ORIGINAL ARTICLE

We examined antioxidant actions in 73 patients undergoing coronary artery surgery by assessing mitochondrial damage and oxidative stress in ventricular biopsies obtained at preischemia and postreperfusion. Those patients who received antioxidant therapy benefited by less oxidative stress and mitochondrial damage.

Oxidative Stress and Mitochondrial Damage in Coronary Artery Bypass Graft Surgery: Effects of Antioxidant Treatments

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INTRODUCTION

Heart reperfusion injury is a complex phenomenon leading to biochemical and ultrastructural changes in myocardial cells which in turn may lead to electrical and hemodynamic cardiac dysfunctions, which are expressed as myocardial stunning and reperfusion arrhythmias.[1,2]

Almost ten years ago, a study from our group provided the first evidence of oxidative stress associated with ultrastructural damage in the human heart during reperfusion and suggested the involvement of oxygen free radicals.[2] That study compared oxidative stress, assayed as hydroperoxide-initiated chemiluminescence, with mitochondrial damage, determined by electron microscopy, in pairs of biopsies obtained before ischemia and after reperfusion. The study concluded that "the presence of oxidative stress during reoxygenation...may play a major role in the genesis of reperfusion injury".[2]

Previous work in experimental models, had shown that a variety of antioxidants and scavengers can ameliorate the histological changes and functional disturbances produced by the ischemia-reperfusion in the heart.[3-5] These results suggested that better heart protection could be achieved with the addition of antioxidants or related substances to the cardioplegic solution. In our experience, supplementation of the cardioplegic solution with mannitol[6] or deferoxamine,[7] or the preoperative intravenous infusion of taurine[8] reduced tissue damage and oxidative stress. In addition, the preoperative administration of vitamins A and E significantly decreased heart oxidative stress.[9] We also observed that blood cardioplegia supplemented with mannitol showed better results than plain crystalloid cardioplegic solutions in patients with normal ventricular mass.[10]

REPRINTS

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Numerous studies, including our own, have demonstrated oxidative stress during coronary revascularization. However, some derived subjects need to be addressed. 1) The occurrence of irreversible mitochondrial damage; 2) the existence of oxidative stress secondary to oxygen free radical overproduction; 3) the beneficial effect of antioxidants; and 4) the degree of myocardial protection afforded by these drugs. We present a review of our experience in this field aimed at defining the effective antioxidant actions for coronary artery surgery.[2,6-10]

MATERIAL AND METHODS

All the data from previous studies[2,6-10] were grouped and analyzed. The data consist of a control ischemia reperfusion group and treatment groups (mannitol, deferoxamine, taurine, vitamins A and E, and blood.[6-10]

Patients. Seventy patients who underwent coronary artery surgery were studied.[6-10] In order to obtain a homogeneous group, the patients selected for the studies fulfilled the following criteria: 1) ejection fraction greater than 45%; 2) absence of recent (less than 4 weeks) myocardial infarction; 3) absence of associated valve disease; and 4) achievement of a complete or satisfactory surgical revascularization. Written informed consent for participation in the study was obtained from each patient and the institutional review board approved the study protocol.

Cardiopulmonary bypass was instituted and the temperature of the perfusate was lowered to 28° C. Topical hypothermia with crushed-iced saline solution was applied. Before the aorta was cross-clamped, two full-thickness biopsies were obtained from the apex of the heart, and were designated as preischemia or basal samples. The biopsy sample for chemiluminescence assay was immersed in saline solution and frozen. The second biopsy sample, for electron microscopy, was immersed in cold 3% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4).

After placement of the aortic clamp, 500 mL of Saint Thomas-type cardioplegic solution, at a temperature of 4° C, was injected at 80–100 mm Hg into the aortic root. A needle thermistor was inserted into the septum for continuous monitoring of the myocardial temperature, which ranged from 18° to 22° C. After completion of the distal anastomoses, the aortic clamp was removed and the heart defibrillated. In most instances, countershocks were necessary to return the heart to sinus rhythm (mean value of applied countershocks = 1.6 $\pm$ 0.3). Ten minutes after reperfusion and with the patient rewarmed, two new biopsies were obtained, designated as reperfusion samples and processed in a similar manner.

The main clinical and surgical characteristics of the patients in the study groups are shown in Table 1. There was no significant difference in age, ejection fraction, aortic cross-clamp time, and number of grafts among groups.

Antioxidant Treatments.

- Mannitol group (n = 6): the cardioplegic solution contained 59.8 mmol mannitol (Laboratory FADA, Buenos Aires, Argentina).[6]
- Deferoxamine group (n = 7): the cardioplegic solution was supplemented with 1 g/L of deferoxamine (Ciba-Geigy, Buenos Aires, Argentina).[7]
- Taurine group (n = 6): 1–3 hours before surgery, patients received a rapid intravenous infusion of 5 g of taurine (O-due, Istituto Farmacochimico Nativelle, Florence, Italy).[8]
- Vitamins A and E group (n = 8): patients received an oral administration of 400 mg of vitamin E and 100,000 IU of vitamin A daily for five days before surgery.[9]
- Blood group (n = 10): myocardial protection was achieved using blood cardioplegia supplemented with 40 mmol/L mannitol and 20 mmol/L KCl.[10]

Heart Oxidative Stress. Oxidative stress was indirectly studied using the hydroperoxide-initiated chemiluminescence assay.[11,12] Biopsy samples (approximately 20 mg) were homogenized in 2 mL of 140 mmol KCl and 20 mmol phosphate buffer (pH 7.3). The protein homogenate was adjusted to 1 mg/mL and 3 mmol of tert-butyl hydroperoxide was added to the suspension, and was immediately assayed for chemiluminescence in a scintillation counter in the out-of-coincidence mode for single photon counting. Chemiluminescence was expressed as counts/minute per milligram of protein of the homogenate.

The oxidative stress index was calculated dividing the chemiluminescence yield after reperfusion by the chemiluminescence yield before reperfusion. This adimensional number expresses induced oxidative stress by any number higher than one.[12]

Mitochondrial Damage. The biopsy tissues used for transmission electron microscopy were fixed in cold 3% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4), postfixed in 1% osmium tetroxide, dehydrated and embedded in Epon. Three blocks were selected from different depths of each biopsy sample. From each block, a 1 µm section was cut, stained with 1% toluidine-borax, and examined by light microscopy to select appropriate

TABLE 1

Clinical and Surgical Characteristics of Patients

Groups	n	Age (years)	Ejection Fraction (%)	Aortic Cross-clamp Time (min)	Grafts (number)
Control	33	54 ± 4	53 ± 3	53 ± 5	2.9 ± 0.3
Mannitol	6	54 ± 2	53 ± 2	54 ± 2	3.0 ± 0.2
Deferoxamine	7	54 ± 3	52 ± 3	47 ± 5	2.8 ± 0.7
Taurine	6	57 ± 2	56 ± 2	49 ± 2	3.0 ± 0.2
Vitamins A and E	8	50 ± 3	53 ± 2	49 ± 5	3.0 ± 0.3
Blood cardioplegia	10	56 ± 1	52 ± 3	52 ± 5	2.9 ± 0.2

areas for thin sectioning. Five ultrathin sections were obtained from each of the three blocks. Ultrathin sections were mounted in copper grids, stained with uranyl acetate and lead citrate, and examined in an electron microscope.

Electron micrographs were taken systematically at magnifications of 5000× and mitochondrial damage was determined in a blind manner by two different observers who assigned a single score to each of the areas using criteria based on Kloner et al.[13] A numerical value of 0 through 4 was assigned for each mitochondrion, depending on the degree of morphologic damage. The grading scale was as follows: 0—normal mitochondria; 1—early swelling as manifested by separation of cristae and clearing of matrix density; 2—more marked swelling than in grade 1; 3—massive swelling with architectural disruption; and 4—the same as 3 with rupture of inner and outer mitochondrial membranes. The average obtained from the two observers was expressed for each grade (0 to 4) as a percentage of the total number of mitochondria counted per sample. Approximately 500 mitochondria per biopsy were graded in this manner. Mitochondria graded 3 and 4 were considered irreversibly damaged.[13] The mitochondrial damage index induced by reperfusion was calculated by dividing the percentage of irreversibly damaged mitochondria (grades 3 and 4) after reperfusion by the same percentage before reperfusion. This adimensional number expressed induced damage by any number higher than one.

Statistical Analysis. Comparisons between groups were done with an analysis of variance with post-hoc tests (Dunnett). Values in tables are presented as mean values ± SEM, with an assigned significance level of .05.

RESULTS

Clinical Outcome. The patients had a satisfactory postoperative outcome. No evidence of perioperative myocardial infarction was observed by the appearance of new Q waves in the electrocardiogram (ECG) or by increased values for serum creatine phosphokinase. Four patients in the control group and 3 in the mannitol group required inotropic support 4–18 h postopertively. One patient in the control group had uncontrolled gastrointestinal bleeding and died on the 13th postoperative day. Two patients in the deferoxamine group required electrical conversions of atrial fibrillation within the first 12 postoperative hours.

Oxidative Stress. The oxidative stress index value of 2.13 in the control group clearly indicates the occurrence of oxidative stress[12] during the reperfusion of the heart undergoing coronary revascularization (Table 2). The index value of 2.13 implies a 113% increase in hydroperoxide-initiated chemiluminescence, which corresponds to a decreased content of vitamin E and other related antioxidants in the biopsy. This decreased content of endogenous antioxidants indicates a previously increased rate of oxygen free radical reactions.[12] The oxidative stress indexes for the various treatments indicate protection values of 44 and 94% for the supplementation of the cardioplegic solution with deferoxamine and mannitol, respectively, a protection value of 84% for taurine infusion, and of 100% for pretreatment with vitamin A and E. As the basal samples of the treatment and control groups were not significantly different (± 15%), the lower oxidative stress indices in all the treatment groups were due to significant decreased hydroperoxide-initiated chemiluminescence in the postreperfusion samples. (Figure 1)

TABLE 2

Oxidative Stress in the Heart of Patients Subjected to Coronary Revascularization Surgery

Groups	n	Oxidative Stress Index	Protection (%)
Control	33	2.13 ± 0.18	—
+ mannitol	6	1.06 ± 0.10	94
+ deferoxamine	7	1.63 ± 0.20	44
+ taurine	6	1.18 ± 0.16	84
+ vitamins A and E	8	0.96 ± 0.18	100

Oxidative stress indexes were determined as the ratio of hydroperoxide-initiated chemiluminescence after reperfusion/before reperfusion in the biopsies obtained from each patient. All treatments, except deferoxamine, were statistically ($P < .01$) different from the control ischemia-reperfusion condition. Deferoxamine treatment has a difference with $P = .05$. The mean value for hydroperoxide-initiated chemiluminescence in all the biopsies (control and treatments) was 32.7 x 10^3 cpm/mg of protein.

Tissue Injury and Mitochondrial Damage. *General description.* Basal samples in all the groups showed normal sarcomere structure with normal mitochondrial arrangement and tightly packed cristae and gray matrix density. The morphologic changes in the postreperfusion biopsy specimens of the control group, consisted of focal myofibrillar disorganization with myocytolisis, sarcoplasmic vacuolization, and mitochondrial swelling and disruption. Distension of sarcoplasmic reticulum and T tubules were frequently seen and diffusely scattered in damaged myocytes. Mitochondria showing swelling, clearing of matrix density, and separation of cristae with granular and linear densities or myeline figures and membrane disruption were observed in the most advanced stages.

Mitochondrial damage. The morphologic quantitative analysis of the biopsy specimens showed that the occurrence of mitochondria graded 0, 1 and 2 were not statistically different between groups. The number of severely damaged mitochondria, graded 3 and 4, increased markedly from 10.2% to 26.8% following reperfusion in the control group, resulting in a mitochondrial damage index of 2.63. Supplementa-

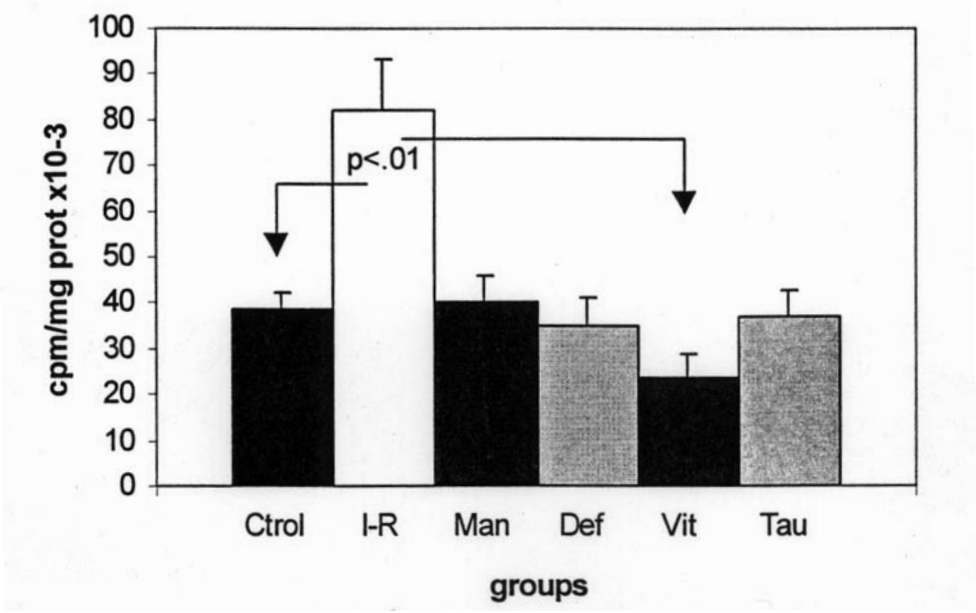

Figure 1.—Hydroperoxide-initiated chemiluminescence in heart biopsies of patients subjected to coronoary revascularization surgery. Ctrol, basal sample; I-R, reperfusion sample without treatment; Man, reperfusion sample of mannitol treatment; Def, reperfusion sample of deferoxamine treatment; Vit, reperfusion sample of vitamins A and E treatment; Tau, reperfusion sample of taurine treatment.

tion of the cardioplegic solution with mannitol or deferoxamine produced protection values of 63% and 47%, respectively, and taurine infusion or the use of mannitol in blood cardioplegia provided protection values of 85 and 95%, respectively. (Figures 2–5) Regarding mitochondrial damage, statistical differences were observed in the percentages of injured mitochondria between the postreperfusion control samples and the basal samples of the control group and the postreperfusion samples of all the treated groups. Furthermore, significant differences were observed between the postreperfusion samples of untreated and treated patients. (Figure 6 & Table 3)

DISCUSSION

In the 1970s, Hearse created the concept of "the oxygen paradox",[14] and thereafter, its pathophysiology has been extensively researched. Hearse described that after a period of ischemia, ultrastructural damage to the heart increases during reperfusion. This is associated with functional alterations of the myocardial contractility and arrhythmias, and is a major concern during surgical procedures requiring cardiopulmonary bypass.[1] Thus, various substances have been evaluated to lessen myocardial damage during ischemia-reperfusion.

In our experience the use of mannitol,[6] deferoxamine,[7] taurine,[8] vitamins A and E,[9] and blood cardioplegia supplemented with mannitol[10] seemed to ameliorate reperfusion damage by decreasing oxidative stress and mitochondrial damage. Experimental studies in rabbits[15-18] also demonstrate the overproduction of oxygen free radical and the occurrence of oxidative stress in coronary artery

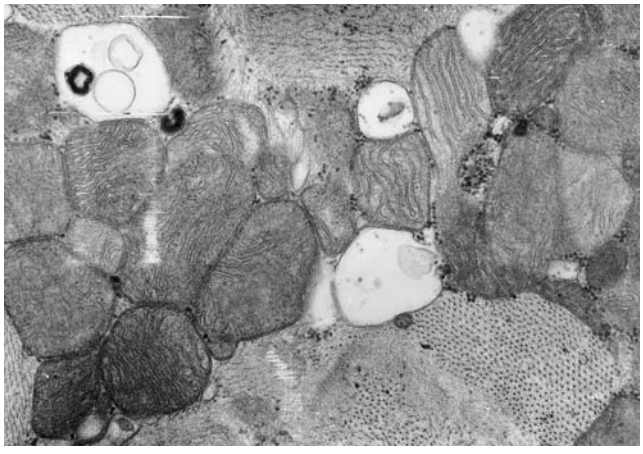

Figure 2.—Mitochondria show tightly packed cristae and intact membranes. Basal sample specimen, only some of them present mild swelling. Transmission electron micrograph 10,000×.

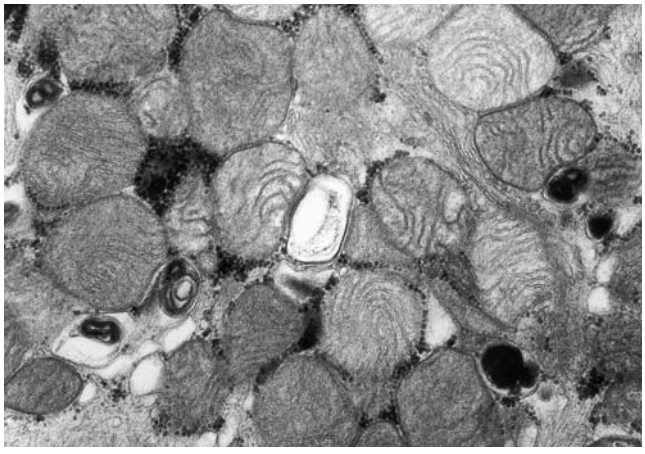

Figure 4.— Reperfusion sample protected with mannitol. Mitochondrial structures are preserved and only mild and moderate swelling can be observed.

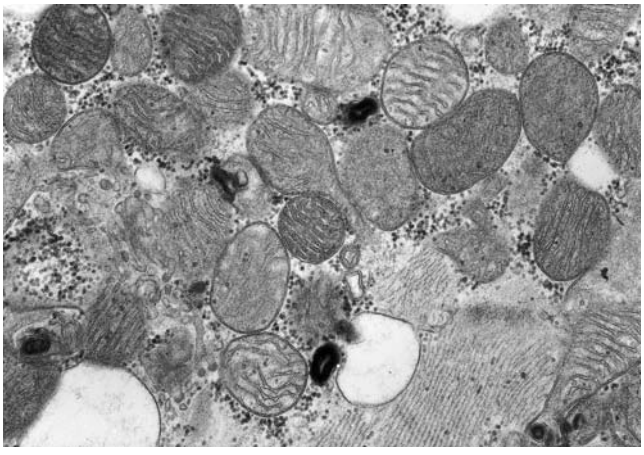

Figure 3.—Basal sample from a specimen of the mannitol group. In general, mitochondria are well preserved as in figure 2 10,000×.

Figure 5.—Reperfusion sample specimen showing severe mitochondrial damage with massive swelling and disruption of cristae, rupture of membranes and deposits of amorphous bodies. Transmission electron micrograph 10,000×.

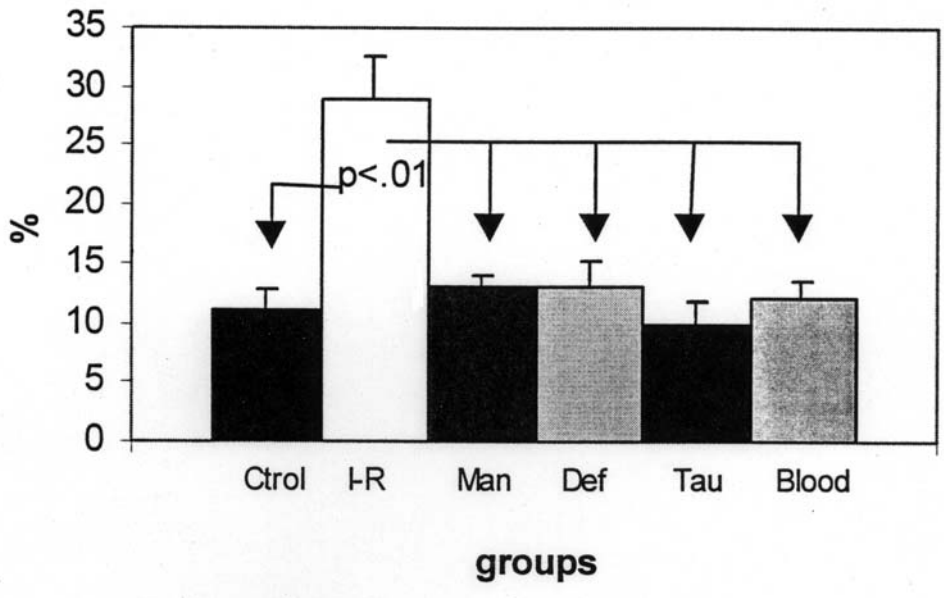

Figure 6.—Irreversible damaged mitochondria in heart biopsies of patients subjected to coronary revascularization surgery. Ctrol, basal sample; I-R, reperfusion sample without treatment; Man, reperfusion sample of mannitol treatment; Def, reperfusion sample of deferoxamine treatment; Tau, reperfusion sample of taurine treatment; Blood: reperfusion sample of blood cardioplegia plus mannitol treatment.

surgery and other clinical situations in which ischemia-reperfusion takes place.

In the present study, we grouped the data of various clinical trials[6-10] to compare the degrees of myocardial protection afforded by the different treatments.

The indices of oxidative stress, taken from the hydroperoxide-initiated chemiluminescence of the biopsies, and of mitochondrial damage, determined by counting damaged mitochondria in the electron micrographs taken for the different treatments showed a statistical correlation ($r = 0.95$; $P < .001$). (Figure 7) A similar correlation between oxidative stress and mitochondrial damage was previously reported in individual heart biopsies.[12]

The mechanism by which oxygen free radicals are generated during reperfusion[19] is still debated. The role of xanthine oxidase as a source of oxygen free radicals has been reported in animals. However, little if any xanthine oxidase activity occurs in the human heart.[8] Alternately, it has been suggested that injured endothelial cells from damaged vessels could be a source of oxygen free radicals in the heart.[20]

TABLE 3

Mitochondrial Damage in the Heart of Patients Subjected to Coronary Revascularization Surgery

Groups	n	Mitochondrial Damage Index	Protection (%)
Control	19	2.63 ± 0.28	—
+ mannitol	6	1.30 ± 0.20	63
+ deferoxamine	7	1.86 ± 0.24	47
+ taurine	6	1.25 ± 0.23	85
+ blood cardioplegia	10	1.08 ± 0.21	95

Mitochondrial damage indexes were determined as the ratio of the percentage of irreversibly damaged mitochondria after reperfusion/before reperfusion in the biopsies obtained from each patient. All treatments, except deferoxamine, were statistically ($P < .01$) different from the control ischemia-reperfusion condition. Deferoxamine treatment was different with a $P < .05$. The mean value for the percentage of mitochondria showing grades 3 and 4 damage in all biopsies (control and treatments) was 10.2%.

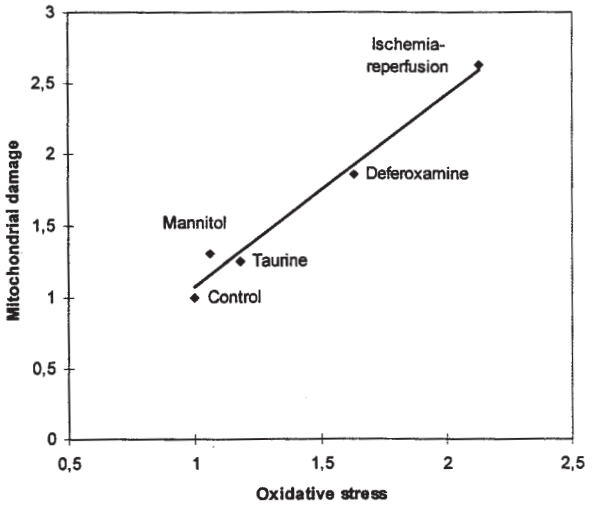

Figure 7.—Relationship between the indexes of oxidative stress and of mitochondrial damage in heart biopsies of patients subjected to coronary revascularization surgery with different treatments.

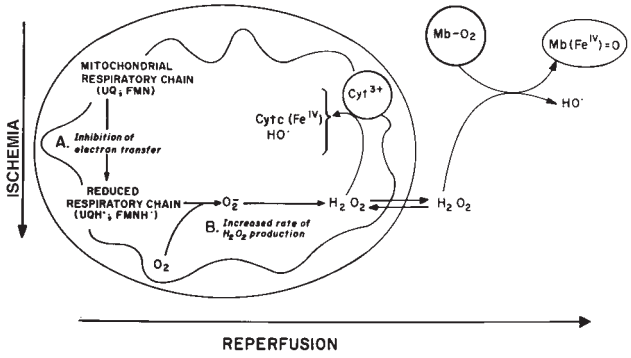

Figure 8.—Scheme of the proposed mechanism for O_2^- / H_2O_2 increase associated to ischemia-reperfusion in heart. Statements A and B represent mitochondrial changes during ischemia (A) and reperfusion (B). UQ and UQH•, oxidized and reduced forms of ubiquinone; FMN and FMNH•, oxidized and reduced forms of NADH dehydrogenase flavoprotein; Mb–O_2, myoglobin–O_2; Cyt c, cytochrome C; $Fe^{IV} = O$, perferryl hemoprotein.

Another hypothesis suggests that during reperfusion, oxygen encounters a highly reduced mitochondrial respiratory chain that triggers an overproduction of superoxide anion at the inner mitochondrial membrane.[21,22] After dismutation of the superoxide anion at the mitochondrial matrix, the resulting hydrogen peroxide diffuses out of the cytosol, reacts with myoglobin, and produces hydroxyl radicals that initiates free radical chain reaction. (Figure 8) The results of each antioxidant treatment will be addressed separately.

Mannitol. Mannitol has been reported to improve myocardial function after ischemia. Bernier et al.[4] found a decreased incidence of ventricular fibrillation associated with a reduction of myocardial edema in the isolated perfused rat heart when 50 mmol/L mannitol was added to the cardioplegic solution. In addition, we found that mannitol also decreased the number of countershocks required to return the heart to sinus rhythm, reduced rhythm disturbances in the postoperative period, appreciably reduced oxidative stress and mitochondrial damage.[6] Two mechanisms can explain these effects: 1) an action as a hydroxyl radical scavenger;[6] and 2) an osmotic effect through the hyperosmotic activity of the extracellular medium.[6]

Deferoxamine. Iron is an important catalyst in the generation of oxygen free radicals through the Fenton reaction, with formation of hydroxyl radical leading to lipid peroxidation. For this to occur, suitable chelated forms of iron are needed. Deferoxamine diminishes free iron toxic effects by forming a high-affinity, catalytically inactive, chelate[7] that prevents reperfusion injury.[23,24]

Ambrosio et al.[25] reported good recovery of myocardial function and energy metabolism. However, in isolated rat hearts, no effects were seen during preconditioning[26] and deferoxamine provided poor protection in ischemia-reperfusion.[27] In iron loaded rats, deferoxamine prevented the generation of ventricular fibrillation and normalized contractility.[28] Rat hearts pretreated with deferoxamine showed better recovery of adenosine triphosphaye and aortic blood flow.[29] In our experience, the addition of deferoxamine to the cardioplegic solution significantly reduced oxidative stress and mitochondrial damage of the myocardium during reperfusion.[7]

Vitamins A and E. These natural antioxidants that scavenge peroxyl radicals generated during the propagation phase of lipid peroxidation. In addition, vitamin A effectively quenches singlet oxygen.[9] Several trials have demonstrated the benefit of vitamin E for the prevention of coronary heart disease, but few report its use as an antioxidant in cardiac surgery. Sisto and coworkers,[30] evaluated 40 patients undergoing coronary artery surgery. The patients were randomized into two groups: 1) placebo and 2) 4 weeks pretreatment with vitamin E 600 mg/day plus allopurinol 600 mg for two days before surgery. The group receiving vitamin E and allopurinol required less dopamine perioperatively and had fewer ECG events.

We observed a marked decrease in the oxidative stress index in patients receiving vitamins A and E preoperatively, suggesting that these substances

effectively counter free radical overproduction in myocardial cells during reperfusion.[9]

Taurine. Taurine is one of the most abundant intracellular amino acids in mammalian hearts and its deficiency is implicated in various pathologic states of the heart. Aside from its other effects, taurine may have antioxidant and scavenging properties.[8,31,32] The cardioprotective action of taurine, both in vitro and in vivo, is well documented in the literature.[8] Irrespective of the cardioplegic solution used, patients undergoing coronary artery surgery, showed a fall in heart tissue taurine, possibly influencing the extent of recovery following surgery.[31]

Taurine maintains mechanical heart function through a calcium concentration dependent biphasic action.[8] In the isolated rat heart, taurine prevents both the large decline in ATPase activities and the associated increase in sarcolemmal calcium binding.[8] In hamsters with cardiomyopathy, oral pretreatment with taurine decreased the accumulation of calcium and the severity of lesions.[8]

Taurine has shown beneficial actions in patients with congestive heart failure,[8] possibly through a modulatory action on calcium entry at the sarcolemmal level. Taurine, in our experience, was able to prevent oxidative stress and mitochondrial damage showing a definite cardioprotective action during cardiovascular surgery.[8]

Blood Cardioplegia Supplemented With Mannitol. In 1989, a new concept in myocardial protection was initiated as an alternative to the standard use of hypothermia.[33] This technique involved continuous, normothermic blood-potassium perfusion during the cross-clamp period, which provides an uninterrupted supply of oxygenated blood to the myocardium.[34] Crystalloid cardioplegic solutions have at least three major disadvantages compared with blood cardioplegic solution: 1) It supplies only small quantities of oxygen to the myocardium; 2) it significantly hemodilutes the patient; and 3) it decreases the oncotic pressure.[35]

Mullen et al.[36] compared blood cardioplegia with crystalloid cardioplegic solutions after coronary artery operations. Blood cardioplegia provided excellent protection for the left ventricle, whereas crystalloid cardioplegic solutions produced colder right ventricular temperatures and better postoperative right ventricular function. Blood cardioplegia appears superior to crystalloid solutions and the benefits of adding mannitol to blood cardioplegia were observed in patients with normal ventricular mass in whom an adequate preservation of the ultrastructure in the reperfusion samples was achieved.[10] In a current study, we are comparing the myocardial protection afforded by warm blood to cold crystalloid cardioplegic solution in patients undergoing elective coronary artery surgery. Significant rises in thiobarbituric acid reactive substances (increase lipid peroxidation) in coronary sinus plasma were found with both crystalloid and blood cardioplegia. Increased mitochondrial damage was found in both groups, but statistical significance was only observed within both groups between preischemia and reperfusion. In conclusion, it is apparent that blood cardioplegia may afford better protection than crystalloid cardioplegic solutions.

CONCLUSIONS

Several alternatives may be taken into consideration to improve myocardial protection in patients undergoing coronary artery surgery, namely: 1) the administration of warm blood cardioplegia; 2) the use of combined anterograde/retrograde cardioplegia; 3) the addition of oxygen free radical scavengers to the cardioplegic solutions; and 4) pretreatment of the patients with vitamins A and E. Although several issues remain unclear, there is no doubt that oxygen free radicals are important contributors to myocardial injury during the reperfusion period of coronary artery surgery.

From our results, the association of preoperative oral vitamins A or β-carotene and vitamin E and the addition of mannitol, deferoxamine and taurine to cardioplegic solutions seems advisable.

In the meantime, trimetazidine, shown to preserve energy balance and reduce intracellular acidosis and free radical induced injury,[37] and normothermic blood cardioplegia are currently being used by our team with promising preliminary results. **CT**

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