

MOLECULAR, HISTOPATHOLOGICAL AND ULTRASTRUCTURAL STUDIES OF LANGENDORFF RAT HEART. THE IMPACT OF ISCHEMIC PRECONDITIONING

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Abstract

The potential clinical relevance of ischemic preconditioning (IP) on ischemia-reperfusion injury is a major object of clinical research to find new methods of cardioprotection in humans. The aim of our study was to characterise the Langendorff model of rat heart investigating different durations of ischemia and reperfusion and (IP) protocol on myocardial ischemia reperfusion injury, to test the potential clinical relevance of (IP) phenomenon on infarct size, to assess genomic DNA integrity and histological and ultrastructural changes in heart muscle. Our results revealed that as ischemia and reperfusion time is extended, the level of infarction is also increased. We concluded that 20 minutes stabilisation and 45 minutes ischemia followed by 30 minutes reperfusion seems to provide a compliant platform to study ischemia reperfusion injury. Protection using (IP) was induced by 2 cycles 5` ischemia and 5` reperfusion prior to lethal ischemia reperfusion protocols. The reduction in infarct size in IP hearts pointed out that inhibition of apoptosis is one of the mechanisms which participate in eliciting the increased resistance to ischemia.

Key words: Langendorff rat heart, ischemia reperfusion, ischemic preconditioning (IP), infarct size, apoptosis, necrosis.

INTRODUCTION

It has been established that atherosclerosis a systemic process is the main factor implicated in the onset of cardiovascular and cerebrovascular disease; it affects vascular territories especially at the branch: of coronary tree with risk for myocardial infarction (MI) as well as carotidian tree with risk for stroke (Revnic et al., 2007).

Cardiovascular disease is the leading cause of death in civilised world, over 3.8.million men and 3.4 million women die annually because of the coronary artery disease (Yellon et al., 2007; Marin et al., 2018b).

The number of known risk factors associated with the development of cardiovascular disease (CVD) are ever increasing and currently include: hypertension (Marin and Goga, 2018a), hypercholesterolemia (Revnic et al., 2007), increased low density lipoprotein (LDL), decreased high density lipoproteins (HDL), obesity, diabetes mellitus, (Revnic et al., 2007), smoking and physical inactivity (Revnic et al., 2007).

Severe abrogation of blood supply (ischaemia) to a region of the heart can cause cell death, which is known as myocardial infarction (MI); this is detrimental and can induce tissue damage and consequently the initiation of ischaemic heart disease.

Clinicians often observed this condition in patients who develop angina, which can be caused by the narrowing or obstruction of coronary arteries due to debris, such as a thrombus and/or atherosclerotic plaque formation. Ultimately, this ischaemic period initiates the process of tissue damage that is associated myocardial infarction (MI) (Revnic et al., 2007). Reperfusion of the ischaemic area is required to prevent further tissue damage however, paradoxically; the act of reperfusing the ischaemic area can itself induce additional damage and can affect cardiac function thereafter damage is referred to as ischaemia-reperfusion or reperfusion-induced injury work load and minimise ischaemia-reperfusion injury.

Current best practice for the treatment of patients suffering with a MI is to restore blood

flow using thrombolytic therapy or to use surgical interventions, such as balloon catheter angioplasty or coronary artery bypass surgery (Laskey et al., 2005). While these interventions have proven successful, some are invasive and may result in further tissue damage and reoccurrence of MI later in life.

Therefore, alternative strategies to enhance tissue viability post MI are required to help address this unmet clinical need (Dauterman and Topol, 2002; Ferdinandy et al., 2007). Much research is directed at investigating the cellular pathways involved in ischaemia-reperfusion injury and the cell death resulting in the formation of infarcted tissue. The period of ischaemia and the reperfusion of an ischaemic area, together, contribute to cell death and to the severity of myocardial injury (Hausenloy et al., 2005).

Myocardial ischemia-reperfusion (IR) injury may result from pathological processes such as atherosclerotic coronary artery disease and acute myocardial infarction and/or be secondary to surgical processes such as operating on the arrested heart or cardiac transplantation.

In contrast to “ischemic injury” which occurs when oxygen demand exceeds the available blood supply and is associated with cell necrosis, IR injury occurs upon return of blood supply after a period of ischemia and is usually associated with apoptosis (i.e. programmed cell death).

When compared to endothelial cells (ECs), cardiomyocytes (CMs) are more sensitive to ischemic injury and have received the most attention in the quest for preventing myocardial IR injury.

However, several studies suggest that ECs are more sensitive to IR injury than CMs and that they might be a critical mediator of IR injury in the heart (Arun et al., 2010).

Patients succumbing after developing this syndrome generally had infarcts of over 30% of the left ventricular mass at autopsy (Bolli et al., 2004). Therefore, reduction in infarct size in the presence of coronary occlusion is potentially of great significance.

Recently it has been shown that necrosis following experimental myocardial ischemia can be reduced by pharmacological or hemodynamic factors. Myocardial ischemia-reperfusion injury occurs upon return of blood supply after a period of ischemia and is usually

associated with apoptosis. Cell-cell interactions between blood cells and vascular endothelial cells and the release of cytokines and generation of reactive oxygen species from activated neutrophils (PMNs), endothelial cells and myocytes during reperfusion have been proposed as triggers in the induction of apoptosis. These interactions are initiated within the early movements of reperfusion, and may continue for hours and days. Apoptosis is a process of programmed cell death and is under strict genetic control, the apoptotic stimuli determine a change in cell suppressive influences, manifested by biochemical modifications that include activation of proteases and nucleases (Revnic et al., 2007).

The phenomenon of ischemic preconditioning has been recognised as one of the most potent mechanisms to protect against ischemia reperfusion injury. Preconditioning of the myocardium with short episodes of sublethal ischemia will delay the onset of necrosis during a subsequent lethal ischemic insult. It seems to involve a variety of stress signals which include activation of membrane receptors and signalling molecules such as PKC, mitogen activating protein kinases, opening of ATP sensitive K channels and expression of many protective proteins (Revnic et al., 2009).

Efforts to prevent ischemic injury have focused on finding ways to block events associated with irreversible ischemic injury. In 1986, Murry et al. (1986) described a classic phenomenon termed (IP) for the first time, as “multiple brief ischemic episodes that might actually protect the heart from a subsequent sustained ischemic insult”. It was originally thought that each ischemic episode caused a cumulative ATP depletion.

While intermittent reperfusion would wash out the ischemic catabolites, surprisingly levels were not depleted by subsequent ischemic challenges and no infarct occurred. This observation led the same scientist group to test the hypothesis that the preservation of highly energy phosphate was due to a slowing consumption during ischemia associated with a rapid and protective adaptation of the myocyte. They tested the hypothesis by subjecting the myocardium to a series of 45 minutes coronary branch occlusion each separated by 5 minutes of reperfusion. This rendered the myocardium

more resistant to a subsequent sustained 45 minutes ischemic insult; the infarct size has been reduced to 25% of that seen in control group.

This phenomenon is called preconditioning with ischemia; the classic IP is short lived and fast decayed with antiischemic effects disappearing completely within 2 hours.

The evolution of necrosis is delayed but not prevented; precondition will limit infarct size during a temporary coronary occlusion, but not during a prolonged or permanent occlusion.

The stimulus for preconditioning is a critical reduction in myocardial blood flow and the end point is infarct size; the optimal duration in ischemia is species dependent also the cellular basis of the mechanism underlined preconditioning is not fully understood. Preconditioning results in activation of a number of receptors such as: adenosine (Cohen et al., 2008), alpha adrenergic, delta opioids and bradikinin (Gross et al., 2008). Preconditioning stages can be applied prior to a planned procedure involving a potentially injurious ischemic insult.

Ischemic preconditioning provides a degree of protection against myocardial ischemia-reperfusion by reducing the number of myocytes damaged by the above mentioned mechanisms and improved ventricular ejection fraction after reperfusion a clear relationship between preconditioning and apoptosis after myocardial ischemia-reperfusion has not been established yet.

One promising approach which considerably decreases infarct size following coronary occlusion as demonstrated by enzymatic and histologic criteria is ischemic preconditioning either mechanically or pharmacologically.

Ischemic preconditioning stands virtually alone in its ability to limit infarct size in the controlled setting of the experimental laboratories. Understanding the basic mechanisms of ischemia reperfusion injury is critical to developing clinically applicable strategies to minimize myocardial reperfusion injuries.

Objectives of paper:

1.To characterise the Langendorff model of rat heart using different durations of ischemia and reperfusion from the ischemic precondition protocol (IPC) assessing the myocardial ischemia reperfusion injury.

2.To test the potential clinical relevance of ischemic preconditioning (IP) phenomenon on the final infarct size, (the golden standard).

3.To assess genomic DNA integrity, the histological and ultrastructural changes of ischemic-reperfused myocardium to get insights into molecular mechanisms of IP to limit infarct size.

MATERIALS AND METHODS

Biological material

This study was conducted on 25 white male Wistar rats aged 12 months old, weighting between 250-300g, kept in standard laboratory conditions, divided into five groups of 5 rats each: group 1- control, group 2 - 45 minutes ischemia followed by 30 minutes reperfusion, group 3 - ischemic preconditioning (2 cycles of 5min of ischemia followed by 5 minutes reperfusion) applied prior to 45 minute ischemia and 30 minutes reperfusion, group 4 - 5 minutes ischemia followed by 120 minutes of reperfusion, group 5 - preconditioning (5 minutes of ischemia followed by 5 minutes reperfusion applied prior to the 45 minutes of ischemia and 120 minutes reperfusion.

The animals were anesthetized i.p. with sodium pentobarbital (60 mg/kg) and then received heparin (300 U IP).

After the reflexes were abolished, hearts were excised and placed in perfusion medium on ice and then were quickly mounted in retrograde Langendorff perfusion system (Fig. 1) at a constant pressure (75 mm Hg) according to the published protocol (Revnicek et al., 2005).

In order to avoid excessive hydration due to crystalline solution infusion, hearts were immersed in the perfusion liquid.

The perfusion liquid was the Krebs Henseleit bicarbonate buffer with the following composition (nmol/l) NaCl 118.5, NaHCO₃ 25, KCl 4.8, MgSO₄ 1.2, KH₂PO₄ 1.2, CaCl₂ 1.7, Glucose 1.2, and pH is 7.4 (Fig.2).

Ischaemia-reperfusion injury: analysis of infarct size

At the end of the reperfusion period, hearts were taken off the perfusion apparatus and perfused, through the aortic cannula, with 1% pre-warmed triphenyltetrazolium chloride (TTC) which acts as a viability dye.

Hearts were subsequently immersed in TTC and incubated at 37°C for 10 min, after which they were weighed and frozen at -20°C for 24h. While still frozen, hearts were sliced from base to apex at a thickness of ≈1mm; the slices were fixed in 10% formalin for 12h to define the stain borders.

The TTC is a redox indicator and can stain areas of viable myocardium red; this is because viable cells (with intact membranes) retain the dye where it can react with dehydrogenases and NADPH.

In the presence of oxygen these enzymes can reduce TTC, causing a conversion into a red colour (Klein et al., 1981).

Areas of tissue containing non viable/infarcted cells have lost their dehydrogenases, due to wash out in reperfusion, and are unable to retain or convert the dye and therefore emerge unstained and white in appearance.

Heart slices were then photographed on a perspex mounting block using a digital EsKape (EsKape, NY, USA) fixed camera.

NIH Image 1.63 software was used to calculate the infarcted areas and the results were expressed as a % of the whole heart at risk of damage induced by ischaemia-reperfusion injury (I/R%) and presented as means ± standard error of the mean (SEM).

A t-test or a one way ANOVA test were used to assess the differences between groups, as described in the statistics section further in this chapter. The results were considered significantly different when $p \leq 0.05$.

Detection of Genomic DNA Fragmentation

For detection and qualitative evaluation of DNA fragmentation, we examined whether genomic DNA isolated from ischemic hearts from groups: 1, 2, 3, 4, and 5 produced a typical "ladder" pattern (≈180-bp multiples) when analyzed on an agarose gel DNA fragmentation was investigated using the DNA laddering kit Cat.Nr.TA 4630, R&D Systems England according to the published method (Revnicek et al., 2004) in all 5 groups of rats.

Methods

TACS apoptotic DNA laddering kit was used for the evaluation of the left ventricular apoptosis by internucleosomal DNA fragmentation and appearance of the DNA laddering.

During apoptosis, the endonucleases produce double chain breaks in DNA, generating DNA fragments, these fragments are isolated from the tissue and optimized.

TACS DNA isolation reagents produce the inactivation of the endogenous nucleases, and the DNA fragments with different molecular weight are rapidly recovered.

The isolated DNA is fractionated according to size by gel electrophoresis and visualized Etd.Br.

Experimental Procedure

Cardiac tissue (left ventricle) of rat hearts from: group 1- controls, group 2 - 45 minutes ischemia followed by 30 minutes reperfusion, group 3- ischemic preconditioning (5 minutes of ischemia followed by 5 minutes reperfusion) applied prior to 45 minutes ischemia and 30 minutes reperfusion, group 4 - 45 minutes ischemia followed by 120 minutes of reperfusion, group 5 - ischemic preconditioning (5 minutes of ischemia followed by 5 minutes reperfusion applied prior to the 45 minutes of ischemia and 120 minutes reperfusion was chopped into small pieces and frozen in liquid nitrogen, then 1-2 g frozen tissue was ground to a powder was obtained which was then re-suspended in 200 µl buffer.

20µl of 10X buffer was added and incubated at 50 C for 12-18 hours with mild shaking.

At 100µl tissue suspension, 100µl lytic solution from TACS apoptotic DNA Laddering Kit was added.

DNA isolation was done in accordance with the instructions in the user guide. We used 1 µl DNA that was diluted in distilled water without DNase.

DNA electrophoresis was done according to the protocol described in the user manual of the kit, and then the gel was stained for 15 minutes with 0.5 µg/l EtidiumBromide.

DNA visualization was performed using a transluminal UV photographs that were taken with yellow filter 22A KodakWratten.

For optical microscopy, myocardial transmural sections from groups 1, 2, and 4 were fixed in formaldehyde (100 ml/L) inclusionated in paraffin. After fixation, frontal sections of the heart, including the ventricles and interventricular septum, were embedded in paraffin (Samir, 2015). Sections were cut at 5

µm and stained with hematoxylin and eosin, periodic acid-Schiff, and Gomori's Trichrome. Sections were scored in a blinded fashion by a veterinary pathologist, using the method as follows: grade 0, no lesions; grade 1, focal lesions of the subendocardial portion of the apex and/or the papillary muscle, composed of fibroblastic swelling or proliferation and accumulation of histiocytes; grade 2, focal lesions extending over a wider area of the left ventricle with right ventricular involvement; grade 3, confluent lesions of the apex and papillary muscles, with focal lesions involving other areas of the ventricles and the auricles; and grade 4, confluent lesions throughout the heart, including infarct-like massive necrosis, with occasional acute aneurysm, myocardial function after ischemia-reperfusion and cut into sections of 5 µm thick and coloured with hematoxylin-eosine.

For electron microscopy studies were collected samples from the anterior and posterior papillary muscle from the same groups (1, 2, 3 and 4), the tissue was fragmented in 1 mm fragments that were fixed in glutaraldehyde (30 ml/L), in 0.1 ml/L of Na cacodilat buffer, pH 7.4, then fixed in 10 g/L osmium tetroxide. Dehydration was done using varying degrees of alcohol, after that the inclusion in Spurr resin with low viscosity was done. Sections were made with Sorval Potter Blum ultramicrotome, using a diamond knife. These sections of 80 nm were collected on copper grids, stained with Uranyl acetate Lead citrate according with the published method and examined in a EM Philips 200 la 60 keV electron microscope for presence or absence of glycogen, interfibrillar edema, nuclear changes, wide I bands, intramitochondrial amorphous dense bodies and breaks within the sarcolemal membrane (Revnic et al., 2002).

RESULTS AND DISCUSSIONS

Study 1: Langendorff control ischemia-reperfusion protocol

Prolonged and unresolved regional myocardial ischemia without reperfusion leads to myocyte death. Although the early restoration of blood flow to the ischemic myocardium is necessary to salvage myocytes from eventual death, abundant evidence indicates that reperfusion

after even a brief period of ischemia has additional deleterious effects on the ischemic myocardium that are not expressed during ischemia. IP refers to a process in which a brief, reversible period of ischemia followed by reperfusion enhances myocardial resistance to a subsequent longer period of ischemia. In every animal species studied, IP has produced the most reproducible reduction in experimental necrosis, studies have been undertaken to confirm its protective effect and possible mechanisms involved since Murry et al. first described this endogenous protective mechanism in limiting infarct size (Murry, 1986).

The evaluation of infarct size

Infarct size was measured as the percentage of infarction to risk area (I/R %), at the end of the reperfusion period, using triphenyltetrazolium chloride (TTC) (white areas represents the infarct and red areas the viable myocardium. Infarct size (I/R% measurement using TTC staining).

Infarction and duration of ischemia reperfusion

Further optimisation experiments were performed using Langendorff rat heart mode in which the hearts were subjected to various durations of ischemia and reperfusion. 35' ischemia was associated with an augmented infarct size to $39.23 \pm 3.5\%$ and 45' global ischemia was associated with an augmented infarct size to $50.26 \pm 2.61\%$, $p < 0.05$.

The infarct size developed in the risk area subsequent to 20 minutes stabilisation and 45' ischemia followed by either 30 minutes of reperfusion or 120 minutes of reperfusion showed a significant increase (60.62 ± 3.16) at 120 minutes. These results account for the negative effect of duration of reperfusion on myocyte. IP consisting of 2 cycles of 5' ischemia and 5' reperfusion applied prior to 45' ischemia and 30 minutes reperfusion significantly reduced the infarct size to $28.24 \pm 2.3\%$ and to $45.24 \pm 2.42\%$ in hearts with 45' global ischemia and 120 minutes reperfusion.

Signal transduction pathways involved in the induction of apoptosis

It is generally accepted that the process of apoptosis involves the activation of death

receptor -dependent and -independent signal transduction pathways.

The binding of pro-apoptotic ligands to their receptors initiates a process that results in an imbalance in regulating proteins (i.e. Bcl-2 family) and an activation of cytosolic proteases (i.e. caspase family) (Revnic et al., 2007).

It has been confirmed that the change in status of caspases from the inactive to the active form by both stimulating pathways is the key step to induction of apoptosis.

Study 2: Evaluation of apoptosis by detection of genomic DNA fragmentation

Apoptosis is a programmed process that develops simultaneously with necrosis principally during reperfusion, but with a time-course that is slower (days) than the development of necrosis (hours). Several mechanisms trigger apoptosis after ischemia and reperfusion: the generation of cytokines and reactive oxygen substances from endothelium, myocytes or cell-cell interactions between inflammatory and endothelial cells, imbalance in regulation of anti-apoptotic and pro-apoptotic proteins; activation of downstream caspases (Revnic et al., 2007), and the release of cytochrome C from mitochondria. Stimulation of PKC isozymes and the opening of mito- K_{ATP} channels have been shown to be associated with a reduction in apoptosis in addition to necrosis (Revnic et al., 2009).

DNA nucleosomal fragmentation of myocyte in non-ischemic control group 1 is no visible DNA 'ladders' were found. In contrast, genomic DNA isolated from the ischemic zone produced a typical 'ladder' pattern from all 5 animals after ischemia and reperfusion (Fig. 1a). The presence of 'ladder' pattern (line 4') in the hearts subjected to ischemia for 45 minutes and 120 minutes reperfusion; hearts subjected to two cycles of 5 minutes ischemia followed by 5 minutes of reperfusion before global ischemia of 45 minutes followed by reperfusion for 120 minutes did not show 'ladder' pattern.

We have found that in a rat model of ischemia and reperfusion, two cycles of 5 minutes of IP preceding 45 min global ischemia significantly reduced the intensity of DNA ladders in the area at risk in preconditioned myocardium.

Myocardial ischemic preconditioning techniques demonstrated the decrease of the

destruction of myocytes by cell apoptosis following ischemia-reperfusion process, this effect is shown by reducing DNA fragmentation in the group of rats that received ischemic preconditioning compared to those which have not received ischemic preconditioning.

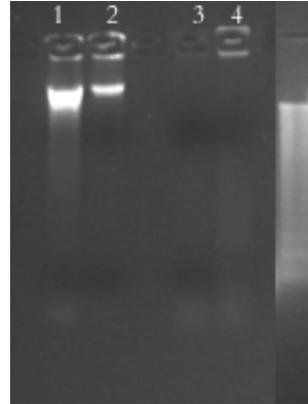


Figure 1a. DNA gel electrophoresis pattern of rat heart from groups: 1, 4, 5, subjected to 45 minutes ischemia and 120 minutes reperfusion and IP. Control-group -1, group 4 with 45 minutes ischemia and 120 minutes reperfusion, group 5 with 45 minutes ischemia and 120 minutes reperfusion and ischemic preconditioning

Ischemic preconditioning reduces the myocardial apoptosis after ischemia-reperfusion by several factors including PKC isoforms, reduced inflammation and oxidative stress and K_{ATP} .

IP induces a biphasic pattern of myocardial protection. Following the acute phase of protection by early IP, a delayed phase, termed the 'second window of protection' appears between 12 and 24 h after the initial IP stimulus, which lasts up to 72 hours. Ischemia-reperfusion induces myocardial apoptosis and this is mediated by translocation of PKC δ in mitochondria and activation of apoptotic effectors implying Cytochrome C liberation and activation of Caspase 3 (Revnic et al., 2007) and fragmentation of DNA. K_{ATP} channels have been implicated in myocardium recovery following ischemic aggression. It has been suggested that phosphorylation of K_{ATP} following activation of protein kinase C via diacyl glycerol, leads to closing the K channels. Activation of protein kinase C abolishes the blocking effect of K channels by glybenclamide in such a way that at the beginning of reper-

fusion cardiac frequency increases above control values leading to a slight decrease in time. Literature data have shown that treatment of hearts with $\delta V1-1$ for inhibiting translocation of PKC δ leads to a marked inhibition of DNA laddering (Revnic et al., 2009).

As mentioned above, PCK plays an important role in ischemic preconditioning, experimental studies have used selective PKC inhibitors have shown an increase in myocardial apoptosis after the ischemia reperfusion cardioprotective effect was abolished.

An imbalance between anti- and pro-apoptotic proteins, an increased release of cytochrome C from mitochondria, increased caspase activation, and activation of protein kinase C isozymes have been proposed as primary signal pathways involved in the induction of apoptosis after initiation of the death signal stimulus. Through altering these pathways, early IP has shown a profound effect on reducing myocardial apoptosis and infarct size.

Although most studies to date have shown that 'classic' or 'early' IP reduces necrotic and apoptotic cell death, more studies are needed to clarify the protective effect of delayed IP on apoptosis and related mechanisms.

From a clinical standpoint, it must be determined whether these interventions delay or permanently reduce apoptosis. However, there are no studies so far showing that early IP permanently reduces apoptosis after a longer period of reperfusion due to the short period of observation in acute experimental studies.

Furthermore, it is not clear whether a reduction in apoptosis contributes to the overall reduction in infarct size after prolonged reperfusion. In addition, it is also unknown whether a short treatment with a caspase inhibitor permanently attenuates apoptosis in the later phase of reperfusion.

Initial studies hold promise of such a translational benefit. If physiological outcomes are improved, then a limitation of apoptosis may offer an opportunity for treatment of ischemic heart disease, heart failure and other cardiac diseases. Although most studies to date have shown that 'classic' or 'early' IP reduces necrotic and apoptotic cell death, more studies are needed to clarify the protective effect of delayed IP on apoptosis and related mechanisms.

Ischemic preconditioning provides a degree of protection against myocardial ischemia-reperfusion by reducing the number of myocytes damaged by the above mentioned mechanisms and improved ventricular ejection fraction after reperfusion a clear relationship between preconditioning and apoptosis after myocardial ischemia-reperfusion has not been established yet.

Ischemic preconditioning stands virtually alone in its ability to limit infarct size in the controlled setting of the experimental laboratories.

Understanding the basic mechanisms of ischemia reperfusion injury is critical to developing clinically applicable strategies to minimize myocardial reperfusion injuries.

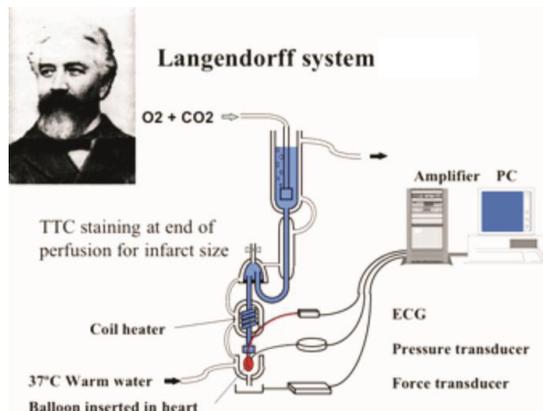
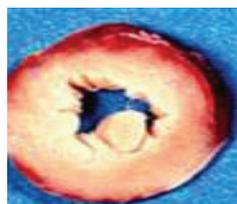


Figure 1b. The Langendorff retrograde perfusion system

Langendorff control ischemia-reperfusion protocol

A: Ischemia reperfusion injury (I/R)



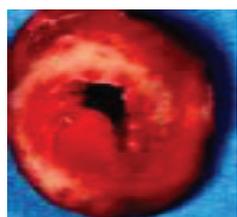
20' stabilization

45' global ischemia

30' reperfusion



B: Myocardial protection (I/R + PC)



20' stab.

5'I

5'R

5'I

5'R

45'ischemia

120' reperfusion



Figure 2. Rat myocardium subjected to: A) Global ischaemia-reperfusion only and B) Global ischaemia-reperfusion with ischaemic preconditioning as a cardioprotective strategy

Study 3: Optical and electron microscopy results

The image presents the myofibrils with normal architecture (Fig. 3).

Perinuclear mitochondria rich in glycogen, with intact double membrane, ordered and compact cristae, dense and homogeneous matrix (Fig. 4). Sarcomeres are uniform with Z bands arranged in register in adjacent sarcomeres.

Mitochondria are intact with regularly arranged cristae. Glycogen granules are abundant in perimitochondrial space. Occasional lipid vacuoles are present (Fig. 5).

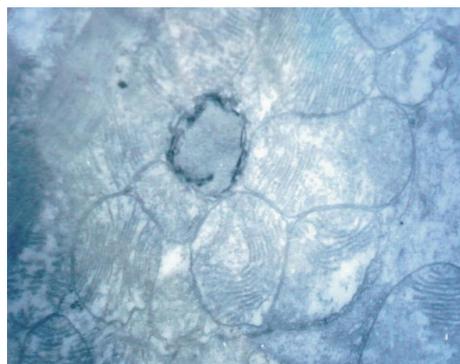


Figure 4. Electron micrograph (22000x) of control non ischemic myocardial tissue

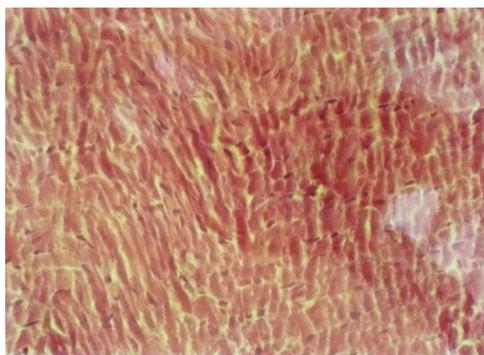


Figure 3. Optical micrograph of rat control myocardium

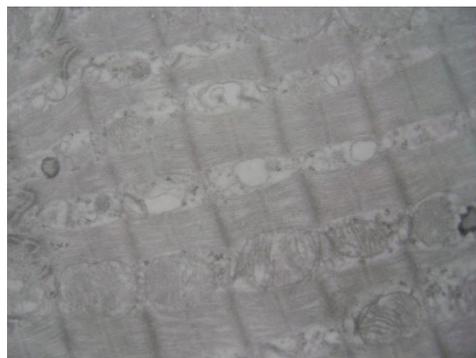


Figure 5. Electron micrograph of control myocardium

Some muscle fibers are vacuolated with pyknotic nuclei or otherwise anucleated, with perimuscular connective tissue destruction. There are accumulations of muscle cells, some muscle cells are poorly colored, which advocates for intracellular edema, scattered as they are form of small islands (Fig. 6). In some areas of the myocardium is less compact with increasing distance between myocytes which advocates for increased extracellular volume. Severe ischemia is evidenced by the complete absence of glycogen and swelling of mitochondria, there is evidence for irreversible damage. Mitochondria show intact double membranes, some have ordered dense criste and compact matrix homogenous, others have disorganised crest (Fig. 7).

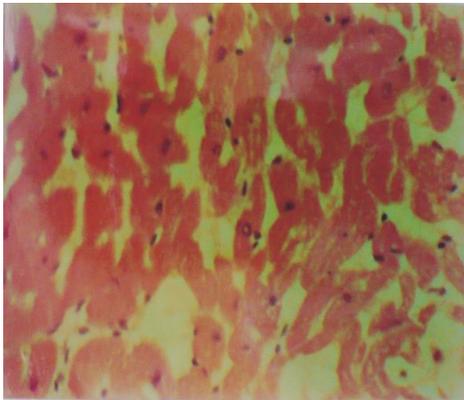


Figure 6. Optical micrograph of myocardium subjected to 45' ischemia and 30 minutes reperfusion.

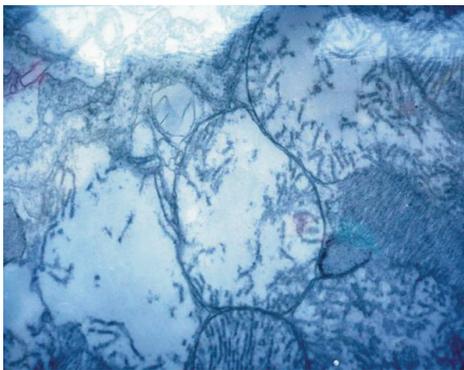


Figure 7. Electron micrograph (22000x) of rat myocardium subjected to 45' ischemia and 30 minutes reperfusion

Alterations classified as minimal. There is a decrease in glycogen granules.

The mitochondria are swollen and reveal the loss of matrix density. Cristae are generally intact. The cytoplasm is severely edematous and sarcomeres are broken separated by an increased tissue space.

The tubules T of the transverse tubular system are dilated. Lysosome are present around the mitochondria. Nuclear cromatin clumping and margination, interfibrilar edema, wide I bands suggesting relaxation.

No amorphous intramitochondrial material or sarcolemma breaks were found (Fig. 8).

The visual inspection by optical microscopy on the surface of endocard until the mid-ventricular anterior wall portion, of rat heart subjected to 45' ischemia and 120' reperfusion, the infarct was in some sections transmurally focused (Fig.9).

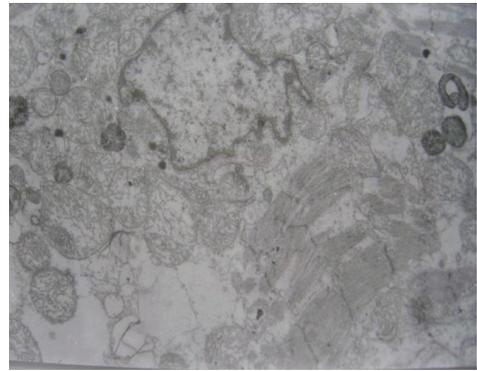


Figure 8. Electron micrograph (11000x) of preconditioned myocardium (group 3)

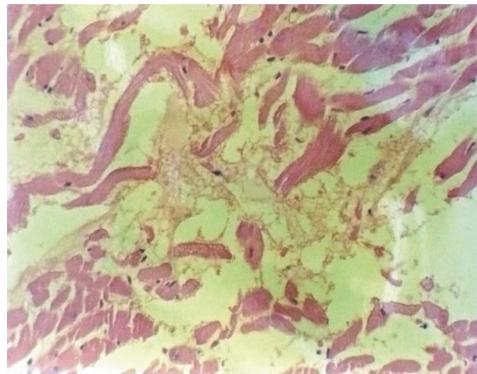


Figure 9. Optical micrograph of cardiac muscle group 4

In the necrotic myocardial tissue, the edema is present together with diffuse haemorrhage and contraction bands (Fig. 10).

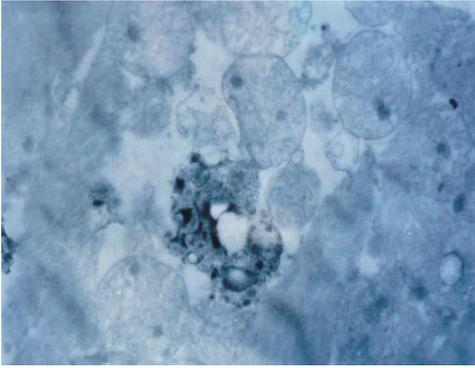


Figure 10. Electron micrograph (11000x) of infarcted myocardium after 45 minutes ischemia and 120 minutes of reperfusion

Myocardial tissue is irreversibly damaged, sarcomeres are expanded and hyperextended, mitochondria are swollen and fragmented, they lack matrix density, and cristae are disorganised, some are vacuolated and contain one or more amorphous dense bodies, which constitute signs of irreversible injury. Cell membrane integrity was disrupted. There are discontinuities in the plasma membranes, the cytoplasm is severely edematiated.

CONCLUSIONS

Ischemia followed by reperfusion in rat heart caused significant changes in the ultrastructure of myocardial cell architecture in particular on cell organelles such as mitochondria that lose their ability to produce ATP, the energy source for cell life. Our results have shown that as ischemia and reperfusion time is extended, the level of infarction is also increased.

A significant DNA “ladder” pattern has been observed in hearts exposed to 45 minutes ischemia and 120 minutes reperfusion accounting for an increased apoptosis process which develops simultaneously with necrosis principally during reperfusion.

Myocardial protection achieved by (ICP) induced by 2 cycles of 5' ischemia and 5' reperfusion prior to lethal ischemia reperfusion protocols, accounted for the reduction in infarct size, the golden standard, accompanied by an inhibition of apoptosis, one of the mechanisms that participate in eliciting the increased resistance to ischemia.

Ischemic preconditioning (IP) provides a degree of protection against myocardial ischemia-reperfusion by reducing the number of myocytes damaged by the above mentioned mechanisms and improved ventricular ejection fraction after reperfusion a clear relationship between preconditioning and apoptosis after myocardial ischemia-reperfusion has not been established yet.

However, the more basic question remains whether a reduction in apoptosis translates into improvement of clinically relevant outcomes such as infarction, incidence of arrhythmias, global contractile performance, or survival.

The potential clinical relevance of IP on ischemia-reperfusion injury is the object of clinical research to find new methods of cardioprotection in humans.

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