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Vitamin E action on oxidative state, endothelial function and morphology in long-term myocardial preservation

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Summary. This study assesses the effects of a vitamin E analogue, Trolox, on the oxidative state, endothelial function and morphology in experimental heart transplantation. Heterotopic heart transplantation was carried out in pigs: untreated after 2 and 24 hours of ischemia and treated with Trolox after 24 hours of ischemia. Prolonged preservation of donor hearts was achieved with continuous perfusion and University of Wisconsin solution, in which acid-base balance and enzymes were determined during the procedure. In recipients, hemodynamic and biochemical parameters were determined at baseline and during reperfusion. Trolox diminished the pH of the preservation solution ($p<0.01$), the left ventricle of the transplanted heart recovered a systolic pressure equaling that of the 2h group and higher than that of the untreated 24h group ($p<0.01$), the antioxidant levels were not decreased and the glutathione reductase level was maintained throughout the first part of reperfusion. In this group also there was a direct correlation between the concentration of this enzyme and the antioxidant levels ($p<0.001$). Although the endothelin concentrations increased, the change was less marked in the Trolox group than in the untreated 24h group ($p<0.01$). Morphologically, mitochondria and myocardial vessels presented a normal structure in the Trolox group, and interstitial edema, inflammatory infiltrate and contraction bands were less prominent than in the untreated group. All these effects indicate that Trolox protected the transplanted heart, at least partially, against ischemia-reperfusion injury.

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Introduction

In heart transplantation, graft failure (primary and nonspecific) is responsible for 40% of the deaths occurring during the first 30 days after transplantation and for 18% of those occurring over the following 11 months (Cropper et al., 2003; Verrier, 2004; Taylor et al., 2008). Although the cause is not well known, and is probably multifactorial, primary graft failure has been related to ischemic time and to ischemia-reperfusion (IR). The production of reactive oxygen species (ROS), the activation of the Na^+/H^+ exchanger (NHE) and intracellular calcium overload are prominent, interrelated aspects, that provoke contractile dysfunction, endothelial damage, apoptosis and necrosis (Castellá et al., 2003; Das, 2003; Muraki et al., 2003; Vinten-Johansen and Mentzer, 2003; Yarbrough et al., 2003; Aker et al., 2004; Klass et al., 2004; Stevens et al., 2004; Toledo-Pereyra et al., 2004; Hool et al., 2005; Kevelaitis et al., 2005; Mallet, 2005; Rabkin et al., 2005).

ROS are considered to be the major contributors to IR injury. Under physiological conditions, endogenous antioxidants protect the myocardium from the attack of ROS but, following IR, the production of ROS exceeds the capacity of this defense system, generating peroxidation, protein denaturation and DNA damage (Beyersdorf, 2004; Renner et al., 2004; Toledo-Pereyra et al., 2004; Luyten et al., 2005; Rabkin et al., 2005).

Strategies to enhance myocardial preservation have focused more on protecting the myocyte than the

endothelial cell, although it is known that the latter may be more vulnerable to damage, since it initiates the inflammatory response and, at the same time, is the target of this phenomenon. The immediate effects of endothelial dysfunction include decreased nitric oxide (NO) production and an increase in endothelin-1 (ET-1), a circumstance that leads to vasospasm, platelet and neutrophil adhesion, capillary obstruction and heterogeneous flow (Castellá et al., 2003; Vinten-Johansen and Mentzer, 2003; Beyersdorf, 2004; Toledo-Pereyra et al., 2004; Verrier, 2004; Stoica et al., 2005). Over the longer term, this dysfunction has been related to rejection episodes and graft vasculopathy (Perrault et al., 2001, 2005; Steen, 2001; Renner et al., 2004; Toledo-Pereyra et al., 2004; Verrier, 2004).

To protect the myocardium from IR injury, cardioplegic and preservation solutions have been supplemented with different substances, mainly NHE inhibitors and antioxidants. Among the latter, glutathione plays an important role as a ROS scavenger and substrate in the redox cycle. The tissue glutathione content diminishes during reperfusion, a decrease that correlates with the duration of ischemia. Glutathione peroxidase (GPX) is the predominant hydrogen peroxide scavenger in the myocardium: it catalyzes the reaction of reduced glutathione (GSH) to the oxidized form (GSSG), decreasing the hydrogen peroxide concentration. The increase in GSSG and GPX and the decrease in GSH and glutathione reductase (GR) are indicators of oxidative stress (Carbonell et al., 2000; Renner et al., 2004; Castillo et al., 2005; Fukai et al., 2005; Luyten et al., 2005; Mallet, 2005).

Although alpha-tocopherol (vitamin E) is a totally exogenous compound that is acquired from food intake, it also plays a role in the endogenous system of cellular defense against oxidative stress. It is the major hydrophobic antioxidant: it interrupts the chain reaction that leads to lipid peroxidation in cell membranes and plasma lipoproteins (Heller et al., 2004), and its plasma levels has been reported to be decreased in heart transplant recipients (Nguyen et al., 2006).

The water-soluble analogues of vitamin E offer the advantage that they do not require bile salts for their absorption when administered orally (Nguyen et al., 2006); moreover, they can be acutely injected via intravenous infusion (Rubinstein et al., 1992; Sagach et al., 2002). One of these analogues, Trolox, has been reported to have an antioxidant action similar to that of vitamin E, although it appears to provide greater protection against oxidative damage to the myocyte than to the endothelial cell (Mickle, 1993; Heller et al., 2004). The purpose of this study was to assess the capacity of Trolox to counteract the changes in the oxidative status and endothelial function in an experimental model of heterotopic heart transplantation.

Materials and methods

The Institutional Animal Care and Use Committee approved the study. The investigation conformed to Spanish Law and to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Forty-six heterotopic heart transplantations were performed in Landrace x Large White pigs (weighing 19 to 22 kg), which were randomly assigned to the following groups: 2h (n=7), in which the donor heart was transplanted after two hours of ischemia. 24h UW (n=6), in which the donor heart was subjected to 24 hours of ischemia, during which the heart, suspended by the aorta, was preserved in a gravity-driven continuous perfusion system (maximum pressure 15 cm H_2O) with recirculation of the solution, hypothermia (4°C) and oxygenation (95% O_2, 5% CO_2) (Wicomb et al., 1984), using University of Wisconsin solution (UW, Viaspan®) as the preservation solution (total volume 2 L). And 24h UW+TR (n=7), in which the preceding procedure was followed, with the addition of Trolox (TR, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Aldrich®) at 0.5 g/L (2 mmol/L) to the preservation solution. In addition, in this last group, 3 g of this substance, diluted in saline, were injected i.v. into the recipient immediately before the start of the surgical procedure.

Anesthetic and surgical procedures

Donors and recipients were sedated with ketamine (20 mg/kg bw), diazepam (0.1 mg/kg bw) and atropine (0.02 mg/kg bw). Anesthesia was induced with an i.v. bolus of propofol (2 mg/kg bw), midazolam (0.6 mg/kg bw) and fentanyl (5 μg/kg bw). After endotracheal intubation, anesthesia was maintained with continuous i.v. infusion of propofol (9 mg/kg/h), midazolam (0.6 mg/kg/h), fentanyl (5 μg/kg/h) and pancuronium bromide (0.4 mg/kg/h). An Adult Star® ventilator (Infrasonics, Inc.) was used for mechanical ventilation. Catheters were inserted in right (Swan-Ganz) and left jugular veins, and in the carotid artery for hemodynamic assessment, blood sampling, and drug and serum infusion.

In donors, via median sternotomy and following systemic heparinization (3 mg/kg bw), cardiac arrest was induced by injection of a crystalloid cardioplegic solution (K^+=30 mEq/L) through the aortic root. The heart was then excised, including the aorta up to the descending segment. The pulmonary artery was transected at the level of its bifurcation and anastomosed to the left atrial appendage, in order to permit the drainage of the right chambers. After the ischemic period corresponding to each group, heterotopic heart transplantation was carried out according to the technique described by Matsui et al. (1988), modified by our group (Roda et al., 2004). In no case inotropic support was utilized.

Data and sample collection

In the groups in which UW was employed (24h UW

and 24h UW+TR), samples were collected 10 minutes and 6 and 24 hours after the initiation of continuous perfusion to determine pO_2, pCO_2, pH, lactate, glucose, lactate dehydrogenase (LDH), creatine kinase (CK) and calcium.

The electrocardiogram and heart rate, mean arterial pressure (mAP), pulmonary artery pressure, pulmonary capillary pressure and central venous pressure were recorded by a PM8060 Vitara monitoring unit (Dräger), and cardiac output (CO) was measured by thermodilution, using an SAT-2™ monitor (Baxter Healthcare Corporation), at baseline and after 5, 60 and 120 minutes of reperfusion. The systolic and diastolic pressures of the left ventricle of the transplanted heart (LVs and LVd, respectively), obtained via apical puncture, were measured after 60 and 120 minutes of reperfusion.

The levels of hemoglobin, alpha- and gamma-tocopherol and LDH were measured in peripheral venous blood at baseline and at the initiation of reperfusion (5 minutes) and 60 and 120 minutes after the transplantation had been completed. In addition, CK levels were measured in the recipient coronary sinus at baseline and after 120 minutes of reperfusion, and in that of the donor 5, 60 and 120 minutes after the completion of transplantation.

Blood samples were also obtained from left atrium of the recipient animals at baseline and from the coronary sinus of native and transplanted hearts after 5, 30, 60, 90 and 120 minutes of reperfusion to determine the concentrations of total antioxidants (TA), superoxide dismutase (SOD), GPX and GR (kits from Randox Laboratories Ltd Co, Antrim, UK, run on a Hitachi 717 automated analyzer from Boehringer-Mannheim), malondialdehyde (MDA) (kit LPO-586 Bioxytech, Oxis International Inc, Portland, Oregon, USA), ET-1 (kit from Biomedica Gruppe), and nitrite concentrations (kit from R&D Systems, Inc).

Once this phase was concluded, that is to say, after 24 hours of preservation and 2 hours of reperfusion, two samples of 2 cm^3 were taken from the free wall of both ventricles for light microscopy and transmission electron microscopy studies.

For light microscopy, samples of 5 mm^3 were fixed in 10% formaldehyde, embedded in paraffin and cut into 5-micron-thick slices in a Micron HM360 microtome. Sections were stained with hematoxylin-eosin, periodic acid Schiff (PAS), Van Giesson and Azan to evaluate together myocyte glycogen content, contraction bands, necrosis, edema, hemorrhage and fibrosis, studied under a Zeiss Axiophot 2 microscope and photographed by an Axiocam HRc camera.

For immunohistochemical studies, sections were deparaffinized and rehydrated before blocking endogenous peroxidase activity with H_2O_2 (0.3%) in methanol. The slides were rinsed with PBS and incubated with primary antibodies in a moist chamber at room temperature. The primary antibodies used were: Muscle Specific Actin Monoclonal Antibody (A 7811 Novocastra) at a 1:100 dilution and Desmin Monoclonal Antibody at a 1:50 dilution (DE-R11 Novocastra). Sections were subsequently incubated with biotinylated anti-rabbit IgG and LBA (DAKO) for 25 min at room temperature, rinsed with PBS and immersed for 25 min in avidin peroxidase. The immunostaining reaction product was developed using diaminobenzidine. Counterstaining was performed with hematoxylin. The specificity of the immunohistochemical procedure was checked by incubation of sections with nonimmune serum instead of primary antibody.

For the ultrastructural study, samples were cut into $\leq 1 mm^3$ blocks and immersed for 2 hours in 2% phosphate buffered glutaraldehyde. They were then washed in phosphate buffer and postfixed with 2% osmium tetroxide for 1 hour. After dehydration in a graded acetone series, they were embedded in Spurr, cut into semithin slices (5.5-1 micron thick) using a Leica Ultracut R ultramicrotome, and stained with Richardson's methylene blue for light microscopy study. Likewise, ultrathin slices (70 nm thick) were stained with a water-based solution of 2% uranyl acetate and lead citrate for study under a JEOL (JEM-1010) electron microscope.

For each group of studies (light microscopy, immunohistochemical and ultrastructural), samples were evaluated by a single pathologist who was blinded to the details of each specimen.

Statistical analysis

Descriptive variables (mean, standard deviation and range) were calculated for each quantitative variable. The hypothesis of a normal distribution was verified using the Kolmogorov-Smirnov test. To assess the differences in the variables according to duration of ischemia and group, repeated measures analysis of variance (ANOVA) was applied, followed by the Student-Newman-Keuls test for multiple comparisons. Those results with a p value of less than 0.05 were considered significant. To prevent the inclusion of spurious associations (data dredging), in the analysis of the linear correlations between variables, only those in which the correlation coefficient (r) was equal to or greater than 0.580 ($p<0.001$) were considered significant. The data were analyzed by the Biostatistics Section of our hospital, using the SPSS statistical software package (v. 10.0).

Results

During the ischemic period, both the 24h UW and the 24h UW+TR groups showed similar increases in pO_2, pCO_2, LDH and CK, and there were no changes in calcium concentrations. The pH decreased progressively in both groups, but the values were significantly lower in the 24h UW+TR group from the start and throughout the entire study, a difference that can be attributed to Trolox, which is an acid. The glucose concentration increased

significantly in the 24h UW group, in contrast to that observed in the group treated with Trolox. The lactate concentration increased in both groups, although in the 24h UW+TR group, the increase was significant only at the end of the preservation period, and the values were lower than those observed in the 24h UW group at previous time points (Table 1).

At the end of the ischemic period, the ventricles were soft to the touch in all three groups. The heartbeat was restored during the initial minutes of reperfusion in all the hearts, either spontaneously or by electric shock. The heart rate was stabilized within 3 to 4 minutes in every case.

Decreases in mAP and CO were observed from the start of reperfusion in the 24h UW group and after the first hour in the 24h UW+TR group, but the differences between the two groups were not significant, possibly because the circulation was mainly maintained by the recipient heart. The LVs pressure was decreased in the 24h UW group during the second hour of reperfusion

Table 1. Modifications in the preservation solution during continuous hypothermic (5°C) and oxygenated perfusion.

	24h UW	24h UW+TR
pO_2 (mmHg)		
10 min	156±76	184±66
6 h	368±97[b]	416±109[b]
24 h	595±100[b]	663±50[b]
pCO_2 (mmHg)		
10 min	2.3±0.3	2.5±0.5
6 h	5.8±1.8[b]	6.7±1.6
24 h	15.2±1.9[b]	16.0±1.5[b]
pH		
10 min	7.65±0.13	7.39±0.05[c]
6 h	7.38±0.11[a]	7.18±0.07[b,c]
24 h	7.10±0.04[b]	6.96±0.04[b,c]
Glucose (mg/dl)		
10 min	4.8±4.1	0.3±0.5
6 h	12±3.3[b]	1.1±1.4[c]
24 h	24.7±6.1[b]	1.8±2.1[c]
Lactate (mmol/L)		
10 min	0.29±0.12	0.01±0.01[c]
6 h	0.60±0.15[b]	0.15±0.07[c]
24 h	0.50±0.29[b]	0.30±0.26[b]
LDH (U/L)		
10 min	10±7	5±4
6 h	30±12[a]	19±10
24 h	139±44[b]	92±41[b]
CK (U/L)		
10 min	10±12	1±1
6 h	75±42	19±8
24 h	334±175[b]	129±95[b]
Calcium (mg/dl)		
10 min	1.5±0.3	1.4±0.1
6 h	1.6±0.6	1.2±0.3
24 h	1.4±0.4	1.5±0.1

[a]: p<0.05; [b]: p<0.01 vs 10 min; c p<0.01 vs 24h UW; LDH: Lactate dehydrogenase; CK: Creatine kinase.

Table 2. Hemodynamic parameters.

	2h	24h UW	24h UW+TR
mAP (mmHg)			
Basal	83±18	83±14	75±8
5 min	64±10	62±8[b]	72±8
60 min	75±14	67±7[a]	86±13
120 min	76±16	55±14[b]	78±12
CO (L/min)			
Basal	2.3±0.6	2.5±0.4	2.6±0.9
5 min	2.4±0.6	2.0±0.5[a]	2.4±0.6
60 min	2.8±0.7	2.1±0.3[a]	2.0±0.5[b]
120 min	2.3±0.7	1.7±0.3[b]	1.7±0.4[b]
LVs (mmHg)			
60 min	91±17	66±13	98±18[c]
120 min	89±25[d]	47±13[b]	91±22[d]
LVd (mmHg)			
60 min	6±2	5±5	5±3
120 min	3±5	2±3	5±5

[a]: p<0.05; [b]: p<0.01 vs basal; [c]: p<0.05; [d]: p<0.01 vs 24h UW; mAP: Mean arterial pressure; CO: Cardiac output; LVs: Left ventricular systolic pressure; LVd: Id. diastolic.

Table 3. Tocopherols and enzymes.

	2h	24h UW	24h UW+TR
α-tocopherol (μg/dl)			
Basal	193±55	156±36	148±24
5 min	144±38[b]	128±52[a]	132±30
60 min	138±50[b]	105±54[b]	120±38
120 min	141±49[b]	84±51[b]	108±29[a]
γ-tocopherol (μg/dl)			
Basal	14±6	26±16	14±12
5 min	17±6	18±3	16±12
60 min	19±5	18±6	21±12[a]
120 min	15±6	17±7	26±15[b]
LDH (U/L)			
Basal	712±88	674±129	805±204
5 min	674±129	782±163	1021±211
60 min	879±247	1181±422[b]	1487±602[a]
120 min	957±286[b]	1109±330[b]	1528±695[a]
CK-recipient (U/L)			
Basal	906±308	948±471	983±326
120 min	2324±768[b]	3385±1400[b]	3672±1816[b]
CK-donor (U/L)			
5 min	1510±430	2697±1015	2665±544
60 min	2693±1419[b]	3750±1488	4204±1965[a]
120 min	2734±1000[b]	3671±1551[a]	3739±1845[a]

[a]: p<0.05; [b]: p<0.01 vs basal; LDH: Lactate dehydrogenase; CK: Creatine kinase (coronary sinus blood).

and was lower than that of the other two groups, between which no significant differences were observed. Changes in LVd pressure were not recorded in any of the groups (Table 2).

The alpha-tocopherol concentrations decreased during reperfusion in all the groups, which did not differ from one another. The gamma-tocopherol levels did not change in the 2h and 24h UW groups, while they increased in the 24h UW+TR at the end of reperfusion, although there were no statistically significant differences among the groups. LDH increased in all the groups throughout reperfusion, but no significant differences were observed among them. CK also increased, but with no significant differences between groups or between donor and recipient hearts (Table 3).

There were no statistically significant differences between recipient and transplanted hearts in terms of TA, SOD, GPX, GR, ET-1, or nitrite concentrations (Table 4).

The TA levels decreased progressively during reperfusion in the 24h UW group, did not change in the 2h group and increased at the initiation of reperfusion in the 24h UW+TR group, although there were no significant differences among them. GPX increased after one hour of reperfusion in the 2h group, an increase that was observed at the start in the 24h UW+TR and was not detected in the 24h UW group; again, there were no significant differences among the groups. GR was reduced in all three groups: from the initiation of reperfusion in the 2h and 24h UW groups and after 90 minutes in the 24h UW+TR group; in the latter group, the GR concentrations were higher than those of the other two groups at the initiation of reperfusion (Table 4).

No significant differences were detected in the MDA concentrations in the recipients in any of the groups. In contrast, there was a significant increase at the initiation of perfusion in the donor hearts subjected to 24 hours of ischemia; there were no significant differences between these two groups, but the 24h UW donor hearts did differ significantly with respect to the recipient hearts (Table 4).

The ET-1 concentrations increased during reperfusion in the groups that underwent prolonged perfusion; the values recorded in the 24h UW group were higher than those of the other two. The nitrite

Table 4. Oxidative state.

	2h	24h UW	24h UW+TR
TA (mmol/L)			
Basal	0.69±0.09	0.81± 0.21	0.61±0.06
5 min	0.69±0.14	0.72±0.22[b]	0.76±0.17[a]
30 min	0.63±0.13	0.7±0.23[b]	0.60±0.07
60 min	0.7±0.21	0.69±0.24[b]	0.59±0.08
90 min	0.65±0.13	0.64±0.23[b]	0.56±0.07
120 min	0.65±0.15	0.61±0.27[b]	0.56±0.09
SOD (U/gHb)			
Basal	1035±266	906±152	975±269
5 min	1066±415	974±319	1035±174
30 min	987±293	991±235	1073±152
60 min	934±283	998±237	1120±222
90 min	1045±397	1075±268	1054±98
120 min	949±332	881±233	1159±159
GPX (U/gHb)			
Basal	444±47	536±299	466±118
5 min	480±81	607±289	547±114[a]
30 min	482±68	606±303	477±137
60 min	519±63[b]	534±208	557±121[a]
90 min	436±93	514±191	554±208[a]
120 min	463±36	435±141	534±60[a]
GR (U/L)			
Basal	73±10	73±16	77±18
5 min	65±10[b]	61±17[b]	78±14[c,d]
30 min	59±10[b]	56±18[b]	71±15
60 min	60±13[b]	51±21[b]	69±15
90 min	58±10[b]	48±17[b]	64±14[b]
120 min	59±12[b]	43±18[b]	60±13[b]
MDA-recipient (μmol/L)			
Basal	1.68±0.33	1.34±0.4	1.46±0.58
5 min	1.68±0.24	1.47±0.24	1.88±0.79
30 min	1.7±0.33	1.49±0.34	1.73±0.76
60 min	1.62±0.29	1.47±0.27	1.67±0.31
90 min	1.62±0.38	1.45±0.25	1.52±0.25
120 min	1.61±0.3	1.39±0.27	1.9±0.58
MDA-donor (μmol/L)			
Basal	1.68±0.33	1.34±0.4	1.46±0.58
5 min	1.66±0.29	2.73±0.58[b,e]	2.7±1.0[b]
30 min	1.64±0.39	1.58±0.32	2.17±0.41
60 min	1.75±0.32	1.55±0.28	2.06±0.49
90 min	1.65±0.24	1.4±0.31	1.7±0.23
120 min	1.72±0.47	1.58±0.5	1.75±0.3

[a]: p<0.05; b p<0.01 vs basal; [c]: p<0.05 vs 24h; [d]: p<0.05 vs 2h; [e]: p=0.002 transplanted vs native heart; TA: Total antioxidants; SOD: Superoxide dismutase; GPX: Glutathione peroxidase; GR: Glutathione reductase; MDA: Malondialdehyde.

Table 5. Endothelial function.

	2h	24h UW	24h UW+TR
ET-1 (fmol/ml)			
Basal	4.28±1.86	5.95±2.21	3.80±1.73
5 min	4.7±1.26[d]	8.53±3.56[a]	4.57±2.12[d]
30 min	4.56±1.35[d]	7.90±3.44[b]	5.73±2.05[b,c]
60 min	4.42±1.23[d]	9.28±4.19[b]	7.08±2.18[b,e]
90 min	4.6±1.22[d]	9.02±3.29[b]	6.55±2.23[b,c,e]
120 min	4.5±1.19[d]	9.28±3.53[b]	6.12±1.41[b,d]
Nitrite (μmol/ml)			
Basal	5.6±2.4	5.5±3.3	3.3±1.2
5 min	4.0±2.3[a]	5.1±3.6	3.7±3.3
30 min	4.6±3.2[a]	3.7±2.7	3.5±1.9
60 min	4.5±3.3[a]	3.4±2.6[a]	3.2±2.3
90 min	3.7±3.1[b]	5.1±3.7	4.2±2.2
120 min	4.2±3.1[b]	5.1±3.9	4.0±3.6

[a]: p<0.05; [b]: p<0.01 vs basal; [c]: p<0.05; [d]: p<0.01 vs 24h; [e]: p<0.05; ET-1: Endothelin-1.

concentrations decreased during reperfusion in the 2h group, while no changes were detected in the other two groups, nor were there differences between groups (Table 5).

In the 24h UW group, in contrast to the other two groups, there was a direct correlation between the TA and GPX concentrations (r=0.7634; p<0.001) and an inverse correlation between the nitrite and GPX concentrations (r=-0.5813; p<0.001). On the other hand, in the 24h UW+TR group, there was a direct correlation between the TA and GR (r=0.6874; p<0.001), while in the 2h and 24h UW groups, the relationship between these two variables did not reach statistical significance (Fig. 1).

The light microscopy, immunohistochemical and ultrastructural studies provided us with an overall view of the changes in the myocardium originated by ischemia-reperfusion (Table 6), which we describe here.

No differences were observed between the two ventricles. In every case the morphological changes were irregularly distributed, with normal areas adjacent to others with important lesions within the same field of view.

The small and medium-sized vessels demonstrated no signs of alteration nor changes in the cell structure of the walls in the 24h UW+TR group. In some cases polymorphonuclear leukocytes were adhered to the endothelial cells, but in smaller numbers than in the 24h UW group. Interstitial edema and inflammatory infiltrate was mild in both groups, but always less prominent in the 24h UW+TR group (Fig. 2).

In both groups we observed circumscribed areas of necrosis in contraction bands: thick, irregular bands transversely oriented in the cytoplasm, which stained intensely under the light microscopy techniques. The ultrastructural study showed them to be composed of contracted sarcomeres with widened Z lines. The degree of affectation was also less prominent in the 24h UW+TR group, in which mitochondria did not present

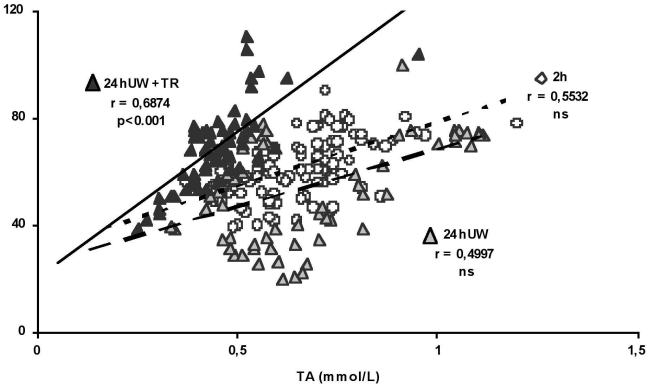

Fig. 1. Direct correlation between the total antioxidants (TA) and glutathione reductase (GR) concentrations in the 24h UW+TR group. In the 2h and 24h UW groups, the relationship between these variables did not reach statistical significance.

Table 6. Structural damage produced after 24 hours of preservation and 2 hours of reperfusion. Comparison between the preservation solutions.

	24h UW	24h UW+TR
Vasoconstriction	–	–
Leukocyte adhesion	+	–/+
Edema. Inflammatory infiltrate	++	–/+
Necrosis. Contraction bands	+/++	–/+
Mitochondrial degenerative changes	++	–
Glycogen decrease	+++	+++
Changes in intercalated discs	++/+++	++
Loss of the actin pattern	+	+

-: None; +: Mild; ++: Moderate; +++: Severe.

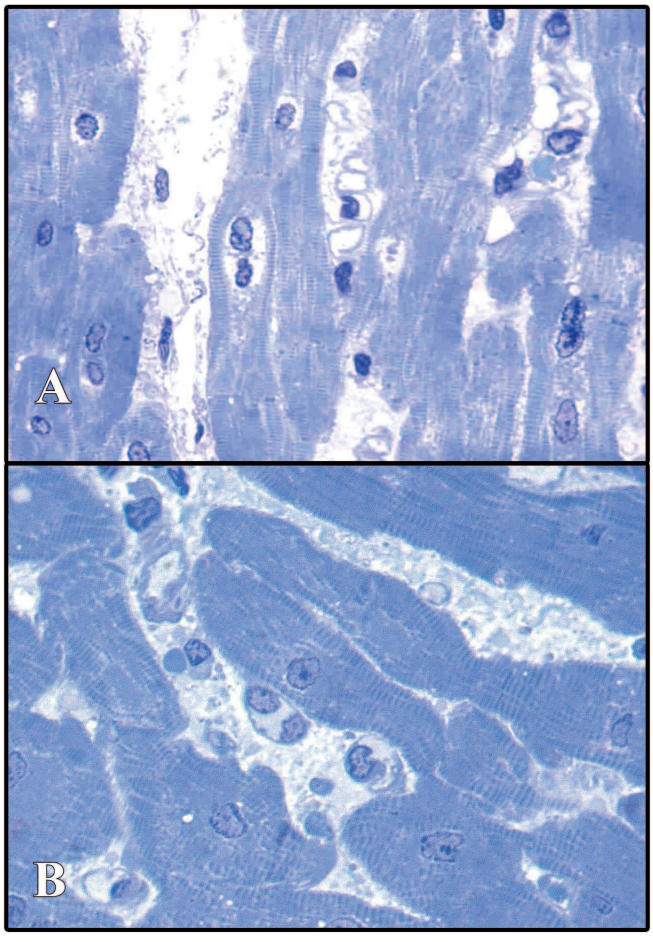

Fig. 2. A. 24h UW group. **B.** 24h UW+TR group. Under light microscopy techniques the small-sized vessels demonstrated no signs of alteration in the wall cell structure of any of the groups. Interstitial edema and inflammatory infiltrated was mild in both groups, but less prominent in the 24h UW+TR group. Semithin. A, x 400; B, x 1,000.

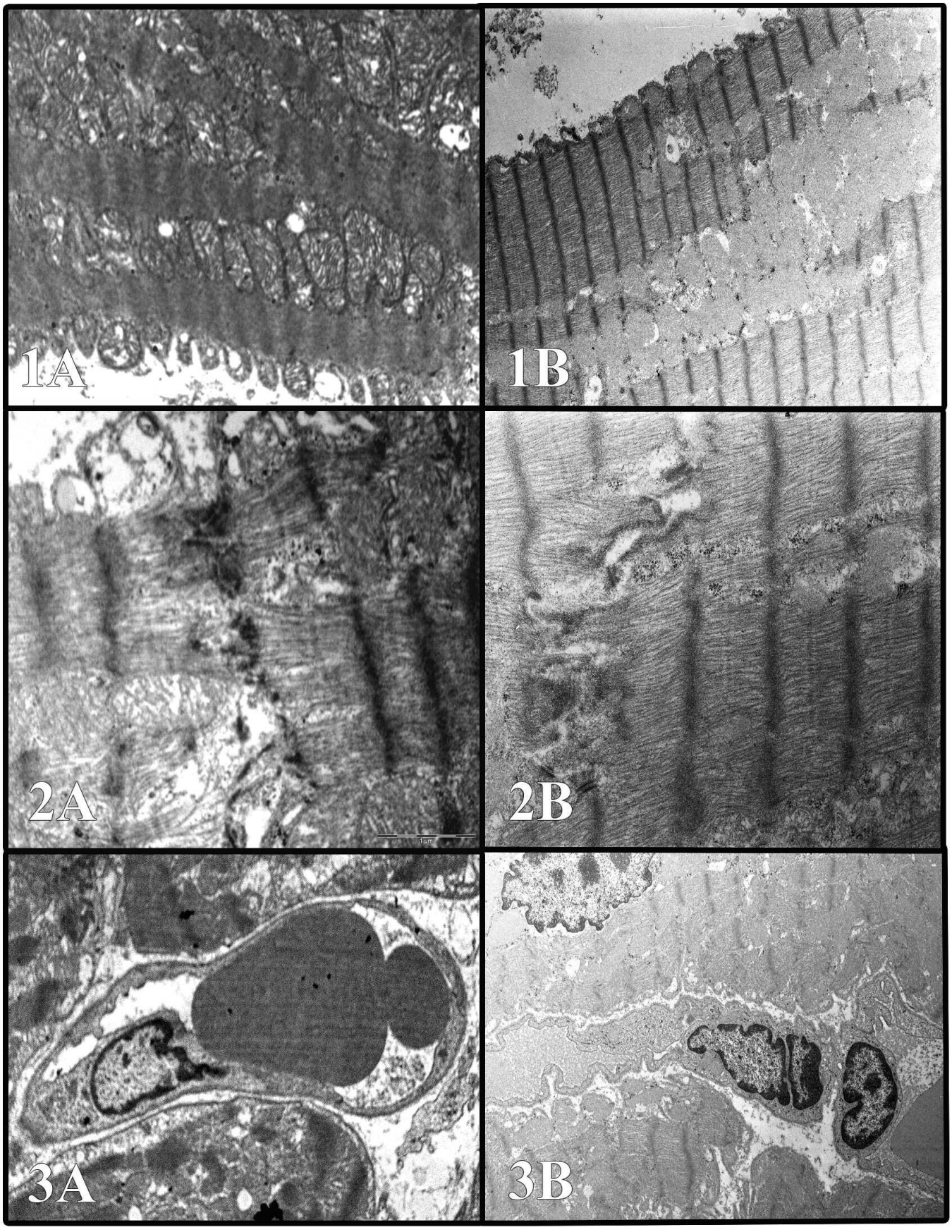

Fig. 3. A. 24h UW group. **B.** 24h UW+TR group. Mitochondrial structure damage was less prominent in the 24h UW+TR group **(1B)** than in the 24h UW group **(1A)**. There were more contraction bands and worse conserved intercellular junctions in the 24h UW group **(1A, 2A)** in contrast with the 24h UW+TR group **(1B, 2B)**. Blood vessels had no significant changes in any group **(3A, 3B)**. EM. **1A**, x 8000; **2A**, x 20000; **3A**, x 8000; **1B**, x 5000; **2B**, x 15000; **3B**, x 3000

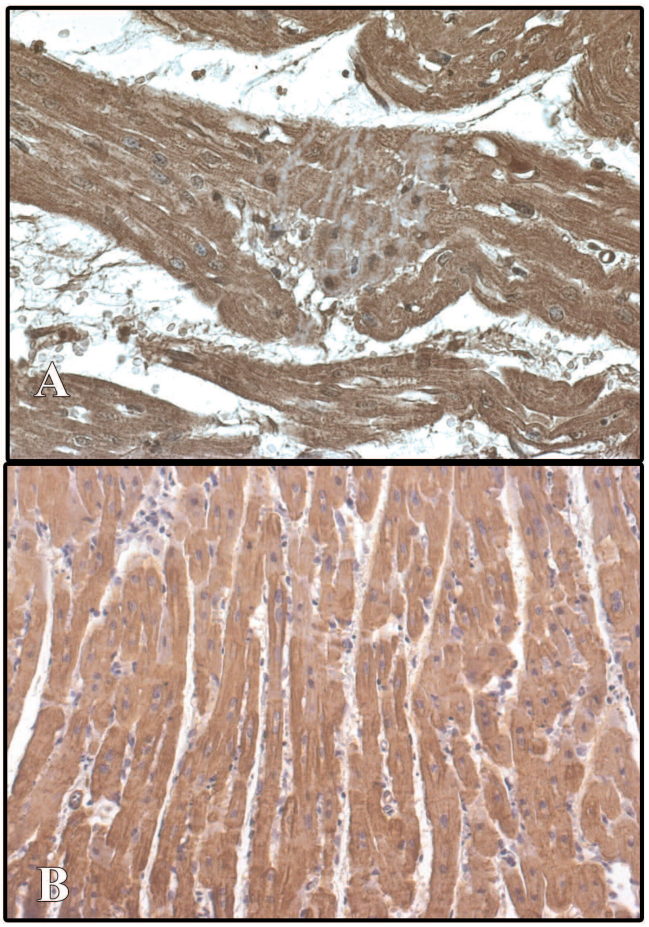

Fig. 4. A. 24h UW group. **B.** 24h UW+TR group. Immunohistochemical labeling for actin demostrated irregular areas with loss of actin expression in both groups. PAP anti-actine, x 200

changes, neither in the matrix density nor in the cristae architecture (Fig. 3).

Intracellular glycogen was severely reduced in both groups, the granules being nearly absent.

Immunolabelling and electron microscopy techniques revealed that the intercellular junctions were badly conserved and disrupted in both groups, but somewhat worse in the 24h UW group (Fig. 3).

In respect to changes in the actin pattern, qualitative or quantitative differences were not observed among the solutions employed in myocardial preservation: there were more or less extensive irregular areas with loss of actin expression in both groups (Fig. 4).

Discussion

Three aspects of the design of our experimental model warrant special consideration: the performance of heterotopic transplantation, the method of prolonged myocardial preservation and the choice of the ischemic times.

The implantation of the heart in heterotopic position enabled us to perform the procedure without cardiopulmonary bypass. When the latter technique is employed, the contact of the blood components with artificial surfaces triggers a systemic inflammatory response, oxidative stress that precedes the inflammation and is perpetuated with it, and endothelial dysfunction (Cooper et al., 2000; Verrier, 2004; Christen et al., 2005; Lamarche et al., 2005; Luyten et al., 2005). Myocardial IR generates the same type of response and, when added to the aforementioned events, it is impossible to demarcate the contribution of each to the outcome. Given that the purpose of this study was to analyze the changes in oxidative status, endothelial function and morphology following IR, it was decided to avoid the use of cardiopulmonary bypass, despite the fact that heterotopic transplantation was not the ideal model for hemodynamic evaluation.

In heart transplantation, a graft ischemic time of more than 6 hours can severely reduce contractility (Fedak et al., 2005; Taylor et al., 2008). A number of experimental studies have employed continuous hypothermic perfusion with different preservation solutions, in the attempt to achieve an acceptable proportion of viable hearts after 24 hours of ischemia: this technique enables uniform cooling, the delivery of oxygen and substrates and maintains anaerobic glycolysis (Mullen et al., 2001; Tsutsumi et al., 2001; Poston et al., 2004; Oshima et al., 2005). In aerobiosis, the myocardium utilizes fatty acids as a source of energy, while in anaerbiosis, it utilizes glucose, obtained from myocardial glycogen stores; the accumulation of metabolites interrupts glycolysis and adenosine triphosphate (ATP) synthesis and increases intracellular proton, calcium and sodium concentrations (Stoica, 2004; Toledo-Pereyra et al., 2004; Oshima et al., 2005). Taking into account the fact that UW solution contains no glucose, the increase in the level of this monosaccharide may indicate the utilization of this metabolic pathway during preservation of the 24h UW hearts, a hypothesis that would be further supported by the increase in lactate concentrations, which peaked after 6 hours of ischemia. In contrast, in the Trolox-treated group, the glucose concentrations did not change, and those of lactate increased only at the end of ischemia, suggesting that glycogen and ATP stores may be better maintained and that the intracellular accumulation of protons, sodium and calcium may be reduced in this group. Although the morphological study did not find that glycogen was better preserved in any of the groups, in the 24h UW+TR group the delayed increase in the lactate concentration, the less prominent cardiac myocyte necrosis and the whole recovery of the left ventricle systolic pressure suggested a better preservation of the high-energy stores.

One noteworthy aspect of IR-induced myocardial damage is intracellular calcium overload (Castellá et al., 2003; Das, 2003; Muraki et al., 2003; Vinten-Johansen

and Mentzer, 2003; Yarbrough et al., 2003; Aker et al., 2004; Klass et al., 2004; Stevens et al., 2004; Toledo-Pereyra et al., 2004; Hool et al., 2005; Kevelaitis et al., 2005; Mallet, 2005; Rabkin et al., 2005). During ischemia, the accumulation of H^+ activates the NHE, producing intracellular Na^+ overload; as the ATP-dependent Na^+/K^+ pump is inoperative under these conditions, the increment in intracellular Na^+ reduces Ca^{2+} efflux or increases its influx due to reverse mode Na^+/Ca^{2+} exchange, leading to an overload of intracellular Ca^{2+}. All these circumstances are intensified by reperfusion since the possible block of the NHE by extracellular acidosis is suddenly alleviated. Extracellular acidosis reduces Na^+/H^+ and Na^+/Ca^{2+} exchange and attenuates NHE activity (Das, 2003; Wang et al., 2003; Kevelaitis et al., 2005); thus, during reperfusion, Ca^{2+} overload is limited, intracellular realkalinization delayed and postischemic diastolic stiffness (unrelated to edema) reduced, without affecting systolic function. In our study, the addition of Trolox to the UW solution produced a decrease in pH, when compared to the untreated group, throughout the entire preservation period and, according to the aforementioned findings, this may have had a decisive influence on the fact that the systolic pressures in 24h UW+TR hearts were restored to levels similar to those of the 2h group.

IR injury is directly related to the duration of ischemia (Beyersdorf, 2004; Toledo-Pereyra et al., 2004; Taylor et al., 2008). For this reason, we chose to compare two widely differing degrees of injury, one equivalent to or milder than that associated with human heart transplantation (2 hours of ischemia) and another involving severe injury due to prolonged ischemia (24 hours).

With the exception of MDA concentrations, there were no statistically significant differences between the recipient and donor hearts in terms of the enzymes and metabolites measured in coronary sinus blood in any of the groups. Given that the first blood sample was taken after 5 minutes of reperfusion, this finding may reflect the rapid distribution throughout the bloodstream of the metabolites released by the transplanted heart or a systemic response to the reperfusion: in addition to the local effects, IR has systemic effects, manifested in the form of a systemic inflammatory response and multiple organ dysfunction syndromes (Seal and Gewertz, 2005).

During reperfusion, the injury in 24h UW hearts, in contrast to those of the 2h group, was reflected by a progressive decrease in TA levels, an early increase in MDA concentration in the transplanted heart and a progressive increase in ET-1 concentrations, with no concomitant increment in nitrite concentrations, findings that suggest free radical production, with lipid peroxidation and endothelial dysfunction (Sharma et al., 1999; Beyersdorf, 2004; Renner et al., 2004; Lerman and Zeiher, 2005).

In the Trolox-treated group, the behavior of the TA level was similar to that of the 2h group; that is, there were no significant decreases. In fact, it increased during the first minutes of reperfusion. The increase in TA concentrations during the first 10 minutes after the establishment of cardiopulmonary bypass was an unexpected finding in Luyten's et al. paper (Luyten et al., 2005), who attributed it to interference in the determination of TA owing to the presence of free hemoglobin secondary to hemolysis. Since cardiopulmonary bypass was not employed in our study, the production of hemolysis is highly improbable, and any artifact in the measurement of TA could have occurred both in the treated group and in the untreated groups. Thus, we consider that the increase in TA in the 24h UW+TR group at the initiation of reperfusion may express a certain capacity to respond to oxidative stress that was not detected in the untreated groups and/or a lower production of ROS, which has been related to an inhibition of the NHE and low pH (Wang et al., 2003).

In our study, during this initial phase of reperfusion, the GR concentrations did not decrease in the group treated with Trolox, in contrast to the other two, and its decrease was not detected until minute 90 of reperfusion. In the 24h UW+TR group, the TA levels were directly correlated with those of GR, while in the 24h UW group, the TA were found to correlate with GPX. These findings are consistent with the affirmation that the administration of antioxidants prior to or at the beginning of the ischemic period influences glutathione metabolism and attenuates IR injury. While GPX is the first line of defense against ROS and an elevated GPX concentration indicates oxidation of the glutathione that combines with them, GR activity indicates the recovery of the glutathione consumed and the capacity to modify oxidative stress (Carbonell et al., 2000; Renner et al., 2004; Castillo et al., 2005; Fukai et al., 2005; Luyten et al., 2005; Mallet, 2005).

Trolox is considered to be more active than alpha- and gamma-tocopherol in interrupting the chain reaction that leads to lipid peroxidation, reducing the production of thiobarbituric acid reactive substances (Bello-Klein et al., 1997; Sagach et al., 2002). However, in our experience, the elevation of the MDA concentrations at the start of reperfusion in the 24h UW and 24h UW+TR groups was similar. This apparent disparity may be a consequence of differences in the experimental conditions: the 10 minutes of warm ischemia described by the other authors may not be comparable to the 24 hours of cold ischemia employed in our study, in which the prolonged ischemic time had a greater influence than the antioxidant therapy.

With respect to ET-1 concentration, the changes in that of the 24h UW+TR group range between the those observed in the 2h and 24h UW groups: the levels increase, but to a lesser degree than those of the latter group. Trolox has been reported to be effective in the protection of the myocyte, but not in that of the endothelial cell (Mickle, 1993; Widlansky et al., 2003), in which it acts only in the presence of high intracellular GSH concentrations (Heller et al., 2004). Although we

did not measure these intracellular levels, the maintenance of plasma GR concentrations in the treated group for a longer period of time than in the untreated groups points toward this mechanism of protection, albeit partial, of endothelial cells. On the other hand, the preservation during the ischemic period may have also played a role since, as has been reported elsewhere (Kevelaitis et al., 2005), the inhibition of the NHE during IR mitigates endothelial dysfunction. Vasoconstriction was not present in any group, but in Trolox-treated cases leukocyte adhesion, interstitial edema and inflammatory infiltrate were less prominent than in the untreated group.

In short, under the experimental conditions described here, treatment with Trolox alleviated morphological damage of myocardium, maintained TA concentrations, delayed the decrease in GR levels and mitigated the increase in ET-1 during reperfusion, effects that could be the consequence of the inhibition of the NHE due to the decrease in pH produced in the preservation solution when this vitamin E analogue was added to it. The capacity of Trolox to protect, at least partially, against IR injury could be of use in clinical practice to reduce the incidence, for example, of primary graft failure in hearts procured from marginal or suboptimal donors.

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