

Journal of Clinical and Basic Cardiology

An Independent International Scientific Journal



Journal of Clinical and Basic Cardiology 1998; 1 (1), 37-42

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Ischaemic preconditioning of rat myocardium: effects on postischaemic coronary endothelium hyperpermeability and microcirculatory damage

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Short cycles of ischaemia followed by reperfusion may exert a protective effect on ischaemic myocardial injury. Several pathophysiological mechanisms have been postulated to explain this phenomenon termed preconditioning (P). The aim of this study was to evaluate the effects of P on postischaemic endothelial hyperpermeability, an important factor involved in reperfusion injury and coronary microcirculatory alteration.

Isolated Wistar male rat (250–300 g) hearts were subdivided into three groups: Group A: control hearts subjected to 20 min global normothermic ischaemia; Group B: hearts subjected to preconditioning (three phases of 3 min ischaemia, each one followed by 2 min Langendorff reperfusion) before ischaemia; Group C: hearts subjected to preconditioning and hypertonic reperfusion in order to increase the effects on postischemic interstitial fluid accumulation (osmotic forces balance). A 65 min working heart reperfusion was also performed to assess functional response. Haemodynamic data, reperfusion arrhythmias, heart weight changes (as wet weight/dry weight ratio, ww/dw), myocardial enzyme release, microcirculation permeability changes (FITC-albumin diffusion) and ultrastructural morphometry have been evaluated. In Group B, a significant reduction of reperfusion injury (functional recovery, enzyme release, arrhythmias and ultrastructural morphometry) was detected, compared with Group A. In Group C this reduction was significantly more evident than in groups A and B. In Groups B and C, a significant reduction in myocardial reperfusion oedema (ww/dw: A: 5.9 ± 0.5 ; B: 4.9 ± 1.1 , $p < 0.02$ vs A; C: 4.4 ± 0.6 , $p < 0.01$ vs A) and FITC-albumin diffusion (A: 32.8 ± 5.9 % area; B: 16.3 ± 6.1 % area, $p < 0.01$ vs A; C: 13.3 ± 4.5 % area, $p < 0.01$ vs A), especially in perimyocytic space was also observed.

Data show that preconditioning may reduce endothelial postischaemic dysfunction (vascular permeability and ultrastructural alterations) and reperfusion interstitial oedema. The importance of fluid diffusion within the interstitium in the development of reperfusion damage is supported by better postischaemic recovery in Group C, in which interstitial oedema was reduced by altering intravascular osmotic load. *J Clin Bas Cardiol 1998; 1: 37–42.*

Key words: Preconditioning; endothelium; ischaemia-reperfusion damage; microcirculation; endothelial permeability

Several experimental and clinical studies [1–5] performed in the last ten years have shown the cardioprotective effects of short cycles of ischaemia and reperfusion against reperfusion injury; this phenomenon has been termed preconditioning (P). Various pathophysiological mechanisms have been hypothesised to explain this phenomenon and it is generally thought to have a multifactorial genesis. *In vivo* and *in vitro* experimental models have shown how the myocardial protection exerted by P with regards to ischaemic injury could also be dependent on its effects on the coronary endothelium. A reduced ATP use induced by P has been largely demonstrated, as also proven by the reduced accumulation of adenine nucleotide degradation products (ipoxanthine, xanthine). This lower utilisation of high energy phosphates could be due to the action of a mitochondrial ATPase specific inhibitor produced during acidosis [1, 2] or it could also be caused by myocardial stunning [3, 4], induced by the short ischaemic periods during P. However, stunning seems to be insufficient to cause, by itself, the whole protective effect of P [5]. The Nitric oxide (NO) could mediate the effects of P, mainly by reducing reperfusion arrhythmias and endothelial alterations [6]. However, studies with NO synthesis inhibitors (such as L-NAME) appear conflicting [7]. A rapid expression of a genetic product similar to stress proteins produced during acute ischaemia has also been postulated [8]. Vegh et al. demonstrated that the myocardial protective properties of P could be reduced by administering a cyclo-oxygenase inhibitor, hence postulating a mediator role for prostacyclin [9]. P could also reduce oxygen free radical

production [10, 11] stimulating tissue superoxide dismutase (SOD) activity [12]. The role of the superoxide anion as a NO inactivator [13] could contribute to explaining the P effects. On the other hand, oxygen free radicals could be produced during P and activate several enzymatic systems (NO synthesis, heat shock proteins, 5-nucleotidase activity modulation, protein kinase-C activity) [14, 15]. P may derive from increased adenosine production through greater 5-nucleotidase activity [16, 17] and A₁-receptor interaction [18]. The A₁ purinergic activation leads to translocation of protein kinase-C (PKC) from the cytosol to the membrane, following phosphorylation and ATP-sensitive potassium channel opening, potassium release and membrane hyperpolarisation causing reduced intracellular input of calcium, known to be involved in ischaemia-reperfusion damage [19, 20]. Several sarcolemmal receptors proposed to be PKC-coupled, such as angiotensin II, α -1, bradykinin and endothelin receptors, appear also to be involved in P phenomenon [21, 22]. Recently, a possible protective role of bradykinin has been detected [23, 24]. Ultimately, the lower catabolic ischaemic overload following P [25–28] due to reduced ATP utilisation (slowed neoglycogenesis and anaerobic glycolysis) [29] and degradation of macromolecules, such as glycogen, during P could contribute to explaining this phenomenon [30–32]. In spite of several pathophysiological hypotheses, the possible effects of P upon the coronary microcirculation permeability are not yet clear; the microvascular permeability alterations are endothelial damage markers and also participate in the postischaemic

myocardial oedema genesis by amplifying the reperfusion damage (mechanical dysfunction, no-reflow phenomenon). The aim of this study was to evaluate the effects of P on post-ischaemic alterations of coronary endothelial permeability and following interstitial oedema using an isolated working rat heart model. Our model excludes the interference of haematic pro-inflammatory factors on ischemia-reperfusion damage and focuses on ischaemia-induced coronary endothelial lesion as well as water-electrolyte exchange disturbances and cellular-interstitial osmotic balance.

Materials and Methods

Animals and perfusion technique

Forty-eight adult male Wistar rats were anesthetized with a mixture of ether and air. After injection of 1000 IU of heparin in the femoral vein, hearts were quickly excised, weighed and perfused using the working heart technique [33]. Modified Krebs-Henseleit solution (KH: 108 mM NaCl, 25 mM NaHCO₃, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 11 mM glucose; 287 mOsm) was used as the perfusion medium. The buffer was bubbled in 95 % O₂ and 5 % CO₂, maintained at 37 °C and pH 7.4.

Pre-load (height of atrial chamber) and after-load (height of aortic chamber) were set at 20 and 72 cm H₂O respectively. Aortic and coronary flows (ml/min) were measured by collecting aortic chamber overflow and heart chamber effluent into graduated cylinders. Aortic pressure (mmHg) was monitored through a membrane transducer (TNF-R, Viggo-Spectramed, Oxnard CA) connected to a side arm of the aortic cannula. Heart rate (bpm) and rhythm were determined with an epicardial ECG (Cardioline 350/1, Milan, I). Minute work (joule) was computed as the product of cardiac output (sum of aortic and coronary flows) and peak aortic systolic pressure.

Experimental protocol (see fig. 1)

All rat hearts were divided into 3 groups: Group A, control (n = 15): hearts subjected to 20 min of global ischaemia; Group B, preconditioning (n = 15): hearts subjected to ischaemic preconditioning before ischaemia, by means of 3 x 3 min of global ischaemic periods, each one followed by 2 min of Langendorff reperfusion; Group C, preconditioning + hypertonic reperfusion (n = 15): after preconditioning and global ischaemia hearts were subjected to reperfusion with hypertonic KH solution (hKH, obtained by adding 80 mM Sucrose to KH, 345 mOsm).

Control hearts were subjected to 25 min of working heart stabilization, followed by 20 min of global ischaemia, 5 min of Langendorff reperfusion and 65 min of working heart reperfusion. Groups B and C were subjected to preconditioning during stabilisation. Group C was reperused with hKH from the first minute of Langendorff reperfusion.

Analysis of reperfusion arrhythmias

Heart rate was monitored and paper registered before ischaemia (25 min) and during reperfusion (50, 55, 65, 75, 85, 95, 105, 115). For statistic evaluation an arrhythmic score was utilised according to the Lambeth Convention [34] (0: no arrhythmias, 1: single BEV, 2: couple or salves of BEV, 3: ventricular tachycardias, 4: sustained ventricular tachycardias, 5: ventricular fibrillation).

Heart weight changes

All hearts were weighed before and re-weighed after experimental procedures. Myocardial reperfusion oedema was estimated by per cent heart weight gain (heart weight before/heart weight after ischemia-reperfusion x 100). Four hearts from

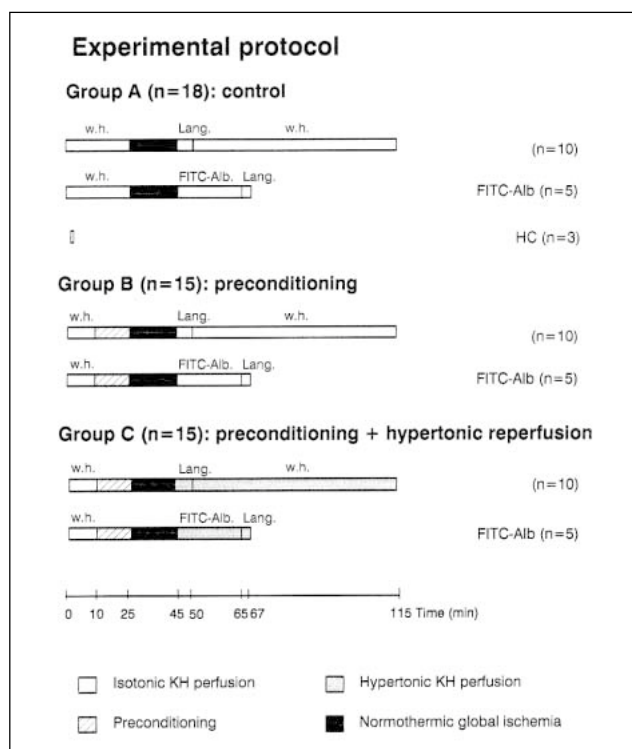


Figure 1: Experimental protocol. w.h.: working heart perfusion. Lang.: Langendorff reperfusion. FITC-Alb.: Fluorescein Isothiocyanate-Albumin. HC: Histologic control.

all groups were dried overnight at 105 °C and re-weighed to determine the wet weight/dry weight ratio (ww/dw).

Enzyme release evaluation

Myocardial necrosis enzyme assay of coronary effluent was performed during stabilisation (10 and 25 min), Langendorff reperfusion (45 min), and working heart reperfusion (55, 65, 75, 85, 95, 105 and 115 min). Creatine kinase (CPK) and lactate dehydrogenase (LDH) activities from effluate samples were determined using commercial kits.

Endothelial permeability

Five rat hearts from groups A and B were subjected, after ischaemia, to 20 min Langendorff reperfusion with 75 mg FITC-albumin (Sigma, Milan, I) dissolved in 200 cc KH to assess the microvascular permeability changes. Hearts were then reperused with KH Langendorff for 2 min, in order to eliminate intravascular fluorescence and re-weighed. Five rat hearts from group C were subjected to the same procedure but with hKH Langendorff reperfusion. Microvascular permeability changes were determined on FITC-albumin hearts by means of fluorescent microscopy. Ventricles were cut transversally into 4-5 blocks. Tissue blocks were immediately embedded in medium (O.C.T. Compound, Miles, Elkhart, IN) and stored at -80 °C. Tissue blocks were mounted on a specimen holder in a Slec microtome-cryostat and maintained at -35 °C and orientated so that capillaries and muscle fibers could be cross-sectioned. Ten 5 μm sections were obtained from each tissue block and placed on a labeled slide, which was pre-warmed on a hot plate. The slides were immediately returned to the hot plate to dry, placed in a dark box for at least 1 hour and then viewed and photographed at 40 x under fluorescent light. FITC-albumin extravasation was quantified per section using an image analysis system. Results were expressed as follows: area of FITC-albumin diffusion/total area of myocardium x 100. A site diffusion analysis in interstitial

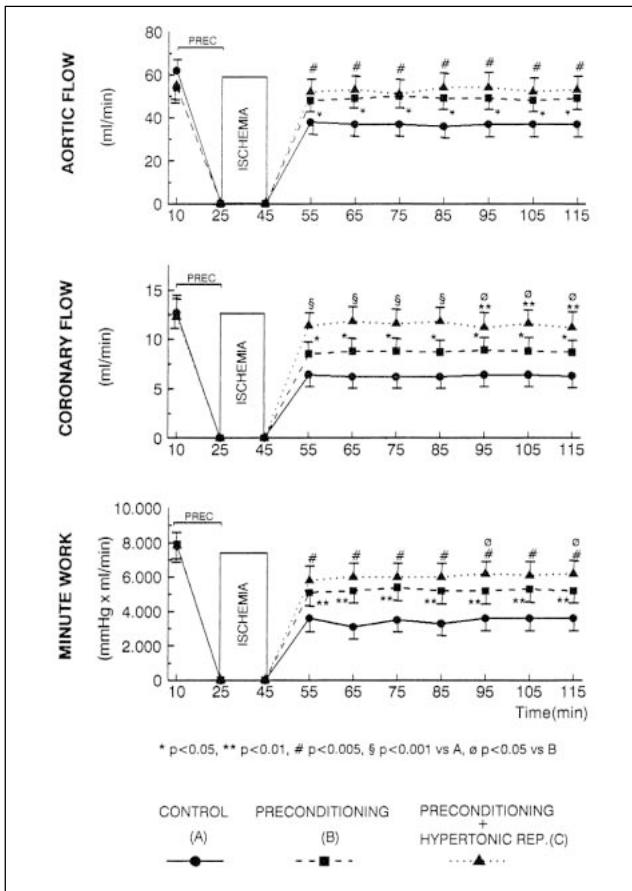


Figure 2: Haemodynamic parameters (aortic flow, coronary flow, minute work). Data are expressed as mean \pm SD.

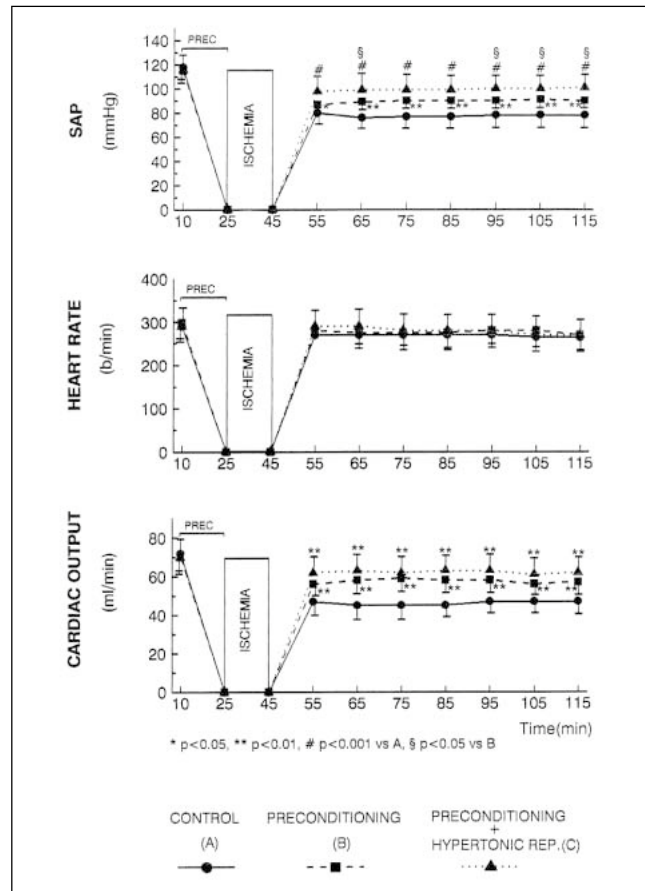


Figure 3: Haemodynamic parameters (systolic aortic pressure, heart rate, cardiac output). SAP: systolic aortic pressure. Data are expressed as mean \pm SD.

space (perivascular and perimyocytic areas) was also performed and expressed as percentage value of total FITC-albumin diffusion area.

Ultrastructural evaluation

Myocardial specimens were fixed in 2.5 % glutaraldehyde for at least 3 hours. Specimens were post-fixed in 1.33 % osmic acid, dehydrated in a graded ethanol series and embedded in Epon 812 Resin. Subsequently, thin sections were carried out (30–40 sections/specimens) stained with uranyl acetate and lead citrate, and studied by electron microscopy. Three rat hearts were used as a histological control. An average of 30 fields in each section were examined and photographed at 4.500 x.

Statistical analysis

Results are expressed as mean \pm SD. Statistical analysis was performed using Student's t test and two-way analysis of variance (ANOVA) where indicated. Probability of null hypothesis of < 5 % ($p < 0.05$) was considered statistically significant.

Results

Haemodynamic parameters

Functional responses to ischaemia and effects of P alone or associated with hypertonic reperfusion are reported in Figure 2 and 3. In isolated working heart, ischaemic preconditioning produced a significant improvement of all functional parameters, particularly, if associated with hypertonic reperfusion; coronary flow was significantly higher in B and C groups with

respect to controls (at 55 min, group A: 7 ± 2.1 ml/min; group B: 8.3 ± 1.8 ml/min, $p < 0.05$ vs A; group C: 11.3 ± 2 ml/min, $p < 0.001$ vs A and $p < 0.05$ vs B). A similar trend was observed in all analysed parameters. Heart rate was not significantly different between all groups before or after ischaemia (Fig. 2).

Release of myocardial necrosis enzymes

Enzyme release (CPK and LDH) in coronary effluent is reported in Figure 4. In group B and C a significant reduction of CPK and LDH release was observed in the coronary effluent during Langendorff reperfusion (at 45 min, CPK: group A: 47 ± 5.3 UI/g ww; group B: 25 ± 5.4 UI/g ww, $p < 0.01$ vs A; group C: 16.2 ± 6 UI/g ww, $p < 0.002$ vs A, $p < 0.05$ vs B). During working reperfusion enzyme release was similar in all groups.

Reperfusion arrhythmias

Arrhythmic score is reported in Figure 5. In control hearts a significantly elevated score was observed, because of 20 % of irreversible ventricular fibrillation. Between group B and C arrhythmic score was similar and no ventricular fibrillation was demonstrated.

Postischemic heart weight changes (myocardial oedema)

In group A ischemia produced a significant increase of heart weight (group A: $+ 39.7 \pm 4$) significantly reduced by preconditioning (group B: $+ 27 \pm 4$ %, $p < 0.05$ vs A); in group C the reduction of postischemic myocardial edema was even more evident (group C: $+ 14 \pm 5$ %, $p < 0.002$ vs A). These data were confirmed by ww/dw ratio (Group A: $5.9 \pm$

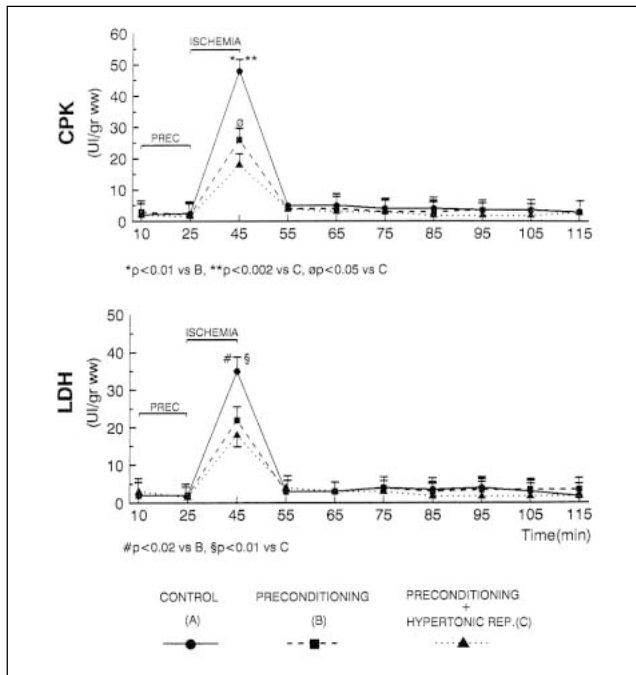


Figure 4: Time-course of enzymatic release in coronary effluent (CPK, LDH). Data are expressed as mean \pm SD.

0.5; Group B: 4.9 ± 1.1 , $p < 0.02$ vs A, Group C: 4.4 ± 0.6 , $p < 0.01$ vs A).

Endothelial permeability changes

In group A a significant postischemic FITC-albumin diffusion in perivascular and perimyocytic spaces (% Area: 32.8 ± 5.9) was detected. In group B albumin diffusion was significantly reduced (% Area: 16.3 ± 6.1 , $p < 0.01$ vs A) especially in perimyocytic spaces. This phenomenon was slightly more

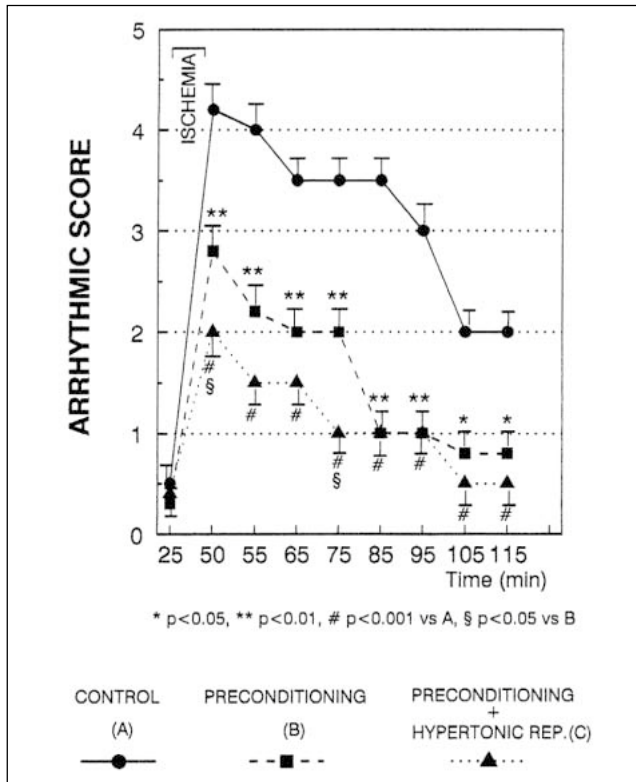


Figure 5: Arrhythmic score.

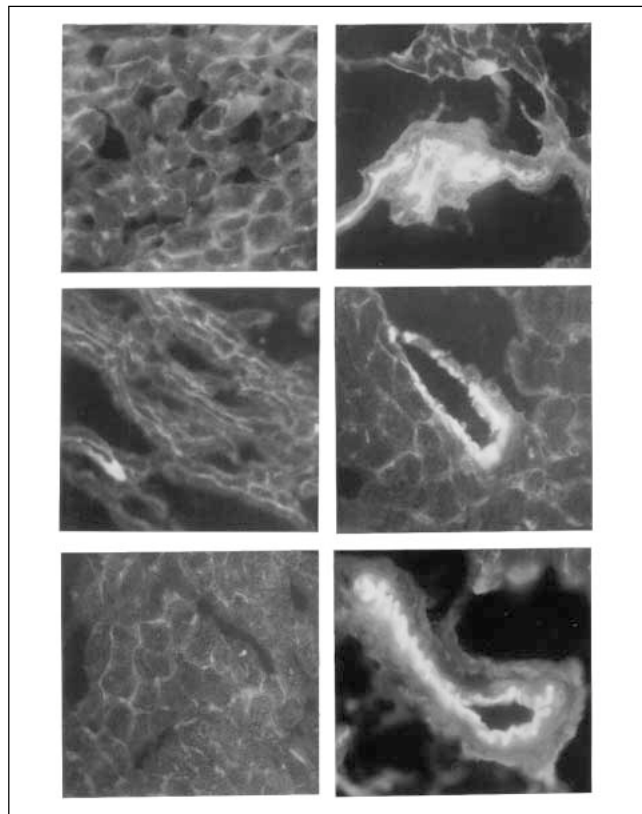


Figure 6: Histological samples (200X, fluorescent light) showing the FITC-Albumin diffusion in perimyocytic space (left) and perivascular space (right). Top: control. Middle: preconditioning. Bottom: preconditioning + hypertonic reperfusion. In control group fluorescence is strongly diffused around myocytes and perivascular space. After ischaemic preconditioning, albumin extravasation is limited; adding hypertonic reperfusion FITC-albumin diffusion is detected in the only endothelial surface of blood vessels (bottom, on the right).

evident in group C (% Area: 13.3 ± 4.5 , $p < 0.01$ vs A) where preconditioning was associated with hypertonic reperfusion (Fig. 6).

Ultrastructural damage

An increase of interstitial area (interstitial oedema), especially in the perivascular space was observed in group A in comparison with group B. Addition of sucrose to KH (C group) resulted in a more evident reduction of oedema in comparison with group A and B.

Morphological evaluation of group A demonstrated signs of ischaemic damage in terms of areas of necrosis with disruption of normal interfibril geometry, swollen cells and mitochondria, with severe alterations of the cristae. Endothelial damage was also evident in terms of swelling and membrane disruption. The damage was decreased in group B, with preserved interfibril geometry and slight focal mitochondrial swelling. Endothelium was mostly preserved with small areas of micropinocytosis. In group C there was a significant further decrease in damage in comparison with group B, with no significant fibril and mitochondrial injury .

Discussion

The preconditioning phenomenon certainly represents one of the most interesting chapters of myocardial ischaemic pathophysiology. The discovery of its protective effect on ischaemia-reperfusion at the beginning of the 80's has been in time

supported by several animal [1, 25, 27, 28, 35] and human [36, 37] experimental studies. As already mentioned, the sequence of pathological stages leading to the cardioprotective action of P has yet to be completely proved. The results of our studies suggest that P may produce its protective effect towards reperfusion myocardial damage by reducing the post-ischaemic endothelial alterations by affecting the microvascular permeability and therefore the interstitial myocardial oedema development.

Functional alteration and cellular damage: Our analysis of functional changes and enzymatic release confirms the protective role of P in reperfusion damage genesis. In literature, this result is widely reported in several animal and human models [4, 38, 39]. Although this effect has been variously explained, it could depend upon the reduction of the postischaemic myocardial oedema, which is one of the factors involved in the reperfusion injury. Several studies show that ischaemia is able to induce a myocardial oedema which is directly related to the ischemic damage [12, 15, 17, 38]. Moreover, the established oedema results in further alterations of the capillary-myocyte exchange, and finally it increases the ischaemic damage [38]. However, our experimental model also shows a slight degree of interstitial oedema following normal basal perfusion. Our data [25] demonstrated an 8 % heart weight increase after 100 min of working heart perfusion technique, nevertheless the aforementioned increase was unable to induce a significant functional and ultrastructural myocardial impairment and it was likely to be due to the intrinsic characters of perfusion buffer (as isotonic and isoionic but obviously hyposmotic buffer).

Reperfusion arrhythmias: Our study has also shown a significant arrhythmic score reduction in preconditioned and in particular hypertonic reperfused hearts. This reperfusion arrhythmia reduction after P is variously reported in literature with differing pathophysiological bases. Several studies highlight G proteins [40], adrenergic stimulation [41], nitric oxide [41], oxygen free radicals [42], ATP-dependent potassium channels [43], glycogen depletion [44] and prostacyclines [6] as factors involved in this mechanism; however, the results are conflicting [43, 45]. Our experimental model shows the lowest frequency of reperfusion arrhythmias in hearts subjected to P and hypertonic reperfusion. It is deduced that arrhythmias may be due to the endothelial alteration, and therefore, to the degree of interstitial oedema.

Endothelial function: In recent years endothelial functions have been taking on more importance in our understanding of the genesis of reperfusion damage. The endothelium is considered to be not only a simple blood-interstitium barrier but also a real organ with several endocrine and haemostatic functions able to regulate *in situ* circulating flow and blood-interstitial space-myocytes exchanges [38]. It is one of the first structures to be injured during ischaemia-reperfusion damage as the target of neutrophil infiltration, oxygen free-radical injury and other factors responsible for ischaemia-reperfusion damage; therefore, its importance is comparable to the well known myocyte damage. Hearse et al. [38] have demonstrated that myocytic damage depends on an early ischaemic endothelial injury and its flow regulation properties. Moreover, it is to emphasise its role during reversible ischaemia and myocardial infarction [3, 4, 38]. In the isolated working heart rat model we considered vascular permeability changes, through FITC-albumin diffusion into myocardial interstitial spaces, as impaired endothelial function index. According to the Fick and Starling laws regulating the fluid diffusion between intravascular and interstitial spaces in physiological state, there is slight diffusion of labeled albumin into interstitial space which is drained through venous and lymphatic systems. This process

is severely and quickly altered during ischaemia proportionally to pathophysiological endothelial damage [3, 38]. Albumin accumulation was first observed into perivascular space, and then into perimyocytic space related to higher oedema degree. This perymyocytic oedema acts as a muff around myocytes by significantly altering ventricular systo-diastolic properties, as confirmed by haemodynamic parameters. This diffusion was significantly reduced in preconditioned hearts. Moreover, preconditioned and hypertonic reperfused hearts have shown further reduction of interstitial oedema related to an improvement of functional parameters and lower cellular damage. This reduced diffusion is due to sucrose responsible for hypertonic action of the perfusion buffer; this agent has no scavenger activity unlike mannitol, widely utilised in other experimental studies [3, 18, 42, 43, 46]. Several studies demonstrated that P could prevent endothelial dysfunction following ischaemic injury by direct or mediated mechanisms. De Fily and Chilian [47] reported that P reduces postischemic alterations of endothelial reactivity in *in situ* hearts; a similar experimental protocol has been also utilized by Tsao et al. [39]. However, these studies evaluated vascular reactivity as endothelial dysfunction index. Some authors studied the effects of endothelial NO [7], adenosine [9], energetic state and oxidative stress [4], platelet aggregation [48], neutrophilous adhesion and infiltration [49] on coronary endothelial function during P; on the other hand, many of these factors exert a direct effect on vascular permeability. In our experimental model, the contribution of blood cells and plasmatic proteins on endothelial dysfunction genesis has not been evaluated. Therefore, endothelial permeability changes shown in our model could be mainly due to endothelial alterations (structural lesions, altered NO synthesis, adenosine, tissue kinines) and cellular and interstitial osmotic gradients [50].

Finally, our data showed that P, in an isolated working rat heart, is able to significantly reduce ischaemia-reperfusion damage, at least in part, by reducing coronary endothelial post-ischaemic and microvascular permeability alterations. Although the pathophysiology of this phenomenon is certainly multifactorial, the results of our study point out the importance of microvascular permeability changes and following interstitial oedema in the development of functional and myocytic damages and reperfusion arrhythmias.

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