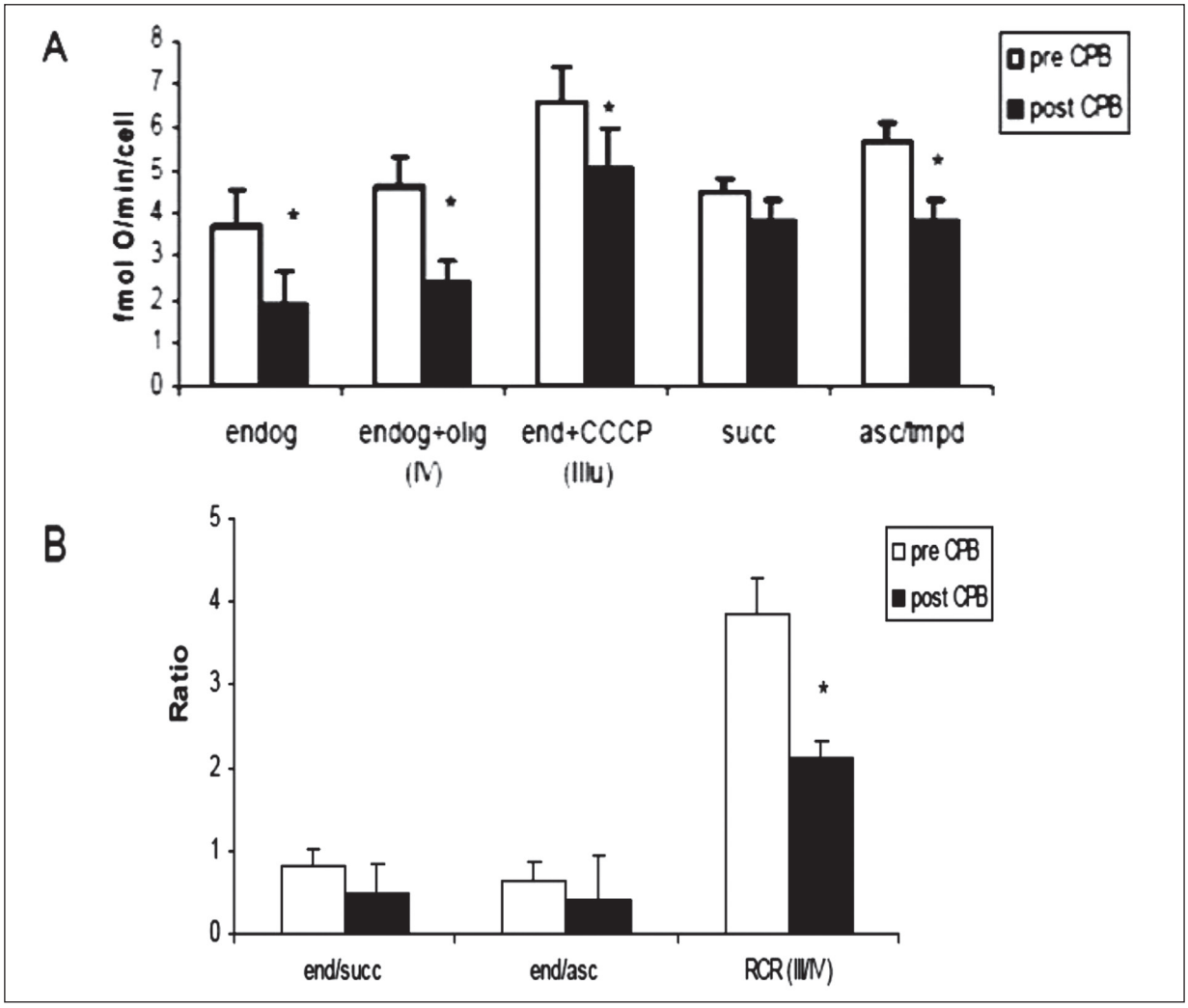

Figure 5. Polarographic analysis of cellular respiratory activity in cellular suspensions of pre-CBP and post-CBP serum-treated human fibroblasts. **A,** Marked reduction in cellular endogenous respiration was observed in fibroblasts incubated with post-CBP serum (p = 0.008); a similar decrease was noted after addition of oligomycin (state IV) and of the uncoupler CCCP (state uIII); an impairment of the state IV and uIII of respiratory chain appeared after post-CBP serum addition (p = 0.018 and p=0.001). Ascorbate/TMPD respiration was reduced (p=0.01) too. *Significantly different with respect to pre-CBP serum addition. **B,** Rates between endogenous activity and Complex II oxymetry (ratio end/succ) and between endogenous activity and Complex IV oxymetry (ratio end/asc) were reduced after CPB with p = 0.015 and p = 0.047, respectively. RCR index (State uIII/State IV ratio) decreased significantly after CPB treatment (p = 0.007). Each bar is the mean ± SEM; the results are analyzed with Student's *t-test*. $p < 0.05$ was considered statistically significant. * Significantly different with respect to pre-CBP serum addition. End= endogenous respiration; oligo = oligomycin; uIII = uncoupled state III; succ = succinate; asc = ascorbate; RCR = respiratory control ratio (Ratio between uIII state and IV state of respiration).

ities of the OXPHOS system and of mitochondrial membrane potential, was observed. Conversely, an increase of the oxidative stress, determined by elevated ROS production, was detected.

When we assayed in a selective approach the activities of single components of the respiratory chain, we observed a reduced activity of mitochondrial complex I and complex V, but not of complex II activity.

It is well known that alterations of mitochondrial efficiency and function are mainly related to alterations in mitochondrial mass, a amount of respiratory enzymes or changes in enzyme activities[28,29]. The relative preservation of citrate synthase activity we found indicates that total mitochondrial content remains largely unchanged and resistant to CPB injury, thus excluding changes in mitochondrial mass. Conversely, the complex

I/complex IV and complex I/citrate synthase ratios show a reduction after CPB indicating a mitochondrial enzymatic complex I defect. Note that the deficiency of complex I activity has been indicated as a major cause of reduced mitochondrial oxygen consumption and of increased ROS production.

The decrease of Ascorbate/TMPD respiration can be associated to an impairment of complex IV activity. The alteration of RCR index indicates a mitochondrial uncoupling, namely a mitochondrial membrane injury with cytochrome c loss, which is an expression of the apoptotic process. Taken together, these results showed that CPB induces damage of some mitochondrial complexes, particularly the Complex I, and even to a lesser extent Complex IV and V. The obtained results add further support to those of Aebert et al[11] and Schmid et al[12] who reported higher proportion of apoptotic endothelial cells in culture plates incubated with serum samples obtained at 6 h after weaning from CPB, when compared to plates incubated with pre-operative samples.

When mitochondrial complex activities have been evaluated in septic patients, similarly to our data, a reduction in Complex I activity was observed, indicating a strong association between septic shock and mitochondrial damage[35-38]. Furthermore, using spectrophotometric assay, cytochrome c function has been evaluated in septic baboons[26,27]. In agreement with our findings, it has been reported that after infusion of *Escherichia coli*, Cytochrome c oxidase activity was found to be reduced, and, similarly to our data, 6 h after application of the noxious stimulus, cytochrome c function was lost[37,38]. Taken together, these findings should be also seen in the light of the whole membrane moiety, including lipids such as cardiolipin and quinones, which plays a key role in the function of the mitochondrial respiratory complexes[39,40].

Proinflammatory cytokines have been linked to depression of myocardial contractility both in patients with acute septic shock and in *in vitro* models employing isolated myocytes exposed to serum from such patients[41,42]. In sepsis such as in post-CPB SIRS is evident an association between proinflammatory cytokines and mitochondrial damage[41].

The loss of efficiency by OXPHOS, as a consequence of the reduced ATPase activity that we observed in post-CPB-treated fibroblasts (Figure 2), might contribute to explain some major post-CPB complications such as acute kidney injury, or pulmonary and neurological dysfunctions.

Traditionally, postoperative complications have been related to the critical role of low oxygen delivery during CPB. Low hematocrit and pump flow have been identified as factors promoting renal dysfunction[43], and the finding of high lactate levels have been viewed as index of low oxygen delivery[8,44,45]. Our data may offer new insights. The target of maintaining an adequate oxygen delivery during CPB may be insufficient to guarantee aerobic metabolism, if CBP determines mitochondrial chain impairment and consequent cytopathic hypoxia. Evidence has been reported that the latter, rather than inadequate oxygen delivery, may play a more important role in the development of multiple organ dysfunction syndrome (MODS)[46-48]. In this connection, as mitochondria are the primary consumers of cellular oxygen, mitochondrial function and dysfunction are crucial in the development of cytopathic hypoxia[49-50]. Certainly, future studies are warranted to deeply understand the relationship between postoperative organ damage and mitochondrial dysfunction.

Conclusions

Mitochondria play a central role in the intracellular events associated with CPB. Altered metabolism can lead to rapid ROS overproduction and damage of the mitochondrial protein with a generalized reduction in the capacity to generate ATP. We have shown that post-CPB SIRS is associated with a condition similar to "cytopathic hypoxia". Although this phenomenon is considered to be self-limiting, an exaggerated and prolonged inflammatory status with mitochondrial dysfunction may be critical in the pathogenesis of post-operative complications. More translational research studies are needed to clarify the role of mitochondrial dysfunction in the various phases of CPB, before testing therapeutic strategies targeting mitochondria.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Authors' Contribution

A.G. conceived and designed the research. R.T., F.T., and A.M.M. performed the laboratory research; S.S. supervised

the manuscript and gave the final approval of the version to be published. G.D., N.S., and L.S. participated in bibliographic research. A.B. contributed to statistical analysis and manuscript revision. F.I. contributed to bibliographic research, reagents, materials, and analysis tools. All authors read and approved the final version of the manuscript. P.F., F.M., and N.B. collected the biological material. All authors read and approved the final version of the manuscript.

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