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Purkinje fibers after myocardial ischemia-reperfusion

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Summary. The purpose of this study was to evaluate the effects of ischemia-reperfusion on Purkinje fibers, comparing them with the adjacent cardiomyocytes. In a model of heterotopic heart transplantation in pigs, the donor heart was subjected to 2 hours of ischemia (n=9), preserved in cold saline, and subjected to 24 hours of ischemia with preservation in Wisconsin solution, alone (n=6), or with an additive consisting of calcium (n=4), Nicorandil (n=6) or Trolox (n=7). After 2 hours of reperfusion, we evaluated the recovery of cardiac electrical activity and took samples of ventricular myocardium for morphological study. The prolonged ischemia significantly affected atrial automaticity and A-V conduction in all the groups subjected to 24 hours of ischemia, as compared to 2 hours. There were no significant differences among the groups that underwent prolonged ischemia. Changes in the electrical activity did not correlate with the morphological changes. In the Purkinje fibers, ischemia-reperfusion produced a marked decrease in the glycogen content in all the groups. In the gap junctions the immunolabeling of connexin-43 decreased significantly, adopting a dispersed distribution, and staining the sarcolemma adjacent to the connective tissue. These changes were less marked in the group preserved exclusively with Wisconsin solution, despite the prolonged ischemia. The addition of other substances did not improve the altered morphology. In all the groups, the injury appeared to be more prominent in the Purkinje fibers than in the neighboring cardiomyocytes, indicating the greater susceptibility of the former to ischemia-reperfusion injury.

Key words: Purkinje fibers, Ischemia-reperfusion injury, Wisconsin solution, Cytoskeletal proteins, Connexin-43

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Introduction

In heart transplantation, graft failure (primary and nonspecific) is responsible for 36% of the deaths occurring during the first 30 days after transplantation and for 17% of those occurring over the following 11 months (Cropper et al., 2003; Verrier, 2004; Lund et al., 2013), a situation that has not changed substantially since 1994. Although the cause is not well known, and is probably multifactorial, primary graft failure has been related to ischemia time and to ischemia-reperfusion. The production of reactive oxygen species, the activation of the Na^+/H^+ exchanger 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).

A wide variety of cardioplegic and preservation solutions are being employed for myocardial protection.

Their composition has continued to change as our understanding of the pathophysiology of ischemia-reperfusion injury has improved, but none of the existing solutions can be considered ideal (Ferrera 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. In previous studies our group examined the effects on cardiomyocytes and endothelial cells of prolonged ischemia and the addition of different substances to University of Wisconsin solution (UWS), demonstrating that it is possible to improve the protection of both cell types simultaneously (García-Poblete et al., 1997; Alvarez-Ayuso et al., 2008, 2009, 2010).

There is a third type of specialized cells, those of the conduction system. We have found no information regarding the damage they may sustain when subjected to ischemia-reperfusion and prolonged ischemia, possibly because their role has been considered much less relevant than that of the myocytes and endothelial cells in the recovery of myocardial function. The effects of ischemia on the Purkinje fibers have been analyzed in classical studies (Gornak and Lushnikov, 1967; Friedman et al., 1973; Thornell, 1974; Eriksson and Thornell, 1979; Thornell and Ericsson, 1981), but the methodology employed does not permit the extrapolation of these results to cardiac transplantation.

For this reason, we have undertaken this study, the purpose of which is to analyze the effects of prolonged ischemia and of reperfusion on the Purkinje fibers, reproducing the conditions under which we studied myocytes and endothelial function, to enable us to compare the behaviors of the different cells, basically from the morphological point of view.

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).

Heterotopic heart transplantation was performed in Landrace x Large White pigs (weighing 19 to 22 kg), which were randomly assigned to the following groups:

2h (n=9), in which the donor heart was transplanted after 2 hours of ischemia during which it had been 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), using University of Wisconsin solution (Viaspan®) as preservation solution (total volume 2 L);

24hUW+Ca (n=4), in which the same procedure was followed, with the addition of calcium (2.4 mmol/L) to the preservation solution;

24hUW+NIC (n=6), in which nicorandil (0.3 mg/L) was added to the preservation solution;

and, 24hUW+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 intravenously (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 ischemic treatment of each group, heterotopic heart transplantation was carried out according to the technique described by Matsui et al. and modified by our group (Matsui et al., 1988; Roda et al., 2004). Data collection was maintained throughout the 2 hours of reperfusion duration. Inotropic support was not used in any case.

Data and sample collection

The electrocardiogram and heart rate were continuously recorded by a PM8060 Vitara monitoring unit (Dräger, Healthcare Corporation) throughout the surgical procedure and 120 minutes of reperfusion. We assessed spontaneous recovery of the electrical activity, the need to apply an electric shock to revert episodes of tachycardia or ventricular fibrillation, restoration of sinus rhythm with or without variable degrees of A-V-block, the need for temporary or permanent pacemaker and the existence of A-V dissociation.

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 at room temperature, embedded in paraffin and cut into 5-micron-thick slices in a Micron HM360 microtome. Sections were stained with hematoxylin-eosin and periodic acid Schiff (PAS) to evaluate together myocyte glycogen content, contraction bands, necrosis, edema, hemorrhage and fibrosis, studied under a Zeiss Axiophot 2 microscope and photographed with an AxiocamHRc camera.

For immunohistochemical studies, histology sections were deparaffinized and rehydrated before endogenous peroxidase activity was blocked 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, desmin monoclonal antibody at a 1:50 dilution (DE-R11 Novocastra) and connexin-43 mouse monoclonal antibody at a 1:50 dilution (cst-3512 Cell Signaling Technology). The 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 non immune serum instead of primary antibody.To assess the degree of Purkinje damage, in each case 12 fields were photographed using a 200x lens, for each study group and for each histological technique. The images obtained were evaluated semi-quantitatively, considering the following degrees: (-): no changes; (+): mild changes; (++): moderate changes; (+++): severe changes.

For each group of studies samples were evaluated by a single pathologist who was blinded to the group to which the sample belonged each specimen.

Statistical analysis

Heart rate differences between groups were assessed using the corrected chi-square test. Those results with a p value of less than 0.05 were considered significant.

Results

Electrical activity

The characteristics of the cardiac rhythm following ischemia-reperfusion in each group are summarized in Table 1.

2h (n=9): Electrical activity was restored spontaneously in all cases. In 8, it evolved to tachycardia - ventricular fibrillation, which was reverted with electric shock. Sinus rhythm was maintained in every case (transient A-V block developed in 1). No animal required pacemaker nor were there any cases of A-V dissociation.

24hUW (n=6):Electrical activity was restored spontaneously in every case. In 3, it evolved to tachycardia – ventricular fibrillation, which was reverted by electric shock. Sinus rhythm was restored in only 1 case, but with A-V block. Five animals required pacemaker, temporary in 3 and permanent in2. A-V dissociation was produced in 3 cases.

24hUW+Ca (n=4): The electrical activity was restored spontaneously in all cases. In 3, it evolved to tachycardia - ventricular fibrillation, which was reverted with electric shock. Sinus rhythm was not restored in any case. Two animals required pacemaker, temporary in 1 and permanent in the other, and all of them had A-V dissociation.

24hUW+NIC (n=6): Electrical activity was restored spontaneously in 5 cases. In 4, it evolved to tachycardia – ventricular fibrillation, which was reverted by electric shock. No animal recovered sinus rhythm. All of them required pacemaker, temporary in 5 and permanent in 1, and A-V dissociation was detected in 5.

24hUW+TR (n=7): Electrical activity was restored spontaneously in every case, after which it evolved to tachycardia – ventricular fibrillation, which was reverted by electric shock. Sinus rhythm was restored in 2 cases, but with A-V block. Four animals required pacemaker, temporary in 3 and permanent in 1, and there was A-V dissociation in 5.

The prolonged ischemia affected atrial automaticity and A-V conduction: the differences in all the groups subjected to 24 h ischemia with respect to 2 h were significant. When the groups with 24-h ischemia were compared between one another, the differences did not reach statistical significance, but the least marked difference was that recorded between the Trolox group and the group that underwent 2 h of ischemia, and the greatest difference was found between the NIC group when compared with the 2-h ischemia group.

Morphological study

Figure 1 (A-E) shows the morphology of the

Table 1. Post-ischemia-reperfusion electrical activity.

	2h n=9	24hUW n=6	24hUW+Ca n=4	24hUW+NIC n=6	24hUW+TR n=7
Spontaneous electrical activity	9	6	4	5	7
Electric shock	8	3	3	4	7
Sinus rhythm / A-V block	9	1^b	0^b	0^c	2^a
Pacemaker	0	5^b	2	6^c	4^a
A-V dissociation	0	3	4^b	5^b	5^a

Chi-square test: [a] p<0.05; [b] p<0.01; [c] p<0.001 vs 2h. UW: University of Wisconsin solution. Ca: calcium. NIC: nicorandil. TR: Trolox.

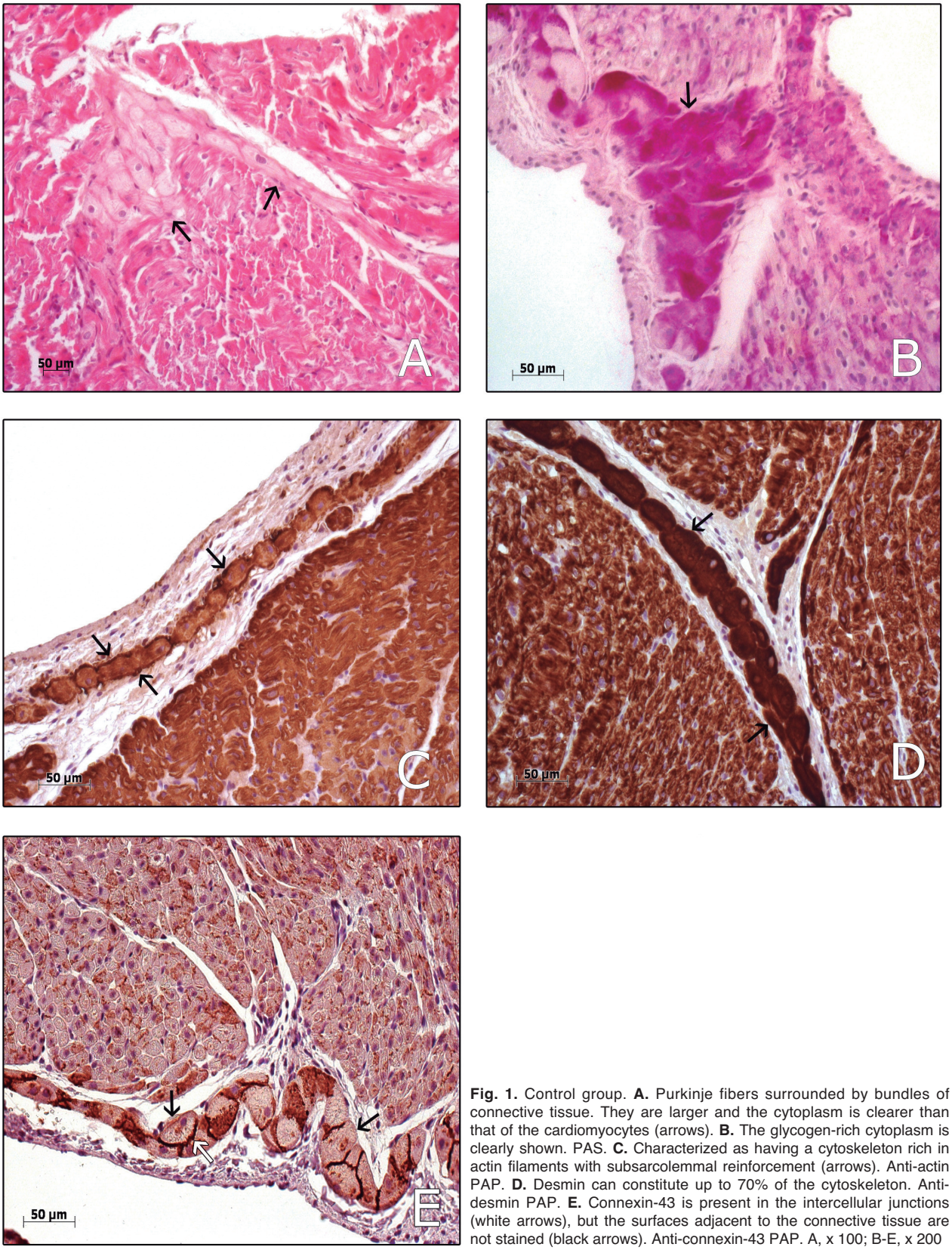

Fig. 1. Control group. **A.** Purkinje fibers surrounded by bundles of connective tissue. They are larger and the cytoplasm is clearer than that of the cardiomyocytes (arrows). **B.** The glycogen-rich cytoplasm is clearly shown. PAS. **C.** Characterized as having a cytoskeleton rich in actin filaments with subsarcolemmal reinforcement (arrows). Anti-actin PAP. **D.** Desmin can constitute up to 70% of the cytoskeleton. Anti-desmin PAP. **E.** Connexin-43 is present in the intercellular junctions (white arrows), but the surfaces adjacent to the connective tissue are not stained (black arrows). Anti-connexin-43 PAP. A, x 100; B-E, x 200

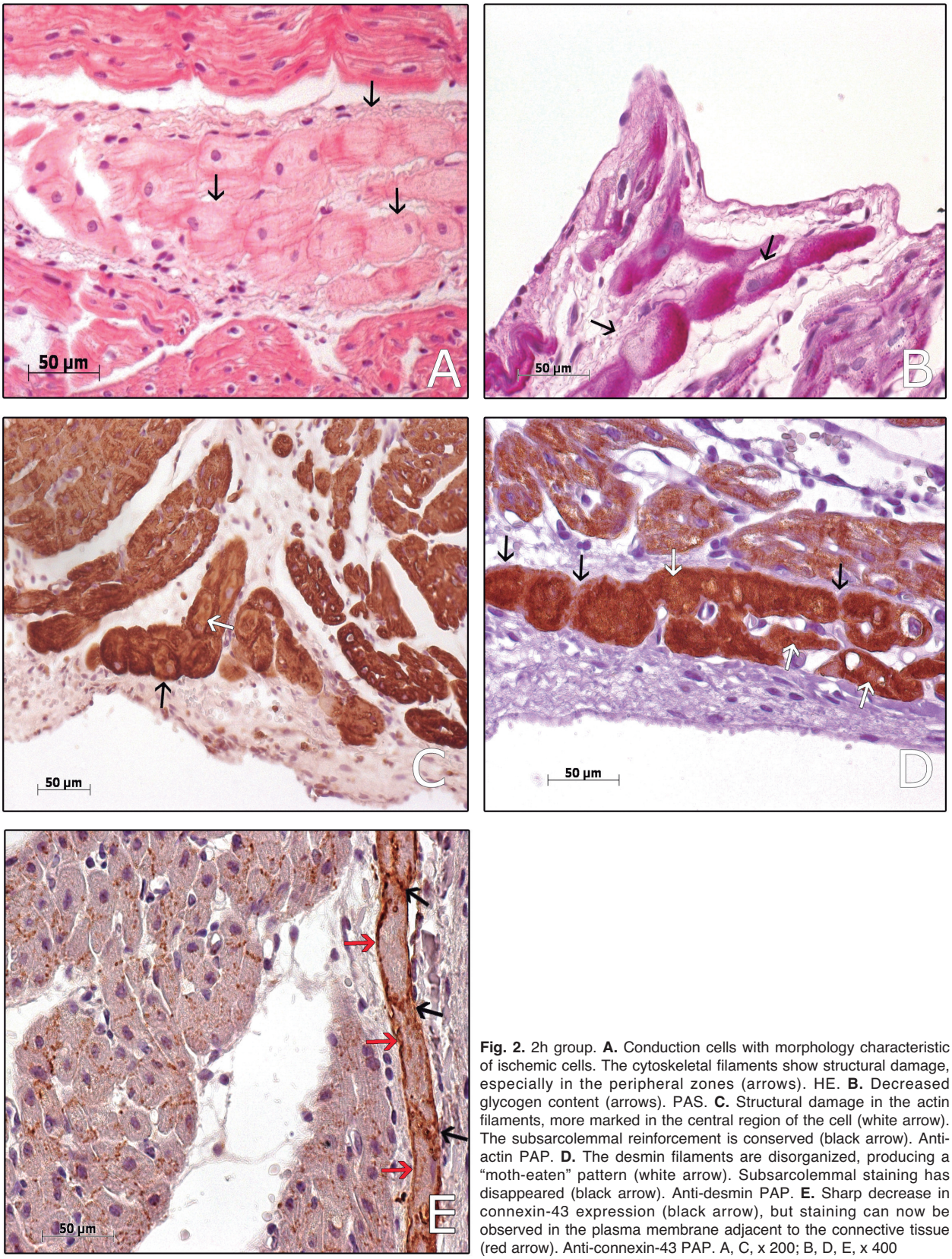

Fig. 2. 2h group. **A.** Conduction cells with morphology characteristic of ischemic cells. The cytoskeletal filaments show structural damage, especially in the peripheral zones (arrows). HE. **B.** Decreased glycogen content (arrows). PAS. **C.** Structural damage in the actin filaments, more marked in the central region of the cell (white arrow). The subsarcolemmal reinforcement is conserved (black arrow). Anti-actin PAP. **D.** The desmin filaments are disorganized, producing a "moth-eaten" pattern (white arrow). Subsarcolemmal staining has disappeared (black arrow). Anti-desmin PAP. **E.** Sharp decrease in connexin-43 expression (black arrow), but staining can now be observed in the plasma membrane adjacent to the connective tissue (red arrow). Anti-connexin-43 PAP. A, C, x 200; B, D, E, x 400

Purkinje fibers in normal hearts; these images were taken as a reference to evaluate the changes observed in the different study groups.

Table 2 summarizes the degree of Purkinje cell damage in each group of study, considering cell size, loss of the actin pattern, loss of the desmin pattern, decreased glycogen content, changes in intercellular junctions.

Findings that were common to all of them were the maintenance of cell size and the marked decrease in the glycogen stored in their sarcoplasm. Other changes can be reported as it follows:

2h: In this group, the conduction cells showed a morphology characteristic of ischemic cells. With respect to the control group, the cytoplasms were clearer, the glycogen was decreased, the actin and desmin were damaged, and connexin-43, in addition to being decreased, underwent a change in its distribution.

The actin cytoskeleton was most affected in the central region of the cell (Fig. 2C), while desmin was more impaired in the subsarcolemmal region (Fig. 2D). This alteration is also observed in Fig. 2A. Altered distribution of connexin-43 was expressed both in the intercellular region (dotted and dispersed immunolabeling) as well as in the plasma membrane adjacent to the connective tissue, negative in normal conditions (Fig. 2E).

24hUW: This is the group in which the cytoskeleton seems best preserved.

Hematoxilyne-eosinrevealed a slight decrease in the cytoplasmic staining, widespread and homogeneous. The cytoskeleton was somewhat unstructured in the peripheral region of the cell (Fig. 3A).

Here the decrease in glycogen was much more accute than in the other groups (Fig. 3B)

The pattern of actin and desmin filaments showed only a slight decrease in staining intensity. Actin damage occurred only in certain very limited areas (Fig. 3C). The reduction in desmin-staining happened in the most peripherical zone of the cells, and was confined and mild (Fig. 3D).

Immunohistochemical analysis with anti-connexin-43 revealed a moderate decrease in staining intensity, with a dotted or beaded distribution.We detected no connexin-43 signal in the outer edges or connective tissue of the cells (Fig. 3E).

24UW+Ca: The most affected morphological parameters in this group were glycogen and connexin-43: glycogen was very sparse (Fig. 4B) and connexin-43 showed an irregular distribution and a weak signal (Fig. 4E).

In the cytoskeleton, the actin filament labeling was decreased widely and homogeneously (Fig. 4C). However, damage to desmin filaments was limited to the central regions of the cell (Fig. 4D).

24U+NIC: In this group, conduction cells had major structural changes (Fig. 5A). Actin filaments were more poorly preserved than those of desmin. The former showed a large decrease in the peripheral regions of the cells (Fig. 5C), while in the latter the structural damage was more localized (Fig. 5D). Glycogen and connexin-43 were reduced in this group (Fig. 5B,E).

24UW+TR: The conduction cells show a slight decrease in the number of cytoskeletal filaments (Fig.6A), also seen in anti-actin and anti-desmin immunohistochemistry. Figure 6C shows a significant

Table 2. Degree of cell damage produced in the Purkinje fibers in each group of animals.

Purkinje fibers	2 h	24hUW	24hUW+Ca	24hUW+NIC	24hUW+TR
Cell size	-	-	-	-	-
Loss of the actin pattern	+/++	+	++	++/+++	+/++
Loss of the desmin pattern	+/++	+	+/++	++	++/+++
Decrease in glycogen content	++	+++	+++	++/+++	++/+++
Changes in intercellular junctions	+++	++	++	+++	++

-: None. +: Mild. ++: Moderate. +++: Severe. UW: University of Wisconsin solution. Ca: calcium. NIC: nicorandil. TR: Trolox.

Table 3. Comparison between the degree of structural damage in the Purkinje fibers and the adjacent cardiomyocytes.

	2h		24hUW		24hUW+Ca		24hUW+NIC		24hUW+TR	
	Purkinje	Cardiomyocytes	Purkinje	Cardiomyocytes	Purkinje	Cardiomyocytes	Purkinje	Cardiomyocytes	Purkinje	Cardiomyocytes
Loss of the actin pattern	++	+	+/++	+	++	+/++	++/+++	+	+/++	+
Loss of the desmin pattern	+/++	++/+++	+	++	+/++	++/+++	++	++/+++	++	++/+++
Decrease in glycogen content	++	+	+++	++	+++	++	++/+++	++	++/+++	++
Changes in intercellular junctions	+++	+/++	++	+	++	+	+++	+	++	-/+

-: None. +: Mild. ++: Moderate. +++: Severe. UW: University of Wisconsin solution. Ca: calcium. NIC: nicorandil. TR: Trolox.

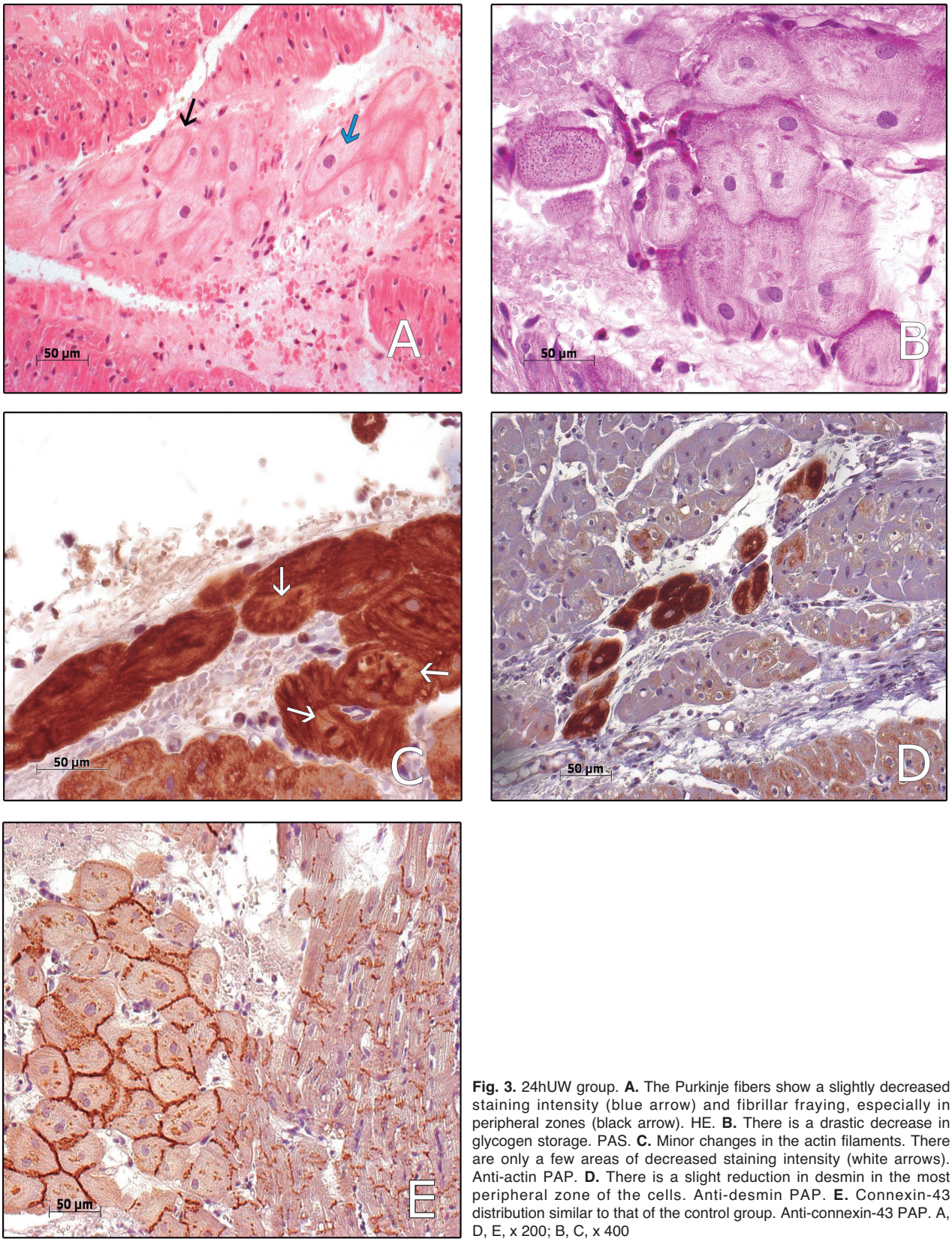

Fig. 3. 24hUW group. **A.** The Purkinje fibers show a slightly decreased staining intensity (blue arrow) and fibrillar fraying, especially in peripheral zones (black arrow). HE. **B.** There is a drastic decrease in glycogen storage. PAS. **C.** Minor changes in the actin filaments. There are only a few areas of decreased staining intensity (white arrows). Anti-actin PAP. **D.** There is a slight reduction in desmin in the most peripheral zone of the cells. Anti-desmin PAP. **E.** Connexin-43 distribution similar to that of the control group. Anti-connexin-43 PAP. A, D, E, x 200; B, C, x 400

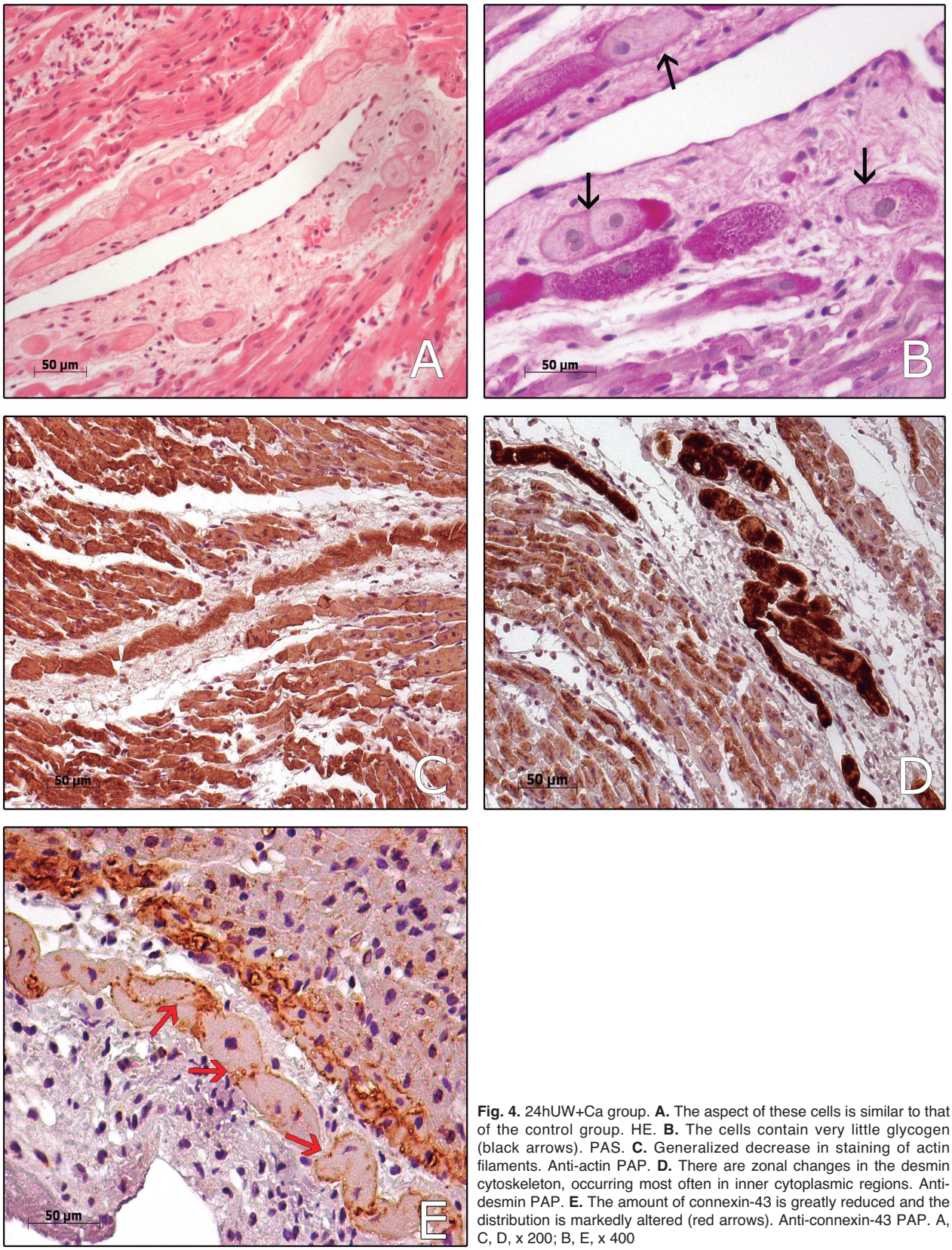

Fig. 4. 24hUW+Ca group. **A.** The aspect of these cells is similar to that of the control group. HE. **B.** The cells contain very little glycogen (black arrows). PAS. **C.** Generalized decrease in staining of actin filaments. Anti-actin PAP. **D.** There are zonal changes in the desmin cytoskeleton, occurring most often in inner cytoplasmic regions. Anti-desmin PAP. **E.** The amount of connexin-43 is greatly reduced and the distribution is markedly altered (red arrows). Anti-connexin-43 PAP. A, C, D, x 200; B, E, x 400

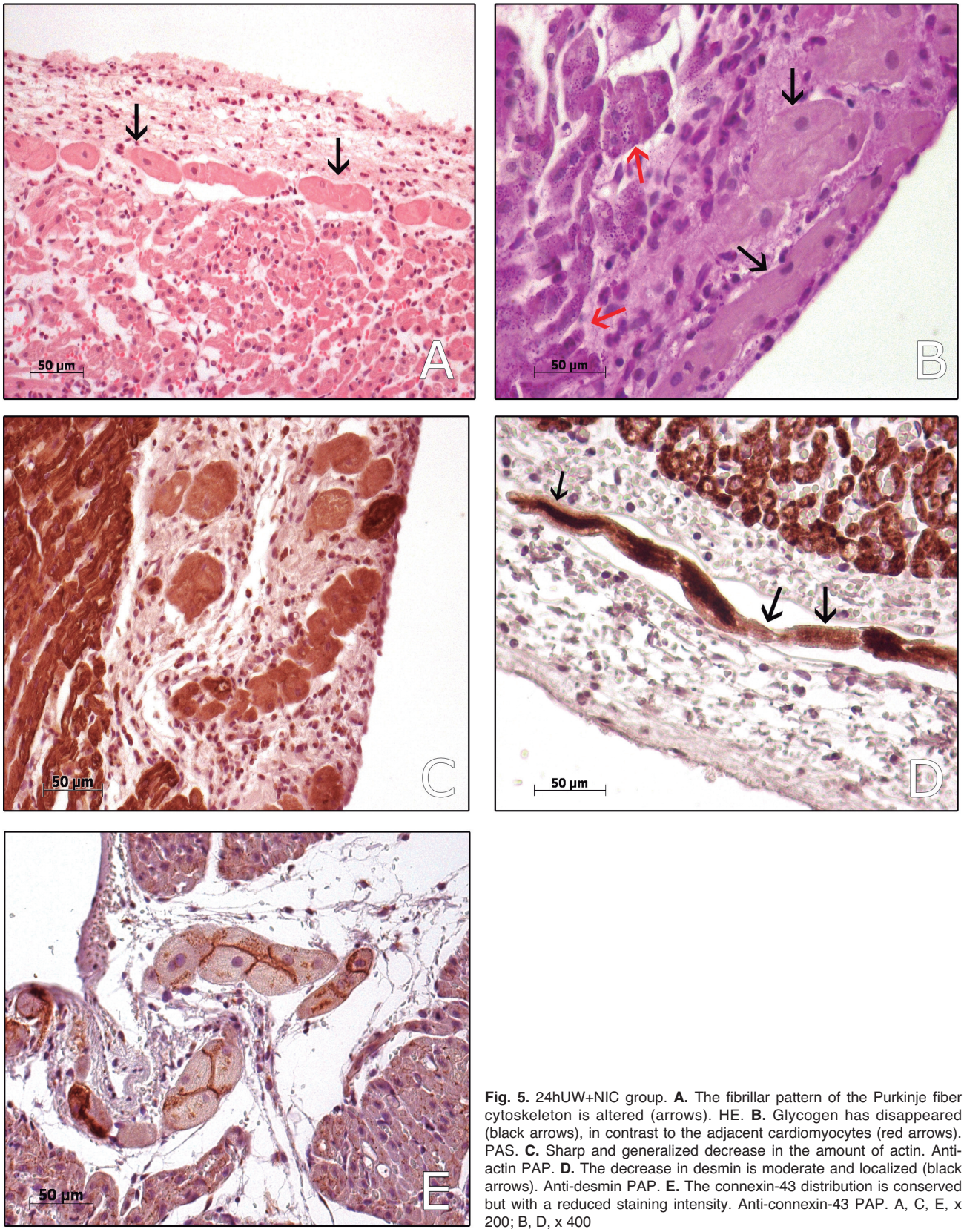

Fig. 5. 24hUW+NIC group. **A.** The fibrillar pattern of the Purkinje fiber cytoskeleton is altered (arrows). HE. **B.** Glycogen has disappeared (black arrows), in contrast to the adjacent cardiomyocytes (red arrows). PAS. **C.** Sharp and generalized decrease in the amount of actin. Anti-actin PAP. **D.** The decrease in desmin is moderate and localized (black arrows). Anti-desmin PAP. **E.** The connexin-43 distribution is conserved but with a reduced staining intensity. Anti-connexin-43 PAP. A, C, E, x 200; B, D, x 400

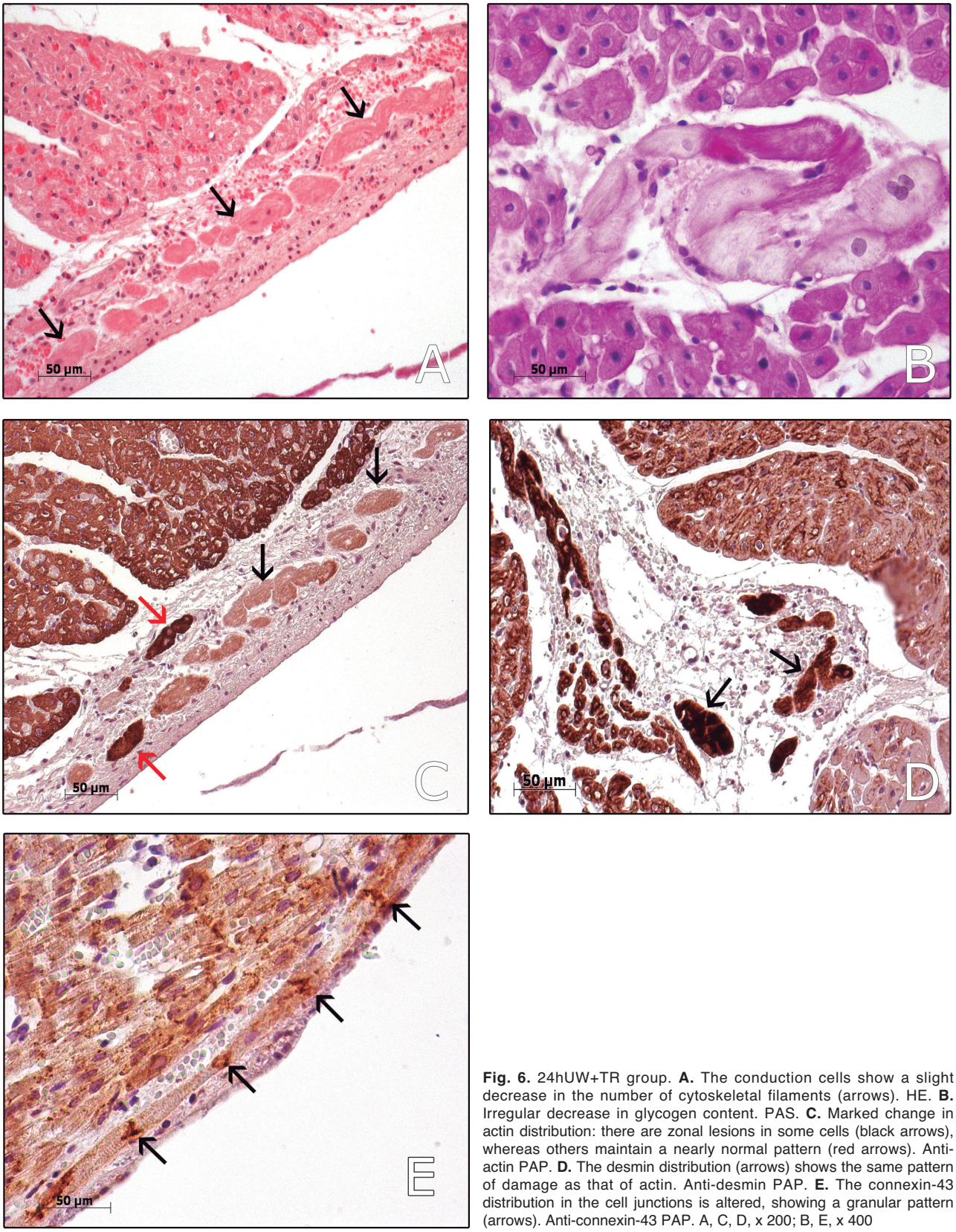

Fig. 6. 24hUW+TR group. **A.** The conduction cells show a slight decrease in the number of cytoskeletal filaments (arrows). HE. **B.** Irregular decrease in glycogen content. PAS. **C.** Marked change in actin distribution: there are zonal lesions in some cells (black arrows), whereas others maintain a nearly normal pattern (red arrows). Anti-actin PAP. **D.** The desmin distribution (arrows) shows the same pattern of damage as that of actin. Anti-desmin PAP. **E.** The connexin-43 distribution in the cell junctions is altered, showing a granular pattern (arrows). Anti-connexin-43 PAP. A, C, D, x 200; B, E, x 400

change in the labeling and distribution of actin filaments: there are zonal lesions in some cells, even with areas of necrosis, whereas others mantain a nearly normal pattern. We observed a decrease in desmin expression, with a loss of structure of the filaments and a granular pattern in the zones most markedly affected (Fig. 6D).

PAS staining showed uneven glycogen distribution (Fig. 6B).

Connexin-43 expression was moderately reduced, and the pattern of labeling showed a granular distribution (Fig. 6E). As in the 2h group, we observed positivity on the outer edges or connective tissue of the cells.

When we compared the changes in the Purkinje fibers with those observed in the neighboring cardiomyocytes, analyzed in previous studies by our groups (García-Poblete et al., 1997; Alvarez-Ayuso et al., 2008, 2009, 2010) we found that, except in regard to the desmin filaments, the damage is greater in the conduction cells (Table 3).

Discussion

Although there are differences between species, the Purkinje fibers are morphologically characterized as being larger than the cardiomyocytes and as having higher contents of glycogen and desmin and few myofibrils, which are more organized in the most peripheral region of the cell. These cells form bundles surrounded by connective tissue, and contain two different domains in their sarcolemmas: one surrounded by the connective tissue that has a basal membrane that separates it from the extracellular matrix, and one that corresponds to the zone by which it is joined to other conduction cells and that lacks a basal lamina (Thornell et al., 1984; Osinska and Lemanski, 1989; Machida et al., 2002; Peirone and Filogamo, 2004; Shimada et al., 2004). The cytoskeleton of the Purkinje fibers is highly developed, more so than that of the neighboring cardiomyocytes (Thornell and Erikson, 1981): the conduction cells are exposed to great mechanical stress and their integrity is indispensable for the maintenance of both the conduction functions and the distribution of the mechanical stress of the cell with each beat.

Since the advent of heart transplantation, a number of studies have dealt with the effects of ischemia-reperfusion on the myocardium, contributing substantially to the development of techniques for the preservation of this organ. These studies have focused their attention on the cardiomyocytes and the endothelial cells, but not on the Purkinje fibers, possibly because their role has been considered much less relevant for the recovery of myocardial function. Our report addresses this information void in part, describing the changes that these cells undergo following ischemia-reperfusion and under different preservation conditions.

The effects of ischemia on the Purkinje fibers have been analyzed in classical studies (Gornak and Lushnikov, 1967; Friedman et al., 1973; Thornell 1974; Eriksson and Thornell, 1979; Thornell and Ericsson, 1981). Although the methodology employed impedes the extrapolation of these results to heart transplantation, they may be a reference in the assessment of the changes produced in conduction cells subjected to ischemia. Gornak and Lushnikov (1967) observed no changes or necrosis in the conduction fibers after 24 hours of ischemia, in contrast to the findings in the cardiomyocytes. They concluded that this circumstance could be due to a lesser need for oxygen on the part of the former cells because of their lower metabolic activity. Friedman et al. (1973) compared the action potentials between infarcted and non infracted regions in histopathological studies, observing electrophysiological alterations in the absence of structural changes, and Thornell (1974) and Thornell and Eriksson (1981), in their comparison of normal hearts and hearts subjected to ischemia found the conduction cells to have a higher glycogen content than the cardiomyocytes and that the particles had a smaller diameter and a stronger connection to the intermediate filaments of the cytoskeleton, a circumstance that would confer a greater resistance to glycogen depletion.

The results of our study do not corroborate these assertions. We have found that ischemia-reperfusion produces less structural damage in cardiomyocytes, previously described by our group (García-Poblete et al., 1997; Alvarez-Ayuso et al., 2008, 2009, 2010) than in Purkinje cells. The animals in which the latter were best preserved were those of the 24hUW group, whereas the damage was most prominent in the 24hUW+NIC group. The distribution of desmin is not evaluable data since the staining pattern of this protein in normal cardiomyocytes is much less intense than that of conduction cells under the same conditions.

With regard to glycogen content, it was lower in the Purkinje fibers in all the groups studied when compared both to their homologues in the control group and to the neighboring cardiomyocytes.

The discrepancy between our results and those of the studies cited above (Gornak and Lushnikov, 1967; Friedman et al., 1973; Thornell, 1974; Eriksson and Thornell, 1979; Thornell and Ericsson, 1981) could lie in the fact that, in our case, the morphological studies were carried out following reperfusion, a condition in which the reoxygenation of the ischemic tissue originates the maximum expression of cell damage (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).

With respect to the restoration of post-reperfusion electrical activity, the group in which we obtained the best results was that in which the hearts were subjected to the shortest ischemia time, 2 hours, although the best preservation of the cell structure was achieved in the 24hUW group. This discrepancy between the electrophysiological and morphological data could

suggest the existence of underlying functional alterations that are not expressed morphologically, a circumstance that has been reported elsewhere (Friedman et al., 1973).

Ischemia produces a reorganization of the gap junctions and, as a consequence, modifies the conduction velocity in the cardiomyocytes: the gap junctions close and the connexin-43 proteins are dephosphorylated and transferred from the intercalated discs to the cytoplasm and other membrane domains, that is, there is a redistribution (García Dorado et al., 2004; Oosthoek et al., 1993; Shimada et al., 2004; Suarez and Bravo, 2006; Zeevi-Levin et al., 2005).

In the Purkinje fibers, and under normal conditions, these junctions are distributed throughout the entire fiber cell membrane, except in that adjoined to the connective tissue. Under conditions of ischemia-reperfusion, not dealt with in the studies we cite, we have observed a decrease in the overall connexin expression, dispersion of these proteins and their presence in the connective tissue borders of the cells, except in the 24hUW group: in addition to the fact that the decrease in overall connexin expression was less marked in those animals than in the other groups, we detected no connexin-43 signal in the outer borders of the cells. The addition of calcium, nicorandil or trolox to University of Wisconsin solution not only did not result in better preservation of this protein, but was also associated with even more pronounced changes. Moreover, concerning the neighboring cardiomyocytes, the alteration in connexin expression was significantly more marked in the Purkinje fibers in all the groups.

In conclusion, in our experience, the Purkinje fibers are less resistant to ischemia-reperfusion than the myocytes. We detected no correlation between the restoration of cardiac electrical activity and cell damage. Both the cytoskeleton and channel proteins (connexin-43) were better preserved with University of Wisconsin solution, even when subjected to prolonged ischemia. Moreover, the addition of other substances to this preservation solution did not improve the morphological parameters.

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References

Aker S., Snabaitis A.K., Konietzka I., van de Sand A., Böngler K., Avkiran M., Heusch G. and Schulz R. (2004). Inhibition of the Na+/H+ exchanger attenuates the deterioration of ventricular function during pacing-induced heart failure in rabbits. Cardiovasc. Res. 63, 273-282.

Álvarez-Ayuso L., García Gómez-Heras S., Roda J.R., Jorge E., Calero P., Torralba A., Millán I., Fernández-García H. and García-Poblete E. (2008). Calcium-supplemented University of Wisconsin solution in long-term myocardial preservation. Histol. Histopathol. 23, 1103-1110.

Álvarez-Ayuso L., García Gómez-Heras S., Jorge E., Guardiola J.M., Torralba A., Millán I., Roda J.R., Calero P., García-Poblete E. and Fernández-García H. (2009). Supplementation of University of Wisconsin solution with Nitroglycerin and Nicorandil in long-term myocardial preservation: Effects on the oxidative state, endothelial function and morphology. Histol. Histopathol. 24, 1487-1498.

Álvarez-Ayuso L., García Gómez-Heras S., Jorge E., Guardiola J.M., Torralba A., Granado F., Millán I., Roda J.R., Calero P., Fernández-García H. and García-Poblete E. (2010). Vitamin E action on oxidative state, endothelial function and morphology in long-term myocardial preservation. Histol. Histopathol. 25, 577-587.

Castellá M., Buckberg G.D., Saleh S., Tan Z. and Ignarro L.J. (2003). A new role for cardioplegic buffering: Should acidosis or calcium accumulation be counteracted to salvage jeopardized hearts? J. Thorac. Cardiovasc. Surg. 126, 1442-1448.

Cropper J.R., Hicks M., Ryan J.B. and Macdonald P.S. (2003). Enhanced cardioprotection of the rat heart during hypothermic storage with combined Na+ - H+ exchange inhibition and ATP-dependent potassium channel activation. J. Heart Lung Transplant. 22, 1245-1253.

Das D.K. (2003). Attenuation of postischemic myocardial injury by cariporide. J. Thorac. Cardiovasc. Surg. 125, 30-31.

Eriksson A. and Thornell L.E. (1979).Intermediate (skeletin) filaments in heart Purkinje fibers. J. Cell Biol. 80, 231-247.

Ferrera R., Michel P. and Ovize M. (2005). Paradoxical toxicity of cardioplegic compounds on ischemic cardiomyocyte using optimal design strategy. J. Heart Lung Transplant. 24, 904-911.

Friedman P.L., Stewart J.R., Fenoglio J.J. and Wit A.L. (1973). Survival of subendocardial Purkinje fibers after extensive myocardial infarction in dogs. Circ. Res. 33, 597-611.

García-Dorado D., Rodríguez-Sinovas A. and Ruiz-Meana M. (2004). Gap junction-mediated spread of cell injury and death during myocardial ischemia-reperfusion. Cardiovasc. Res. 61, 386-401.

García-Poblete E., Fernández H., Alvarez L., Torralba A. and Escudero C. (1997). Structural and ultrastructural study of the myocardium after 24-hour preservation in University of Wisconsin solution. Histol. Histopathol.12, 375-382.

Gornak K.A. and Lushnikov E.F. (1967). Contribution to the morphology of metabolic processes in the fibers of the cardiac conduction system in a normal state and in myocardial infarction. Cor Vasa. 9, 228-236.

Hool L.C., Di Maria C.A., Viola H.M. and Arthur P.G. (2005). Role of NAD(P)H oxidase in the regulation of cardiac L-type Ca2+ channel function during acute hypoxia. Cardiovasc. Res. 67, 624-635.

Kevelaitis E., Qureshi A.A., Mouas C., Marotte F., Kevelaitiene S., Avkiran M. and Menasché P. (2005). Na+/H+ exchange inhibition in hypertrophied myocardium subjected to cardioplegic arrest: an effective cardioprotective approach. Eur. J. Cardiothorac. Surg. 27, 111-116.

Klass O., Fischer U.M., Perez E., Easo J., Bosse M., Fischer J.H., Tossios P. and Mehlhorn U. (2004). Effect of the Na+/H+ exchange inhibitor eniporide on cardiac performance and myocardial high energy phosphates in pigs subjected to cardioplegic arrest. Ann. Thorac. Surg. 77, 658-663.

Lund L.H., Edwards L.B., Kucheryavaya A.Y., Dipchand A.I., Benden C., Christie J.D., Dobbels F., Kirk R., Rahmel A.O., Yusen R.D. and

Stehlik J. (2013). The Registry of the International Society for Heart and Lung Transplantation: Thirtieth Official Adult Heart Transplant Report-2013; Focus Theme: Age. J. Heart Lung Transplant. 32, 951-964.

Machida S., Noda S., Takao A., Nakazawa M. and Matsuoka R. (2002). Expression of slow skeletal myosin heavy chain 2 gene in Purkinje fiber cells in chick heart. Biol. Cell 94, 389-399.

Mallet R.T. (2005). Hypoxic modulation of cardiac L-type Ca2+ current: interaction of reactive oxygen species and beta-adrenergic signaling. Cardiovasc. Res. 67, 578-580.

Matsui Y., Deleuze P., Kawasaki K., Leandri J. and Loisance D. (1988). Experimental model of heterotopic cardiac transplantation for evaluation of graft viability and function. Eur. Surg. Res. 20, 161-167.

Muraki S., Morris C.D., Budde J.M., Zhao Z.Q., Guyton R.A. and Vinten-Johansen J. (2003). Blood cardioplegia supplementation with the sodium-hydrogen ion exchange inhibitor cariporide to attenuate infarct size and coronary artery endothelial dysfunction after severe regional ischemia in a canine model. J. Thorac. Cardiovasc. Surg. 125, 155-164.

Oosthoek P.W., Viragh S., Lamers W.H. and Moorman A.F. (1993). Immunohistochemical delineation of conduction system.II: The atrioventricular node and Purkinje fibers. Circ. Res. 73, 482-491.

Osinska H.E. and Lemanski L.F. (1989). Immunofluorescent localization of desmin and vimentin in developing cardiac muscle of Syrian hamster. Anat. Rec. (Hoboken) 223, 406-413.

Peirone S.M. and Filogamo G. (2004). Caractérisation immuno-phénotypique des cellules de Purkinje du coeur. Morphologie 88, 90.

Rabkin D.G., Curtis L.J., Weinberg A.D. and Spotnitz H.M. (2005). Na+/H+ exchange inhibition and antioxidants lack additive protective effects after reperfusion injury in the working heterotopic rat heart isograft. J. Heart Lung Transplant. 24, 386-391.

Roda J.R., Álvarez-Ayuso L., Téllez G., Cañas A., Castedo E., Ugarte J. and Castillo-Olivares J.L. (2004). Experimental heterotopic heart transplantation: An easier technique. Eur. Surg. Res. 36, 64-66.

Shimada T., Kawazato H., Yasuda A., Noriaki O. and Sueda K. (2004). Cytoarchitecture and intercalated disk of the working myocardium and the conduction system in the mammalian heart. Anat. Rec. (Hoboken) 280A, 940-951.

Stevens R.M., Salik-Jahania M., Mentzer R.M. Jr and Lasley R.D. (2004). Sodium-hydrogen exchange inhibition attenuates in vivo porcine myocardial stunning. Ann. Thorac. Surg. 77, 651-657.

Suarez J.E. and Bravo A. (2006). Conexinas y sistema cardiovascular. Rev. Argent. Cardiol. 74, 149-156.

Thornell L.E. (1974). An ultrastructural study on glycogen in cow purkinje fibres. J. Mol.Cell. Cardiol. 6, 439-448.

Thornell L.E. and Eriksson A. (1981). Filaments system in the Purkinje fibers. Am. J. Physiol. 241, 291-305.

Thornell L.E., Johansson B., Eriksson A., Lehto V.P. and Virtanen I. (1984). Intermediate filament and associated proteins in the human heart: An immunofluorescence study of normal and pathological hearts. Eur. Heart J. 5, 231-241.

Toledo-Pereyra L.H., López-Neblina F. and Toledo A.H. (2004). Reactive oxygen species and molecular biology of ischemia/reperfusion. Ann. Transplant. 9, 81-83.

Verrier E.D. (2004). Activation of the endothelium in cardiac allografts. J. Heart Lung Transplant. 23, S229-S233.

Vinten-Johansen J. and Mentzer R.M. Jr (2003). Attenuation of postcardioplegia injury with inhibitors of the sodium-hydrogen exchanger. J. Thorac. Cardiovasc. Surg. 126, 1265-1267.

Yarbrough W.M., Mukherjee R., Escobar G.P., Mingoia J.T., Sample J.A., Hendrick J.W., Dowdy K.B., McLean J.E., Stroud R.E. and Spinale F.G. (2003). Direct inhibition of the sodium/hydrogen exchanger after prolonged regional ischemia improves contractility on reperfusion independent of myocardial viability. J. Thorac. Cardiovasc. Surg. 126, 1489-1497.

Zeevi-Levin N., Barac Y.D., Reisner Y., Reiter I., Yaniv G., Meiry G., Abassi Z., Kostin S., Schaper J., Rosen M.R., Resnick N. and Binah O. (2005). Gap junctional remodeling by hypoxia in cultured neonatal rat ventricular myocytes. Cardiovasc. Res. 66, 64-73.

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