Histol Histopathol (2009) 24: 1487-1498

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Supplementation of University of Wisconsin solution with Nitroglycerin and Nicorandil in long-term myocardial preservation: effects on the oxidative state, endothelial function and morphology

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Summary. The purpose of this study was to assess the effects of the addition of Nitroglycerin or Nicorandil to University of Wisconsin solution in long-term myocardial preservation. In a model of heterotopic heart transplantation in pigs, the donor heart was preserved for 24 hours by means of continuous perfusion in this solution, in the presence or absence of these drugs. During this period, the oxygenation and pH of the solution were measured, as were lactate concentrations and enzyme release. At regular intervals following reperfusion we measured the concentrations of enzymes, antioxidants, glutathione peroxidase, glutathione reductase, malondialdehyde, endothelin and nitrite, and, two hours later, samples of both ventricles were taken for a morphological study. In the treated groups there was a higher lactate production during preservation and, during reperfusion, the signs of contracture and the elevation of enzyme levels were more marked than in the untreated groups. In contrast, the glutathione reductase concentrations did not decrease during the first phase of reperfusion and were directly correlated with those of antioxidants, endothelin levels increased less than in the untreated groups and, in the case of nitroglycerin, the nitrite concentration was significantly greater than in the remaining groups. We conclude that nitroglycerin and nicorandil improved the oxidative state and endothelial function and did not produce substantial morphological changes, but increased cell necrosis and contracture, possibly due to the duration of ischemia.

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Key words: Nitroglycerin, Nicorandil, Wisconsin solution, Long-term myocardial preservation, Vessel preservation

Introduction

In heart transplantation, primary graft failure 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 (Verrier, 2004; Taylor et al., 2005). In addition to Na^+/H^+ exchanger activation and intracellular calcium overload, the production of reactive oxygen species (ROS) is considered to be the major contributor to ischemia-reperfusion injury: ROS generate peroxidation, protein denaturation and DNA damage, provoking contractile and endothelial dysfunction, apoptosis and necrosis (Das, 2003; Beyersdorf, 2004; Renner et al., 2004; Kevelaitis et al., 2005; Luyten et al., 2005; Mallet, 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 (Beyersdorf, 2004; Verrier, 2004; Stoica et al., 2005). The administration of NO, NO

donors or substances that stimulate its release, prior to ischemia and with the preservation solution, protects the myocardium from ischemia-reperfusion injury, reducing the extent of necrosis and endothelial dysfunction (Tanoue et al., 1999; Das et al., 2001; Schulz et al., 2004).

Nitroglycerin (NTG) is metabolized in the vascular wall, producing NO, which activates guanylate cyclase, increases cGMP and reduces the cytosolic calcium concentration, producing relaxation of vascular smooth muscle. This relaxation is endothelium-independent and, thus, is not affected by the presence of endothelial dysfunction (Simon et al., 2001; Steen, 2001; Barbato and Tzeng, 2004; Thatcher et al., 2004). The inconvenience of NO is that it reacts with superoxide, forming peroxynitrite, a highly reactive species that produces lipid peroxidation, damaging endothelial cells and myocytes. However, NTG acts directly on the myocyte: it stimulates cAMP-dependent protein kinase, increasing contractility, thus counteracting the harmful effects of lipid peroxidation (Tanoue et al., 1999). NO production may be facilitated by the interaction of NTG with the sulfhydryl group of reduced glutathione, one of the components of University of Wisconsin (UW) solution (Baxter et al., 2001).

Nicorandil (NIC) is a drug with a dual action: as a nitrate, similar to NTG, and as a K_{ATP} channel opener. The latter action increases K^+ release by the cell, which produces membrane hyperpolarization, shortening of the action potential duration, inhibition of calcium entry in the cell and vascular smooth muscle relaxation. It decreases preload (nitrate effect) and afterload (K_{ATP} channel opening effect), improves coronary flow, has no negative inotropic effects and is not arrhythmogenic. It increases ATP release by the endothelial cell, which degrades adenosine, inhibiting neutrophil and platelet activation and, thus, the production of ROS in these cells. In the presence of adenosine, another component of UW solution, it reduces the oxidation of actin, and recovery of ATP levels is improved, enhancing contractility (Thatcher et al., 2004; Yang et al., 2005).

Most studies describe the beneficial effects of these two drugs in vitro or after periods of ischemia of less than 16 hours. Given that the prolongation of safe ischemia times could increase the number of donors, by incorporating suboptimal or marginal donors into this pool, our objective consisted of assessing the capacity of these two substances to modify the oxidative state and endothelial function, as well as their relationship to the morphological changes produced in an experimental model of heart transplantation following an ischemic period of 24 hours.

Materials and methods

The Institutional Animal Care and Use Committee approved the study. The investigation conformed 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 in 1996).

Twenty-four 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=6), in which the donor heart was transplanted after 2 hours of ischemia preserved in cold saline; 24hUW (n=6), in which the donor heart was subjected to 24 hours of ischemia; suspended by the aorta, it 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 (Viaspan®) as preservation solution (total volume 2 L); 24hUW+NTG (n=6), in which the preceding procedure was followed, with the addition of nitroglycerin (0.1 mg/mL) to the preservation solution; and 24hUW+NIC (n=6), in which nicorandil (0.3 mg/L) was added to the preservation solution.

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, modified by our group (Matsui et al., 1988; Roda et al., 2004). Inotropic support was not used in any case.

Data and sample collection

Samples of the preservation solution were collected 10 minutes and 6 and 24 hours after the initiation of continuous perfusion to determine pO_2, pCO_2, pH, glucose, lactate, calcium, lactate dehydrogenase (LDH) and creatine kinase (CK).

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 in 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 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 the 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), glutathione peroxidase (GPX) and glutathione reductase (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 $\leq 1mm^3$ 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 Leika 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, 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

The changes in the variables evaluated in the preservation solution (Table 1) were similar in the three groups subjected to 24 hours of ischemia, with the exception of lactate, in which the increase was significantly greater in the groups treated with NTG or NIC. In these two groups palpation of the ventricles at the end of ischemia, prior to transplantation, evidenced a hardness and stiffness that was not detected in the other two groups; at the beginning of reperfusion they appeared to be edematous, congestive and even hemorrhagic in some cases, a situation that tended to improve partially during the two hours of the study period.

Following reperfusion, the functional damage produced in the hearts subjected to prolonged ischemia

(24hUW group) was manifested in the lower systolic blood pressure generated by the transplanted left ventricle (Table 2). The production of free radicals was evidenced by the decrease in TA and GR concentrations, and those of MDA increased as a sign of lipid peroxidation (Table 3). The elevation of the ET-1 concentrations in the absence of a concomitant increase in those of nitrite (Table 4) was indicative of the production of endothelial damage.

In the 24hUW+NTG and 24hUW+NIC groups the CK concentrations in the blood of the coronary sinus of the transplanted heart were significantly higher than in the other two groups and in the corresponding recipient hearts (Table 2). During the first hour of reperfusion, the GR concentrations did not decrease in the 24hUW+NTG group, and although they declined in the 24hUW+NIC group, they were higher than in the remaining groups (Table 3). In the 24hUW group, the antioxidant concentration was correlated with the GPX activity ($r=0.7634$; $p<0.001$), indicating consumption of reduced glutathione (Fig. 1). In contrast, in the 24hUW+NTG and 24hUW+NIC groups, the antioxidant concentrations were correlated with the GR activity ($r=0.8012$ and

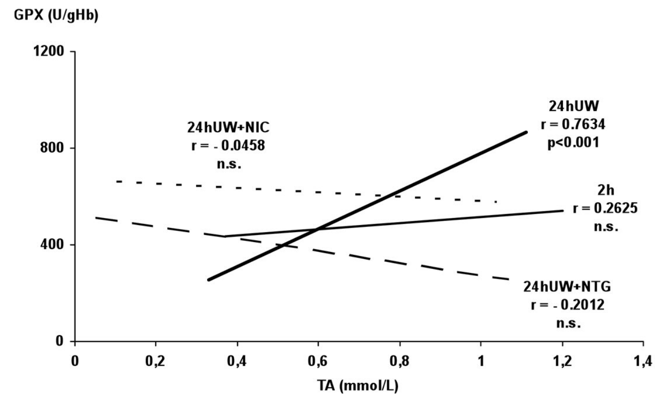

Fig. 1. In the 24hUW group the antioxidant concentration (TA) was correlated with the GPX activity, expressing reduced glutathione consumption to combat the production of free radicals generated during reperfusion.

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

	24hUW	24hUW+NTG	24hUW+NIC
pO_2 (mmHg)			
10 min	160±80	192±55	158±83
6 h	353±107[b]	393±52[b]	361±81[b]
24 h	609±56[b]	622±82[b]	619±61[b]
pCO_2 (mmHg)			
10 min	2.4±0.5	2.5±0.8	2.7±0.6
6 h	5.2±1.9[b]	6.6±0.3[b]	5.6±1.9[b]
24 h	14.9±1.2[b]	15.2±2.0[b]	14.8±1.6[b]
pH			
10 min	7.6±0.13	7.6±0.03	7.7±0.08
6 h	7.4±0.14[b]	7.3±0.02[b]	7.4±0.12[b]
24 h	7.1±0.05[b]	7.1±0.03[b]	7.1±0.04[b]
Glucose (mg/dL)			
10 min	6.6±4.8	7.1±6.6	3.8±2.2
6 h	11.8±3.8[b]	8.3±5.0	10.8±2.2[b]
24 h	28±6.3[b]	27.7±20.1[a]	19.6±3.8[b]
Lactate (mmol/L)			
10 min	0.30±0.16	0.15±0.14	0.08±0.08
6 h	0.57±0.19[b]	0.43±0.17[a]	0.36±0.16
24 h	0.57±0.35[a]	0.96±0.50[bc]	1.25±0.38[bd]
Calcium (mg/dL)			
10 min	1.5±0.3	1.4±0.3	1.5±0.3
6 h	1.4±0.5	1.5±0.1	1.6±0.1
24 h	1.3±0.5	1.6±0.1	1.7±0.2
LDH (U/L)			
10 min	11±7	13±10	6±4
6 h	24±8[a]	41±11[a]	21±6
24 h	140±25[b]	137±46[b]	82±29[b]
CK (U/L)			
10 min	11±7	7±7	3±3
6 h	55±14	50±37	24±12
24 h	299±83[b]	225±175[b]	129±43[b]

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

Table 2. Hemodynamic and biochemical parameters.

	2h	24hUW	24hUW+NTG	24hUW+NIC
mAP (mmHg)				
Basal	83±19	77±8	79±13	75±10
5 min	65±11	64±8	61±18	68±8
60 min	76±14	69±6	68±26	83±11
120 min	76±16	57±17[b]	65±21[b]	73±14
CO (L/min)				
Basal	2.4±0.6	2.4±0.5	2.8±0.6	2.6±0.5
5 min	2.5±0.7	1.9±0.5	2.5±1.0	2.5±0.7
60 min	2.8±0.7	2.0±0.4	2.0±0.5	2.2±0.5
120 min	2.4±0.8	1.8±0.3[b]	2.0±0.3	2.2±0.4
LVs-Don (mmHg)				
60 min	91±17	67±10	48±22[d]	74±16
120 min	90±26	52±10[bd]	40±17[d]	59±17[ac]
LDH (U/L)				
Basal	712±88	675±129	653±96	842±226
5 min	674±130	782±163	1089±549	979±250
60 min	879±248	1181±423[b]	1712±640[bce]	2202±817[bd]
120 min	957±286[a]	1110±331[b]	1813±689[b]	1742±284[b]
CK-Rec (U/L)				
Basal	907±309	948±472	882±350	1275±859
120 min	2325±768[b]	3385±1400[b]	8157±2979[b]	6590±3570[b]
CK-Don (U/L)				
5 min	1511±430	2697±1016	5728±3570[de]	5158±4046[c]
60 min	2693±1420[b]	3750±1488[a]	10447±3308[bdf]	9048±5068[bd]
120 min	2735±1001[b]	3671±1552[a]	9460±3721[bdfg]	7730±4077[bceg]

[a]: $p<0.05$; [b]: $p<0.01$ vs 5 min; [c]: $p<0.05$; [d]: $p<0.01$ vs 2h; [e]: $p<0.05$; [f]: $p<0.01$ vs 24hUW; [g]: $p<0.05$ vs recipient; mAP: Mean arterial pressure; CO: Cardiac output; LVs: Left ventricular systolic pressure; LDH: Lactate dehydrogenase; CK: Creatine kinase (coronary sinus blood); Rec: Recipient; Don: Donor.

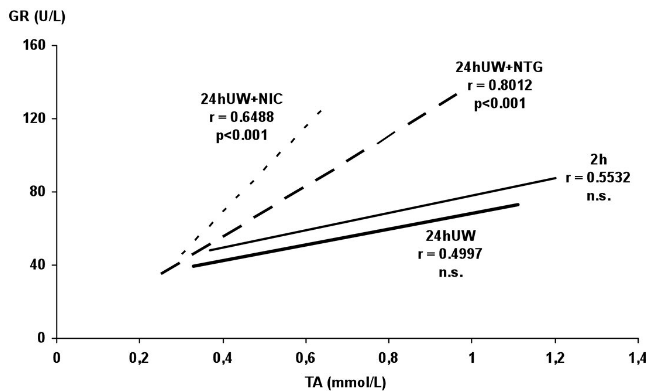

Fig. 2. In the 24hUW+NTG and 24hUW+NIC groups, the antioxidant concentration (TA) was correlated with the GR activity and, thus, with the regeneration of reduced glutathione. This fact shows that both NTG and NIC potentiate the antioxidant capacity.

Table 3. Oxidative state.

	2h	24hUW	24hUW+NTG	24hUW+NIC
TA (mmol/L)				
Basal	0.69±0.09	0.81±0.20	0.47±0.05^{df}	0.47±0.04^{df}
5 min	0.70±0.13	0.71±0.22^{b}	0.45±0.04^{df}	0.42±0.04^{adf}
30 min	0.63±0.12	0.69±0.22^{b}	0.45±0.06^{df}	0.42±0.03^{adf}
60 min	0.66±0.19	0.67±0.21^{b}	0.40±0.09^{adf}	0.41±0.06^{bdf}
90 min	0.63±0.13	0.63±0.22^{b}	0.40±0.07^{adf}	0.37±0.06^{bdf}
120 min	0.62±0.13	0.62±0.25^{b}	0.35±0.09^{bdf}	0.34±0.04^{bdf}
GPX (U/gHb)				
Basal	444±45	536±285	405±31	708±67^{deh}
5 min	473±72	560±245	412±125	615±163^{g}
30 min	478±55	565±270	425±94	632±83^{cg}
60 min	507±61^{b}	549±215	442±103	597±107
90 min	457±87	519±200	387±131	646±134^{ch}
120 min	470±52	466±164	428±79	606±131^{g}
GR (U/L)				
Basal	73±10	73±15	69±8	87±29
5 min	63±10^{b}	59±16^{b}	64±10	77±20^{aceg}
30 min	58±9^{b}	54±18^{b}	62±11	76±18^{adfg}
60 min	60±12^{b}	51±19^{b}	56±12^{b}	69±17^{b}
90 min	58±11^{b}	47±17^{b}	49±13^{b}	61±11^{b}
120 min	58±11^{b}	43±17^{b}	48±13^{b}	53±11^{b}
MDA-Rec (µmol/L)				
Basal	1.68±0.33	1.35±0.40	1.17±0.65	1.65±0.44
5 min	1.69±0.24	1.47±0.24	1.91±0.43^{b}	1.66±0.47
30 min	1.70±0.33	1.50±0.34	1.60±0.22	1.67±0.29
60 min	1.62±0.29	1.47±0.27	1.72±0.21	1.57±0.25
90 min	1.62±0.39	1.46±0.26	1.33±0.15	1.73±0.47
120 min	1.61±0.30	1.40±0.27	1.26±0.30	1.40±0.34
MDA-Don (µmol/L)				
Basal	1.68±0.33	1.35±0.40	1.17±0.65	1.65±0.44
5 min	1.67±0.30	2.73±0.59$^{b\,j}$	3.04±1.58^{bi}	2.70±0.68^{i}
30 min	1.64±0.40	1.58±0.32	2.04±0.31	2.50±0.70^{dei}
60 min	1.75±0.32	1.55±0.28	1.57±0.15	1.98±0.91
90 min	1.65±0.25	1.40±0.32	1.37±0.19	1.75±0.73
120 min	1.72±0.47	1.58±0.51	1.70±0.96	1.64±0.62

a: p<0.05; b: p<0.01 vs 5 min; c: p<0.05; d: p<0.01 vs 2h; e: p<0.05; f: p<0.01 vs 24hUW; g: p<0.05; h: p<0.01 vs 24hUW+NTG; i: p<0.05; j: p<0.01 vs recipient; TA: Total antioxidants; GPX: Glutathione peroxidase; GR: Glutathione reductase; MDA: Malondialdehyde; Rec: Recipient; Don: Donor.

r=0.6488, respectively; p<0.001 in both cases) and, thus, with the regeneration of reduced glutathione (Fig. 2). In these two groups, the elevation of the ET-1 concentrations was less marked than in the 24hUW group, and the 24hUW+NTG group was the only one in which the nitrite concentrations increased during reperfusion (Table 4).

The light microscopy, immunohistochemical and ultrastructural studies provided us with an overall view of the changes in the myocardium originated by ischemia-reperfusion (Table 5), 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 presented the morphological features typical of healthy myocardium: there was no evidence of changes in the diameter of the vascular lumen or in the cell structure of the walls.

Table 4. Endothelial function.

	2h	24hUW	24hUW+NTG	24hUW+NIC
ET-1 (fmol/L)				
Basal	4.28±1.86	5.95±2.11	4.68±1.14	2.30±1.27
5 min	4.70±1.26	8.29±3.42^{ad}	4.82±0.99^{f}	4.97±3.37^{bf}
30 min	4.56±1.35	8.18±3.45^{bd}	5.42±0.90^{e}	5.03±3.01^{bf}
60 min	4.42±1.23	9.06±4.00^{bd}	5.90±1.04^{be}	5.47±3.21^{bf}
90 min	4.60±1.22	9.16±3.09^{bd}	5.52±0.94^{f}	5.24±2.50^{bf}
120 min	4.50±1.19	9.71±3.51^{bd}	5.65±1.12^{af}	4.81±2.15^{bf}
Nitrite (µmol/L)				
Basal	5.6±2.3	5.5±3.1	4.5±1.3	4.7±1.9
5 min	4.4±2.3^{a}	4.6±3.1	17.5±13.7^{bdf}	4.5±2.1^{g}
30 min	4.9±3.1^{a}	4.8±3.3	13.2±5.9^{bdf}	4.7±2.2^{g}
60 min	4.6±3.2^{a}	3.7±2.5^{a}	9.6±3.9^{df}	5.0±2.5^{g}
90 min	3.7±2.9^{b}	5.0±3.7	10.8±6.9^{adf}	4.7±2.5^{g}
120 min	4.2±2.8^{b}	4.9±3.4	8.4±5.5^{c}	5.0±2.6

a: p<0.05; b: p<0.01 vs 5 min; c: p<0.05; d: p<0.01 vs 2h; e: p<0.05; f: p<0.01 vs 24hUW; g: p<0.01 vs 24hUW+NTG; ET-1: Endothelin-1.

Table 5. Structural damage produced after 24 hours of preservation and 2 hours of reperfusion. Comparison among the preservation solutions.

	24hUW	24hUW+ NTG	24hUW+NIC
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.

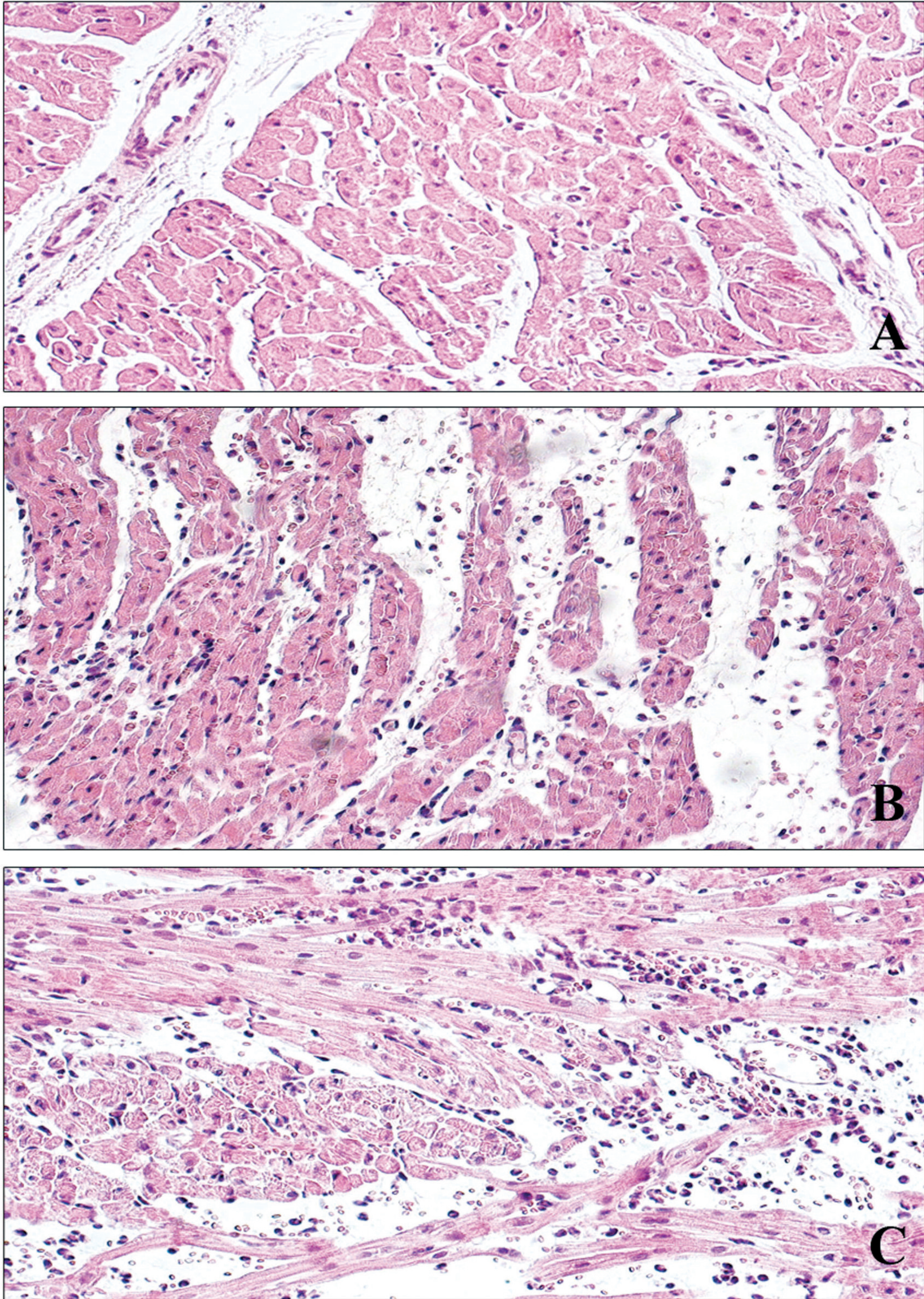

Fig. 3. The interstitial edema and inflammatory infiltrate in the myocardial tissue were less prominent in the 24hUW group **(A)** than in the 24hUW+NTG **(B)** and 24hUW+NIC **(C)** groups. Hematoxylin-eosin, x 100

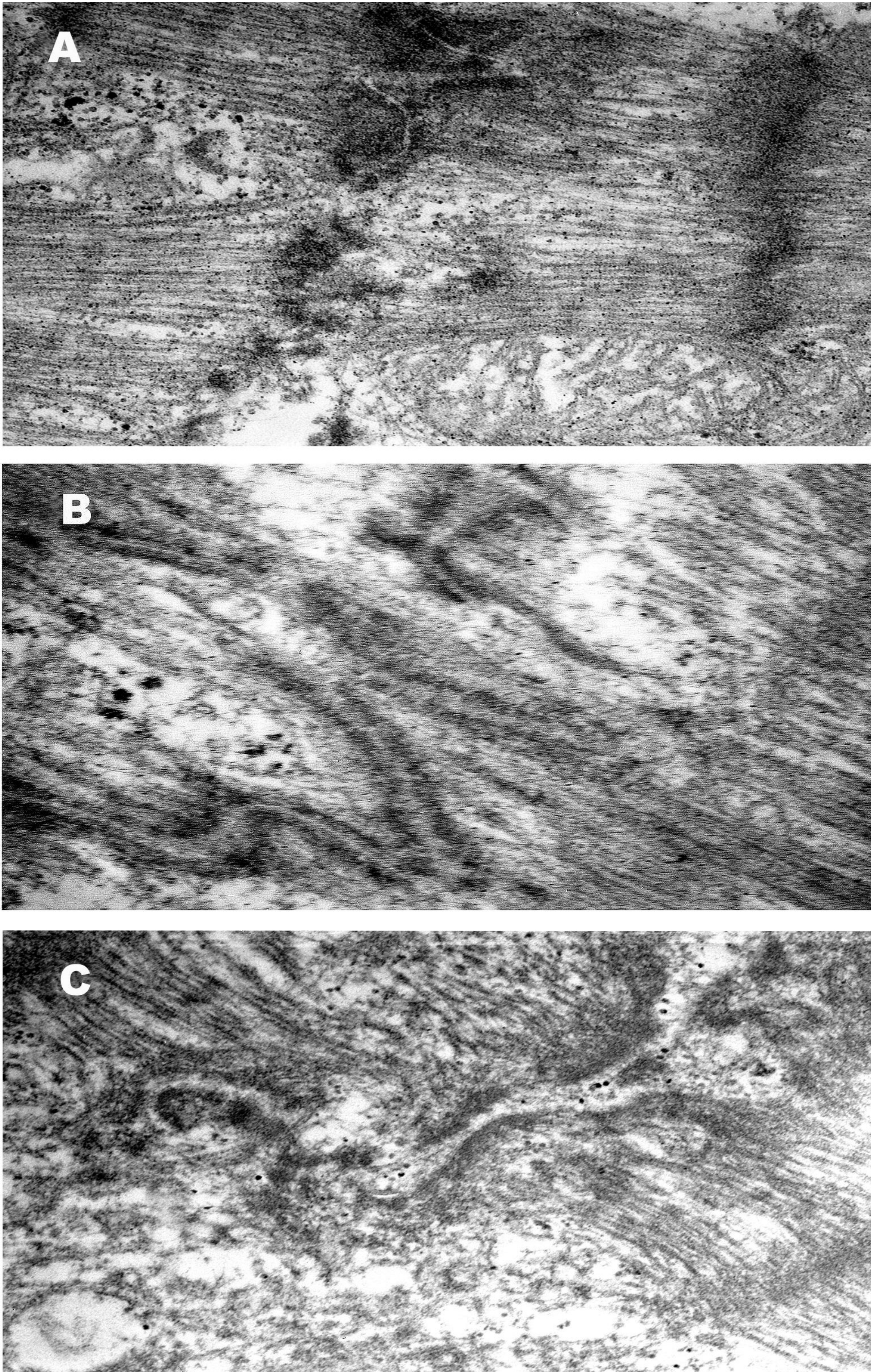

Fig. 4. This figure shows the status of the intercellular junctions in the 24hUW **(A)**, 24hUW+NTG **(B)** and 24hUW+NIC **(C)** groups. The degree of disruption is similar in all three. EM, x 30.000

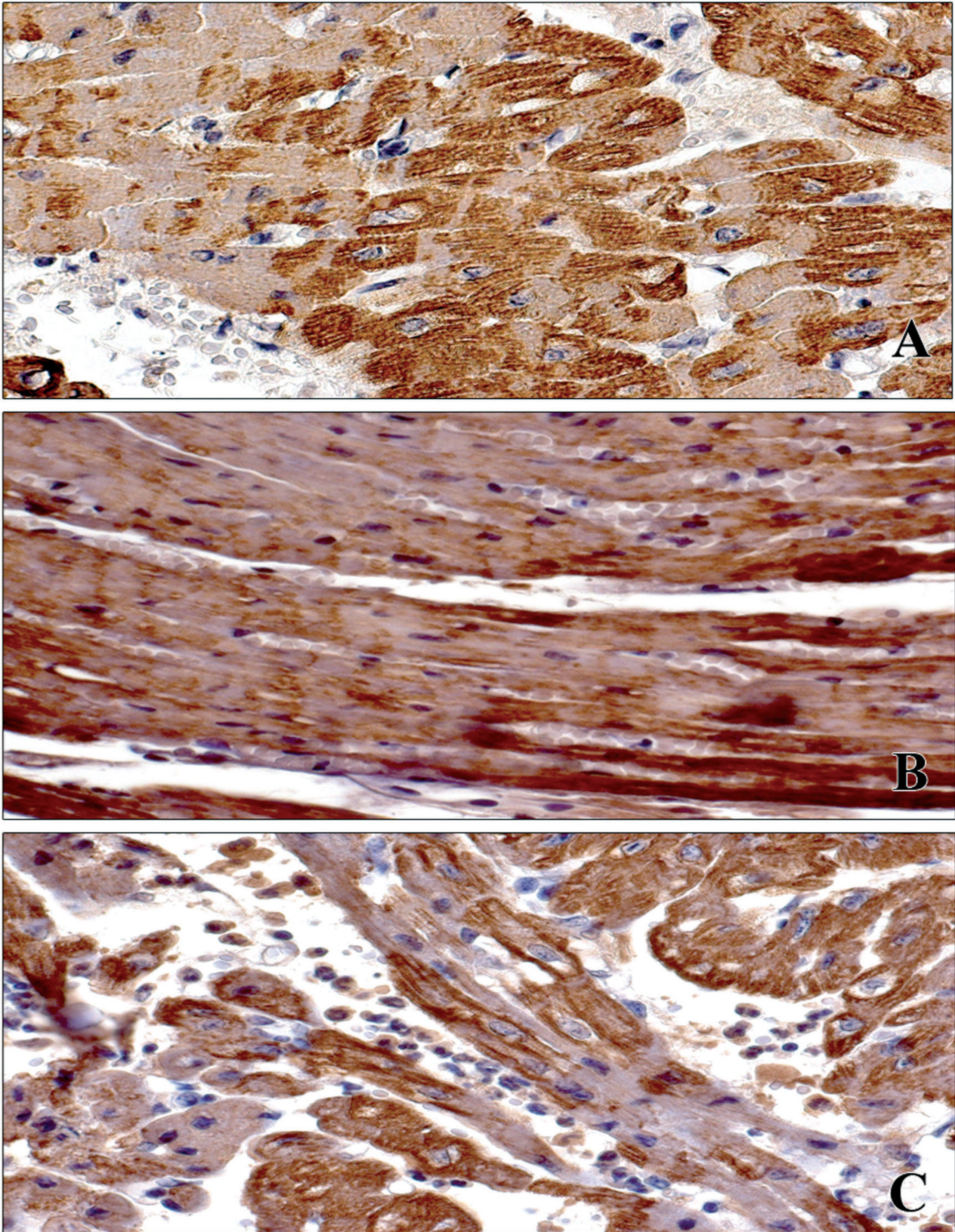

Fig. 5. Immunohistochemical labeling for actin. The loss of actine expression was similar in the three groups: 24hUW group **(A)**, 24hUW+NTG group **(B)**, 24hUW+NIC group **(C)**. The change in the actin pattern showed an irregular distribution, affecting areas of different extension. PAP anti-actin, x 200

Polymorphonuclear leukocytes were adhered to the endothelial cells, and were more abundant in the 24hUW+NTG and 24hUW+NIC groups than in the 24hUW group.

The presence of interstitial edema and inflammatory infiltrate was more marked in the treated groups, being most prominent in the 24hUW+NIC group (Fig. 3).

In all the groups, and without differences among them, we observed circumscribed areas of necrosis in contraction bands: thick, irregular bands, transversely oriented in the cytoplasm, which stained intensely with the light microscopy techniques. The ultrastructural study showed them to be composed of contracted sarcomeres with widened Z lines. In these regions, approximately 50% of the mitochondria presented moderate morphological changes consisting of a reduction in the density of the matrix and moderate disruption of the cristae.

Intracellular glycogen was also reduced in the three groups, and there were nearly no glycogen granules in the myocardial cells.

Both immunohistochemical techniques and electron microscopy revealed that the intercellular junctions were poorly conserved and were disrupted in all the groups (Fig. 4).

With 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 actine expression in the groups (Fig. 5).

Discussion

The duration of ischemia is an independent risk factor in early mortality following transplantation, and the maximum ischemia time considered to be safe in heart transplantation is six hours (Fedak et al., 2005; Taylor et al., 2005; Boku et al., 2006).

In the attempt to achieve viable hearts after 24 hours of ischemia, the authors of some experimental studies have utilized continuous hypothermic perfusion with different preservation solutions. This technique provides uniform cooling, enables 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). With respect to preservation solutions, that of the University of Wisconsin appears to provide better results than others, especially in prolonged preservation (Tedy et al., 1993; García-Poblete et al., 1997, 1998; Boku et al., 2006; Kajihara et al., 2006; Alvarez-Ayuso et al., 2008).

In aerobiosis, the myocardium uses fatty acids as a source of energy; in anaerobiosis, it employs glucose from the myocardial glycogen deposits, a process that is inhibited by the accumulation of its metabolites. This interrupts ATP synthesis and increases the proton, calcium and sodium concentrations, resulting in contraction (Stoica, 2004; Toledo-Pereyra et al., 2004; Oshima et al., 2005). In our study, we observed an increase in the glucose and lactate concentrations in the preservation solution during continuous perfusion; as UW solution contains no glucose, these increases would be an expression of the utilization of anaerobic glycolysis by the myocardium. Although the morphological study did not find that glycogen was better preserved in any of the groups, the fact that the increase in the lactate concentration was significantly more marked in the treated groups, especially in that in which NIC was employed, suggests that there was a greater mobilization of myocardial glycogen in those cases.

When preservation is inadequate, in the first few minutes of reperfusion, surgeons observe myocardial contraction (hard and stiff muscle), attributed to intercellular calcium overload. This is reflected histologically in the presence of contraction bands, with contracted sarcomeres and sarcolemmal rupture. These bands are produced in cells with some type of connection or continuity among them, possibly through the intercellular junctions, and its extension is correlated with the degree of contraction and with enzyme release (Piper and García-Dorado, 1999; Piper et al., 2004). In our study, we did not objectively quantify the extension of the contraction bands in each transplanted heart. Although there do not appear to be substantial differences among the groups, we have observed that those treated with NTG and NIC showed evident signs of myocardial contracture, and that enzyme release was significantly greater than in the other two groups. From the functional point of view, we have observed no differences in the pressures generated by the transplanted left ventricle among the three groups subjected to prolonged ischemia.

NIC has been reported to improve post-ischemic contractile function. Actins regulate cell shape, cytoskeletal integrity, Ca movement and contractility. Ischemia produces oxidation, disruption, polymerization and changes in the localization and aggregation of actin. The oxidation of actin, together with reduced ATP, interferes with Ca movement and depresses contractile function. Through its action on mitochondrial K channels, NIC decreases oxidative damage to the proteins, specifically actin oxidation, and improves the supply of high-energy phosphates (Schwalb et al., 2001; Ono et al., 2004; Steensrud et al., 2004, 2006). In our experience, this beneficial effect was not detected either in the function, as we mentioned above, or the morphology, but in the correlation between AT and GR, both in this group and in the NTG group. Among the antioxidants, glutathione plays a prominent role as a ROS scavenger and a substrate in the redox cycle. Its tissue concentration decreases during reperfusion, a decline that is correlated with the duration of ischemia. GPX is the predominant peroxide scavenger in the myocardium: it catalyzes the conversion of reduced

glutathione to its oxidized form, reducing hydrogen peroxide. The increase in oxidized glutathione and GPX and the decrease in reduced glutathione and GR express recovery of the glutathione consumed and, thus, the capacity to continue to combat ROS production (Renner et al., 2004; Luyten et al., 2005; Mallet, 2005).

During the first three minutes of reperfusion, vasoconstriction is produced in the coronary resistance vessels, which can persist for weeks (Kalweit et al., 2000). In order to produce NO, NTG must be metabolized by an enzyme from the vascular wall that is inhibited during hypothermia and becomes available during reperfusion. The administration of NTG prior to or at the start of reperfusion, as we did in our study, enables its accumulation in tissue, and its conversion is produced immediately during reperfusion (Tanoue et al., 1999; Hillegass et al., 2001). Our results agreed with this description: at the start of reperfusion, we observed a marked elevation in the nitrite concentration, with no increase in that of ET-1 in the 24hUW+NTG group, a situation that was not produced in the remaining groups.

However, the resistance arterioles (≤ 150 microns in diameter), on which coronary flow mainly depends, are not capable of metabolizing NTG to the same extent as the large, nonresistance vessels; thus, this substance is less effective as a vasodilator in microvessels as compared to the large epicardial vessels (Hillegass et al., 2001). The findings of our study are consistent with this description: we did not detect substantial differences among the groups with respect to the diameter and aspect of the small and medium-sized myocardial vessels.

Due to the nitrate action of NIC (Thatcher et al., 2004; Yang et al., 2005), in the group treated with this substance we expected an increase in the nitrite concentration similar to that observed in the NTG group, which, however, was not produced. Although it has not been demonstrated that the activation of the endothelial K_{ATP} channels can modulate endothelial dysfunction, the behavior of the ET-1 concentrations during reperfusion in this group suggests the possibility that NIC inhibits the synthesis and/or release of this vasoconstrictor in a manner similar to that reported for other drugs in this family (Minamino and Hori, 2007; Wang et al., 2007).

In view of these results, we can conclude that under the experimental conditions established in our study, the addition of NTG or NIC to UW solution did not improve myocardial function or the morphological changes following reperfusion, and even increased contracture and the release of intracellular enzymes.

However, on the other hand, it is also evident that these drugs exerted potentially beneficial effects on the oxidative state and, more markedly, on endothelial function. These findings lead us to consider that their use with ischemic periods of 24 hours does not fully reproduce the cardioprotective action reported for both in less prolonged ischemia times (Tanoue et al., 1999; Baxter et al., 2001; Ono et al., 2004; Steensrud et al., 2004, 2006; Yang et al., 2005).

Acknowledgements. This study was financed by grant no. 00/0387 from the Fondo de Investigaciones Sanitarias (FIS), Spain. The authors wish to thank Julio Paredes, Antonio Márquez and Raquel Franco, lab technicians, for their help in processing the histological samples, and M. Messman for her translation of the text.

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Accepted June 12, 2009