Myocardial Ischemia/Reperfusion injury: novel strategies for an old problem

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Abstract
Ischemic heart disease remains the leading cause of death and disability in Europe and worldwide. Thrombolytic therapy and angioplasty, by allowing the recovery of coronary blood flow (reperfusion) after ischemia, greatly improved clinical outcomes and reduced the infarct size in patients. Reperfusion per se, however, can cause serious and fatal cardiac dysfunctions, mainly due to the sudden entry of oxygen, ionic and metabolic disorders that, by overwhelming the endogenous cellular defences, can lead to the death of cardiomyocytes and trigger myocardial injury and dysfunction. Preclinical animal studies have identified and characterized many endogenous pathways that have the potential to protect cardiomyocytes and reduce the infarct size if activated before the ischemic event or early after the onset of reperfusion. However, the improvement in our knowledge and the implementation of adjuvant strategies to provide cardioprotection against short– and long–term ischemia-reperfusion (I/R) induced damage are of great importance and remain a major unmet clinical need. The present review summarizes our current comprehension on the pathophysiology of I/R injury and analyze recent progress in pharmacological and non-pharmacological strategies of cardioprotection. Some critical points that may explain the failure to translate the achievements of preclinical research into clinical practice are also discussed. Perspective suggestions to preclinical research in pursuing deeper understanding, using system biology approaches, of the mechanisms triggered by I/R damage which lead to the deterioration of cardiac function in the medium and long term and in targeting cardioprotective strategies also towards coronary microcirculation, often compromised by I/R, are provided.

Key word: Acute myocardial infarction; Reperfusion injury; Cardioprotection; Coronary microcirculation
1. Introduction
Heart attack, also referred to as acute myocardial infarction (AMI) is the result of occlusion of one or more coronary arteries supplying the downstream myocardial territory. Persistence of the imbalance between myocardial oxygen demand and supply for a long time translates into tissue necrosis if not properly recognized and cured. In patients with AMI, the current gold standard strategy of treatment is a timely and effective restoration of blood flow (reperfusion) to contain the cellular acute ischemic injury or death and to limit the extension of the infarcted myocardial region. The reduction in the patients’ death rate immediately following acute myocardial infarction is one of the most meaningful successes of modern cardiology. This goal has been attained thanks to the rapid reperfusion of the infarcted area with the use of thrombolytic therapy or primary percutaneous transluminal coronary angioplasty (PTCA), implantation of coronary stents helping to maintain the vessel patency, as well as surgical coronary artery bypass graft. However, the process of reperfusion is a two–edged sword. On the one hand, the restoring of blood flow relieves from addition cellular damage or death associated with persistent ischemia. On the other hand, it is responsible of a spectrum of cellular injuries triggered by ionic and metabolic disorders that, by overwhelming the endogenous cellular defences, can lead to a further death of cardiomyocytes, a phenomenon known as reperfusion injury. The most evident clinical outcome of reperfusion damage is arrhythmias and reversible contractile dysfunction (stunning), microvascular no-reflow, and exacerbated myocardial injury. The severity and final size of the tissue damaged depend upon factors of cardiac or extra-cardiac origin such as the entity and duration of O₂ debt, the size of the occluded vessel, the biochemical and biomolecular alterations that modulate the resilience of the tissue to ischemia and reperfusion (I/R) damage, the neuro-hormonal activation and, not least, inflammatory processes. However, clinical outcome of AMI is not decided only during the acute phase and upon the extent of the initial damage, but is concluded in a prolonged time span, during which a complex process of morpho-functional remodeling affects the whole heart and causes extensive structural changes, leading to functional impairment that can progress to heart failure. As far as the necrotic area is concerned, numerous studies have already described the multifaceted sequel of events taking place in the post-infraction period. For instance, the space-time distribution of events such as apoptosis of cardiomyocytes, inflammatory-immunologic response, vasculogenesis and deposition of substitutive fibrosis have already been reported. Hence, although fatality rates during and in the early phase following AMI have decreased in most countries, the post–ischemic structural and functional cardiac remodeling remains a major factor associated with all-cause mortality and hospitalization for heart failure within the following years. To date, the reduction of the infarct size has been considered the main determinant for the long-term prognosis of patients and the majority of studies has focused on this issue to identify or test strategies for cardioprotection,
sometimes neglecting other aspects such as the role of coronary microcirculation in the progression of ischemic cardiomyopathy.\textsuperscript{4,5} However, despite several pre-clinical studies have demonstrated that multiple approaches are capable of attenuating the myocardial I/R injury, there is still no effective cardioprotective therapy in the clinical setting.

This article, after a brief overview of the features characterizing the pathophysiology of I/R, intends to survey the more traditional and innovative therapeutic strategies, emerging especially from preclinical models, useful for I/R cardioprotection.

2. Acute myocardial infarction and ischemia/reperfusion injury

In the heart, ischemia can arise from low flow or total occlusion of a section of coronary artery tree and it may be permanent or transient. In the latter case, ischemia can be successfully reversed by reperfusion after a short or long period of occlusion. In any case, in the myocardial region downstream the ischemic vessel an area at risk (AAR) of potential infarction is outlined. The percentage of myocardium that inevitably faces irreversible damage within the AAR defines the infarcted area, and commonly the wave front of cardiomyocyte death arises in the subendocardial surface and moves transmurally over time towards the epicardium.\textsuperscript{6} The size of tissue damage and/or cell death is certainly determined, in the first instance, by the extent and duration of the interruption of the blood supply but, in the case of I/R, a significant contribution is given by the injury triggered by reperfusion (Figure 1).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Schematic representation of the relationship between infarct size and event of occlusion (ischemia, red bar) of related artery and subsequent reperfusion (green bar) during AMI. The gray dotted area indicates the percentage of injury induced by reperfusion and, also, represents the variable portion to address cardioprotective strategies.}
\end{figure}
Since the discovery of the apparent paradox of damage associated with re–flow in AMI, several studies have been focused to investigate this component of tissue injury as potentially susceptible of therapeutic intervention. Although the numerous studies accumulated over the years have provided much information in this regard and allowed us to know many of the cellular pathways involved in I/R damage, to date the complex and complete picture of the mechanisms underlying this phenomenon is not yet thoroughly understood. Jennings and colleagues in 1960 firstly described the histological features and pathological characteristics of myocardial injury in a canine model of I/R.7 Within the AAR, they found the presence of prominent myocyte swelling, marked contraction bands, membrane disruption and calcium residues in the mitochondria. This irreversible phenomenon, termed contraction band necrosis, developed within 2 minutes after reperfusion.8,9 Since then, many studies carried on experimental models of different species added important information on the cellular and molecular events that characterize I/R damage, as summed up in many valuable reviews on the topic, from the most historical to the most recent ones.2,10-14 Myocardial tissue, unlike other tissues, is particularly sensitive to ischemic insult as it is strictly dependent on aerobic metabolism to generate a sufficient amount of ATP required for the maintenance of contractile function and integrity of cellular and mitochondrial membrane. Indeed, the importance of oxidative metabolic processes is reflected by the consistent content of mitochondria in cardiomyocytes that accounts for at least 30% of the cellular volume.15 During ischemia, the prevalence of anaerobic metabolism leads to depletion of ATP stores, reduction of intracellular pH, lactate accumulation, and activation of Na+/H+ exchanger. Direct consequence of the ATP shortage is the inactivation of Na+/K+-ATPase pump and the decline of cellular Ca++ efflux and reuptake by the endoplasmic reticulum, thereby leading to the increase of intracellular and mitochondrial calcium levels (calcium overload). Furthermore, the low [ATP]/[ADP] ratio removes the allosteric inhibition of cytochrome c oxidase resulting in instability of the inner mitochondrial membrane potential (ΔΨm).16 These cellular changes may trigger the activation of intracellular proteases (e.g., calpains) targeting myofibrils and producing sarcomeric hypercontraction and even contracture band necrosis.17 After the onset of ischemia, the fate of cardiomyocytes of the AAR is dichotomous depending of their resilience: irreversible damage leading to cell death, or reversible damage, in which suffering myocytes remain viable but fragile and are subject to either rescue or further injury upon reperfusion. Indeed, re–introduction of molecular oxygen upon flow restoration precipitates multiple noxious sequelae including generation of reactive oxygen species (ROS) due to the activation of xanthine oxidase, NADPH oxidase and mitochondrial electron transport chain uncoupling, exacerbated calcium overload, mitochondrial dysfunction through disruption of the outer membrane and opening of the mitochondrial permeability transition pore (mPTP).18,19 During reperfusion, ROS can be produced by both
endothelial cells of microvasculature and cardiomyocytes, and in less extent by circulating phagocytes. The imbalance between oxidant agents production (ROS and reactive nitrogen species – RNS) and cellular antioxidant systems leads to an accumulation of radical species able to damage DNA, trigger apoptotic pathways, stimulate the secretion of pro-inflammatory cytokines and chemokines in the course of I/R injury. Another crucial determinant of myocardial injury occurring at reperfusion is the no-reflow phenomenon, associated with microvascular damage. It was firstly described by Kloner in a canine model of I/R and subsequently observed at reperfusion in 10–30% of AMI patients with elevation of ST segment. Several putative causes could underlie the no-reflow phenomenon: vasospasm induced by vasoconstrictor mediators released at reperfusion, presence of platelet and leucocyte aggregates, tissue edema and capillary endothelial damage.

It is evident that reperfusion phase is a very complex network of events and is a dynamic process starting with the re-oxygenation of AAR and continuing for up to several days. Nevertheless, an in-depth knowledge of the main mechanisms underpinning the I/R injury could pave the way for novel and innovative strategies to both potentiate the cellular defences to face ischemic conditions and/or reduce the detrimental effects of reperfusion.

3. Cardioprotective strategies in the acute phase of ischemia/reperfusion
From the first evidence of reperfusion damage, the idea of positively interfering with the re-flow conditions stepped into several studies addressed to revert or lessen tissue damage then leading to smaller size of the infarct area, reduce adverse complications and therefore decrease morbidity and mortality following AMI. Ischaemic time is a critical determinant of cardiomyocyte death and most evidence suggests that cardioprotective pathways must be targeted during the first minutes of reperfusion. Investigations focused on early cardioprotective strategies can be roughly divided into different categories based on the modality, time of intervention and cellular/intracellular target. Among the most studied and practiced modalities, it is noteworthy to mention the controlled application of episodes of brief ischemia and reperfusion (ischemic conditioning), the administration of drug (pharmacological strategies), or the application of physical measures, such as hypothermia or electrical nerve stimulation.

3.1 Ischemic conditioning
In the mid-1980s, a seminal article reported that a series of short episodes of ischemia followed by reperfusion of the circumflex artery in a dog model of AMI could turn into a protective event in the myocardium that was more tolerant to subsequent prolonged occlusion of that vessel, with a 75% reduction of infarct size. The phenomenon, named ischemia preconditioning (IPC), has also been observed in other experimental models and the level of its effectiveness has proven to be species-dependent. This procedure not only reduced infarct size in most models, but also decreased the incidence and duration of arrhythmic events, including premature ventricular beats,
ventricular tachycardia and fibrillation. In the following few years, IPC proved successful even if applied to a vascular bed other than that of the prolonged ischemic event, an occurrence that led to coin the term of remote ischemic precondition (RIPC). Indeed, Przyklenk et al reported that brief episodes of non–lethal I/R of the circumflex artery protected the myocardial tissue downstream of the left anterior descendant coronary artery in the case of its sustained occlusion. These data imply that preconditioning may be mediated by factors activated, produced, or transported throughout the whole heart during repeated episodes of brief I/R.

Ischemic preconditioning has been classified into two types: a) early – or first window of protection – whose onset is immediate, and its infarct-sparing effect is particularly evident if IPC precede ischemic event by 2–3 h; b) late – or second window of protection – whose onset is delayed by 12–24 h after ischemia up to 72 h, and is more successful against post ischemic myocardial stunning.

In the medical practice, a form of myocardial adaptation to ischaemia akin to ischaemic conditioning has been postulated by Tomai in patients with effort angina after their first exercise test. However, even though classical preconditioning may work in a clinical setting such as heart surgery and organ transplantation, it is of little use and not feasible in patients with acute MI because the coronary artery is already occluded at the time of hospitalization.

In 2003, Zhao et al. demonstrated, in a canine model, that intervening at the point of reperfusion with additional brief episodes of I/R could reduce the size of the infarct area, limit arrhythmic events and preserve the endothelial function in an equally effective way of IPC. The phenomenon, called ischemic post conditioning (IPostC), was endeavoured to provide “controlled reperfusion” rather than the abrupt re– introduction of blood typically associated with re–flow vessel therapy. It theoretically allows direct application to the clinical settings, especially during PTCA. Indeed, the repetitive inflation and deflation of the angioplasty balloon used for the reopening of the coronary artery can mimic the recurring brief events of I/R performed in post–conditioned animal models.

In the same way as IPC, IPostC protocols have now been extended to include remote ischemic post-conditioning, whereby sub–lethal I/R episodes are applied in distant regions or organs from that subject to reperfusion after the reopening of the vessel.

The mechanisms of ischemic conditioning are not yet fully defined but several of the putative multi–stage events and their complex interactions have been outlined. Conditioning stimuli trigger the release and production of endogenous substances (e.g., adenosine, bradykinin, cathecolamine, opioids, prostanoids, tumor necrosis factor–α, nitric oxide, cytokines). These substances activate key molecular mediators (kinase cascades) that act to preserve mitochondrial function and integrity and allow cardiomyocyte’s survival (Figure 2).
Figure 2. Schematic representation of the major components of the pathways that promotes cardiomyocyte’s protection against I/R injury. Abbreviations are described in the text.

There are evidence for multiple signaling mediators involved in pre- and postconditioning: protein kinase G (PKG), reperfusion injury salvage kinase (RISK) and survivor activating factor enhancement (SAFE) pathways. The RISK pathway encompasses the activation of phosphatidylinositol 3′-hydroxy kinase (PI3K)/protein kinase B (Akt) signaling and Mitogen-Activated protein kinase kinase (MEK)/Extracellular Signal-Regulated Kinase (ERK). The glycogen synthase kinase 3-β (GSK-3β) is a key downstream target of Akt and is inactive when phosphorylated. Thus, GSK-3β phosphorylation by Akt or other upstream mediators results in inhibition of GSK-3β-activated targets. For instance, inactivation of GSK-3β by Akt reduces mitochondrial Bax (pro-apoptotic) recruitment as well as mPTP opening. Moreover, pathways mediated by PI3K/Akt increase cytosolic levels of nitric oxide (NO) in cardiomyocytes. NO, in turn, activates intracellular signaling pathway mediated by guanylate cyclase(GC)/cyclic guanosine monophosphate (cGMP) and activates protein kinase G (PKG).
monophosphate (cGMP)/protein kinase G (PKG) that, on the one hand stimulates the opening of mitochondrial ATP-dependent K⁺ channels (mitoK<sub>ATP</sub>) and, on the other hand, decreases mitochondrial Ca<sup>2+</sup> overload, thus contrasting the opening of mPTP channels and reducing the incidence of severe arrhythmias and cellular death.

The SAFE pathways is an alternative survival kinase cascade activated by TNFα that, via Janus Kinase (JAK), converges on Signal Transducer and Activator of Transcription-3 (STAT–3). Several targets of STAT–3 have been identified, including proteins involved in cell survival and proliferation. In particular, STAT–3 increases expression of the anti–apoptotic gene Bcl–2 and reduces that of the pro–apoptotic gene Bax. Lacerda and colleagues found that the activation of JAK/STAT–3 pathway was associated with increased levels of phosphorylation of glycogen synthase kinase 3β (GSK–3β), leaving questionable whether GSK-3β is an effective downstream target of the JAK/STAT-3 pathway. Moreover, there is evidence suggesting a possible crosstalk between the two pathways, RISK and SAFE. Maintaining the integrity and function of the mitochondrion by preserving the closure status of the mPTPs at reperfusion is the common key element of both the cardioprotective signal transduction cascades activated by ischemic conditioning.

### 3.2 Pharmacological conditioning

Although it has proven effective, ischemic conditioning, both pre– and post–, either local or remote, entails an intrinsic risk of damage especially for the vessels that are stressed by repeated episodes of occlusion and re–opening. However, the characterization of the major pro–survival pathways, which mediate cardioprotection at the time of reperfusion, has paved the way for the concept of pharmacological conditioning. Indeed, several key molecular points have been highlighted as pharmacological intervention targets to mitigate myocardial reperfusion injury. For this purpose, several endogenous agents or drugs had the ability to activate the signal pathways involved in an ischemic conditioning-like cardioprotection. Numerous preclinical experimental studies successfully tested a number of active endogenous agents. These compounds include adenosine, apelin, catecholamines, bradykinin, acetylcholine, opioids, angiotensin, steroid hormones (estrogens and testosterone), endothelin, atrial natriuretic peptide (ANP), glucagon-like peptide-1 (GLP-1) and its analogous Exenatide e many more. Alongside endogenous compounds, it has been observed that several different classes of drugs, including NO-donors, inhibitors of phosphodiesterase 5, beta blockers, K-ATP channel openers, antioxidants, immunosuppressive agents (cyclosporin A), could also be used to reduce the negative outcome of reperfusion. Many of these agents are agonists of G protein–coupled receptor (GPCR)s and/or trigger intracellular signaling factors, including protein kinases, enzymes, transcription factors associated with signaling pathways (SAFE and RISK), to target structures such as mitochondrion, sarcoplasmic reticulum and nucleus involved in the survival of cardiac cells as described for both pre– and post–ischemic conditioning.
Although the vast plethora of compounds tested demonstrated to be successful in preclinical studies, the results sometimes failed to live up to expectations when they were transferred to the clinical arena. As it will be argued in one of the following sections, in the field of cardioprotection, substantial gaps remain between experimental studies aimed at the identifying the mechanisms and pathways involved, and studies that define and test solid preclinical protocols that justify to be investigated in humans.\(^{55}\)

In the context of pharmacological conditioning, a separate mention must be deserved to volatile anesthetics (VA) such as the halogenate ethers, including isoflurane, desflurane and sevoflurane. Administration of VA before myocardial I/R has been demonstrated a protective strategy in several different animal models and in humans and referred to as anesthetic preconditioning (APC).\(^{56-59}\) The VA–induced cytoprotection shares several common cytosolic signaling paths with RISK and SAFE pathways and are finally mediated at the mitochondrial level. VA are lipophilic, unlike most other cytoprotective drugs and, also, readily penetrate mitochondria to target the more lipophilic protein sites embedded in the membrane structure. In recent years, increasing evidence pointed to the effects of VA on PCK\(\epsilon\) activation, opening of mitoK\(_{ATP}\) channels, closing of mPTP.\(^{60-63}\) Volatile anesthetics also showed post–conditioning effects in terms of reducing the infarct size when administered in the first 30 seconds, but not after 3–10 minutes of reperfusion.\(^{64}\) Use of VA during cardiopulmonary bypass has also been encouraged because of its pre- and post-conditioning effects. However, the main drawback that limits the practical clinical use of VA as cardioprotective agents are co-morbidities and associated medications. Indeed, factors such as advanced age, concentric hypertrophy, diabetes or transient hyperglycemia (common features in patients undergoing the cardiac surgical setting) have been shown to dampen the cardioprotective effects of VA.\(^{65}\)

### 3.3 Hypothermia

Several studies in animal models of AMI persuasively demonstrated the cardioprotective effect of mild hypothermia (MH) (32–34 °C) at the onset or during ischemia.\(^{66-71}\) Chien and colleagues compared infarct sizes at different cardiac temperatures (35–42°C) in rabbits submitted to 30 minutes of coronary artery occlusion; they demonstrated that any decrease in myocardial temperature was linearly correlated to infarct size (\(~8\%\) of the risk zone for each degree decrement).\(^{72}\) Similar findings have been reported by independent investigators in different laboratories and species, providing a high level of evidence of the cardioprotective action of mild hypothermia.\(^{66,73-76}\) The main evidence emerging from studies that have investigated the relationship between optimal timing of initiation of MH and cardioprotective outcomes is that therapeutic hypothermia should be initiated as soon as possible after the onset of ischemia to maximize the benefit provided by MH.\(^{77-80}\) Beyond infarct size reduction, protection induced by MH was associated with
prevention of the no-reflow phenomenon and long-term improvement in terms of left ventricular (LV) remodeling and performance.\(^{74,77,78,80–82}\) Interestingly, no-reflow reduction was not always associated with infarct size reduction. Indeed, cooling started after reperfusion only reduced no-reflow area, but not infarct size.\(^{83,84}\) These findings suggest that hypothermia protects not only cardiomyocytes, but also endothelial cells of microvasculature against ischemic injury.

Unlike the benefit obtained with hypothermic preconditioning, the effects of MH as myocardial postconditioning are not as clear and sometimes they are controversial. Positive outcome is lost if cooling is delayed by as little as 15 minutes after the onset of reperfusion.\(^{85}\) Therefore, this schedule of application has poor clinical relevance, exception for programmed cardio–surgery. In the emergency situation, in fact, both the patient cannot be cooled from the onset of symptoms (preconditioning) and a delay of 15 minutes is almost inevitable during reperfusion, since the setting up of MH in humans takes a long time to use it as post–conditioning strategy.

The mechanism of cardioprotective effect of MH is not fully clarified, but many hypotheses are plausible. Despite hypothermia reduces cellular metabolism – allowing the preservation of high–energy phosphates, accompanied by reduced glucose consumption and lactate accumulation\(^{86,87}\) – this metabolic alteration alone cannot entirely justify the protection afforded by MH. Recently, the activation of pro–survival signaling pathways alike those involved in ischemic pre– and post–conditioning (ERK, NO, and Akt) have been proposed as the mechanisms triggered even by MH.\(^{88–90}\) The final target of MH is the inhibition of mPTP formation to preserve mitochondrial function and biogenesis.\(^{91}\)

### 3.4 Mechanical LV unloading

Most of the reperfusion injury occurs rapidly after coronary flow restoration and therefore an approach that allows intervention and manipulation of ischemic tissue would be desirable. In this perspective, pharmacological interventions are of little use because, by definition, without perfusion of the tissue they cannot achieve their goal. Mechanical unloading of LV is any intervention that reduces the energy expenditure of the ventricle and limits the altered hemodynamic forces that lead to ventricular remodeling after myocardial ischemic insult. The use of ventricular assistance device (VAD) – also known as mechanical circulatory support – is one of the few approaches capable of modulating the stress of the ischemic tissue directly before reperfusion by aspirating blood out of the ventricle directly into the aorta. By restoring the values of cardiac output and mean arterial pressure, it attenuates the hemodynamic unbalance induced by cardiac ischemia.\(^{92}\) Preclinical evidence of the acute cardioprotective effect of LV unloading was reported by Meyns and colleagues.\(^{93}\)

Although the authors described a clear reduction of the size of infarct in a sheep model of I/R, the ventricular support apparatus belonged to the older generation of invasive devices, not particularly appealing for clinical application. The advent of a new generation of miniaturized percutaneous
VADs have made possible a safe and effective ventricular unloading in the clinical arena. After the confirmation of the benefit of their preclinical use in limiting reperfusion damage, the clinical interest in this modality has grown considerably.\textsuperscript{94–96} The mechanism that led to limiting the extension of the infarct area remained unclear. More recently, Esposito and colleagues reported that LV unloading, initiated 30 minutes before reperfusion in a pig model of I/R, had both reduced infarct size in the acute phase and led to a smaller scar size associated with a better–preserved ventricular function in the chronic phase.\textsuperscript{97} The timing of LV unloading was particularly critical, as starting 15 minutes before or after reperfusion did not lead to any beneficial outcome. The authors also found that the expression of stromal-derived factor (SDF)-1α, a key molecule that promotes protective effects through several mechanisms that includes the RISK pathways, was increased under LV unloading and correlated with the infarct size reduction.\textsuperscript{98,99} In addition, the inhibition of the SDF–1α–CXCR4 pathway blunted the positive effect of LV unloading, suggesting the crucial role of SDF–1α and its downstream target in mediating the cardioprotective effect of LV unloading.\textsuperscript{97}

\section*{3.5 Electrical stimulation }

Since the first evidence of RIPC many studies have focused on the possibility to use remote non-ischemic stimuli for cardioprotection from I/R damage, including the stimulation of peripheral nerves, electro–acupuncture and skin nociceptor activation.

\textit{Peripheral nerve stimulation}. Several studies on remote ischemic preconditioning highlighted the relevant role played by afferent nerves in such beneficial outcome.\textsuperscript{100–102} Indeed, it has been demonstrated that prior transection of the femoral nerves abolished the RIPC achieved by lower limb ischemia in a rat model of myocardial I/R, and that stimulation of the femoral nerve likewise could mimic limb RIPC.\textsuperscript{101–103} The emerging hypothesis supported the concept that neural activated pathways shared common features with cardiac conditioning and that remote nerve stimulation could elicit cardioprotection against I/R. In the light of this evidence, numerous nerves and different types of afferent fibers have been tested to use their stimulation for the purpose of cardioprotective preconditioning in I/R. Jones and coworkers reported that stimulation of peripheral nociceptive fibers, by abdominal skin incision or by using topical application of capsaicin on the abdominal skin, resulted in cardioprotection against MI.\textsuperscript{104} According to the authors, nociception triggered neurogenic signaling that activated the sympathetic nervous system in the heart and caused activation of PKCε and inhibition of PKCδ in a type 2 bradykinin receptor–dependent manner. Cardioprotection was finally achieved by the preservation of mitochondrial function through the activation of the mitoK\textsubscript{ATP} channels. Recently, the vagal stimulation has demonstrated effective in reducing the infarct size and improved the ventricular functional recovery in both \textit{ex vivo} and \textit{in vivo} rodent models of infarction.\textsuperscript{105–107} The mechanism at the basis of the beneficial effect was the activation of PKC and the increase of NO.
production, mechanisms similar to those of the RISK signaling pathways.

It is noteworthy that in a murine model of chronic neuropathic pain, the myocardial injury following AMI was reduced.\(^{107}\) The authors' idea was that chronic neuropathic pain had activated the neurons of the anterior nucleus of the paraventricular thalamus, thus increasing the activity of the vagus nerve, which, in turn, led to an increase in the release of acetylcholin and the activation of PKC\(\varepsilon\) with the final result of cardiomyocytes' protection.

Recently, Merlocco and colleagues designed a proof of concept study to validate the effectiveness of nerve stimulation as preconditioning in myocardial I/R damage.\(^{108}\) They used a nerve activation mode that could be easily translated in the clinic – the transcutaneous electrical nerve stimulation (TENS). The authors tested the use of TENS as a possible novel method to release circulating factors in the blood of both rabbits and human volunteers. The blood dialysate of donors was then tested in \textit{ex vivo} Langendorff heart preparation of rabbit and mouse models of I/R injury. The effect of dialysates collected after TENS on the isolated hearts was compared with those obtained in a rabbit model of hind limb RIPC. Results indicated that TENS, alike RIPC, induced the release of a dialyzable and hydrophobic factor that provided cross species cardioprotection, confirming TENS as a novel clinically relevant method of remote precondition.

\textit{Electroacupuncture.} In 1991 Meerson and colleagues demonstrated that a series of transauricular electroacupuncture (EA) in rats improved the resistance to the reperfusion induced tissue necrosis after coronary occlusion in the isolated heart.\(^{109}\) The evidence of EA's protective preconditioning effects formed the basis for the growing interest in this practice, also in consideration of its eventual clinical translability. According to Chinese medicine theory, Neiguan acupoint (PC6, forearm) on the pericardium meridian is considered as the main acupoint for improving heart function and energy metabolism. Preclinical evidence highlighted its efficiency in promoting ischemia tolerance, eliminating free radical, and protecting against cell death.\(^{110–112}\)

Another crucial point to treat AMI is Zusanli acupoint (ST36, leg), whose electrical stimulation has been demonstrated to increase the activation of vagal nerve and inhibit the cardiac sympathetic drive.\(^{113–114}\) Although most animal model studies have shown EA's effectiveness as a preconditioning stimulus, Zhang and colleagues successfully used EA as a postconditioning stimulus in a rabbit model, starting the PC6 electrostimulation during the early reperfusion phase.\(^{115}\) EA also has shown efficacy in protecting against cardiac I/R injury when applied as pretreatment in patients undergoing percutaneous coronary intervention or heart valve replacement surgery.\(^{116–117}\) Despite detailed mechanisms of EA's cardioprotection have not yet been fully elucidated, the hypothesis is that it may act as a preconditioning or remote conditioning through stimulation of skin sensory or other innervation.\(^{118}\) The putative mechanism involves the release of humoral factors that, at least partially, reduce myocardial damage via the activation of endogenous anti-apoptotic signaling and
improve functional recovery in response to prolonged myocardial ischemia.\textsuperscript{112,119}

4. Cardioprotection strategies in the chronic phase of reperfusion

Although timing is crucial in cardioprotective strategies, several early studies in dogs and rabbits suggested that infarct size increases during the early hours of reperfusion up until 48 h, providing evidence of a therapeutic window for targeting the injury triggered by chronic reperfusion.\textsuperscript{120–122} Indeed, beyond the cellular salvage during the early phase of I/R, definitive outcome of acute myocardial infarction depends on complex dynamic mechanisms that, in the first instance, modulate the healing in the infarcted territory. However, subsequently, the residual viable myocardium, as well as the infarcted segment, encounter extensive structural changes including the maturation of a fibrotic scar with shrinkage of the tissues within infarcted area, hypertrophy of the residual myocardium and ventricular dilatation.\textsuperscript{123} Such a post-ischemic remodeling is the final result of molecular, cellular and interstitial processes which involves changes in cardiomyocytes, extracellular matrix and microvasculature. Taken together these alterations may lead to a progressive decline in the overall cardiac function.

4.1 Thyroid system and cardioprotection

Thyroid hormones (TH) are closely linked to the heart homeostasis in a mutual relationship. On the one hand, overt thyroid dysfunction – in its two prevalent manifestations of hypo- and hyper-thyroidism – has an impact on cardiac contractility, myocardial oxygen consumption, cardiac output.\textsuperscript{124–126} On the other hand, homeostasis of TH is affected by acute ischemic cardiac disease and heart failure and, in this context, increasing interest has been devoted to investigate the potential role of hormonal correction in the overall patient outcome of cardiac disease. Early but short-term decrease of circulating triiodothyronine (T3) has been described in 15–20\% of patients with AMI and reproduced in animal models of AMI with similar incidence.\textsuperscript{127–130} Such a low T3 state, occurring in the absence of thyroidal illness, has been called low T3 syndrome (L-T3S), and has been considered for a long time as an adaptive mechanism to reduce the cardiomyocyte’s energy demand during stressful conditions.\textsuperscript{131} However, several clinical and experimental studies pointed out that the L-T3S may have an adverse prognostic impact on various acute and chronic cardiac disorders.\textsuperscript{128,132,133} In addition, cardiac alterations tended to revert upon thyroid homeostasis re-establishment by TH supplementation.\textsuperscript{130,134–137} Therefore, thyroid hormone replacement therapy in AMI patients may represent a potentially useful long-term strategy to reduce infarct size, improve cardiac performance and contrast the post-ischemic adverse remodeling. T3 plays a critical role in regulating mitochondrial function and morphology, modulating antifibrotic and proangiogenic effect.\textsuperscript{137–140} The cardioprotective effect of TH is mediated by regulation of pro-survival pathways, including activation of the PI3K/AKT and PKC signaling cascades, enhancement of heat shock proteins (HSP70 and HSP27) expression, and suppression of
A recent study by Forini and coworkers reported for the first time a wide-ranging profile of T3-modulated mRNA and miRNAs, taking into account two of the major processes affected by I/R, namely mitochondrial function and fibrosis, in a rat model of regional I/R. After 30 min occlusion of the left descending coronary artery followed by 3 days of reperfusion, a replacement dose of T3 was administered for 48-h starting 24h after the ischemic event. T3 replacement dampened the alterations in expression of 67 out of the 87 genes and of 11 out of the 102 miRNAs dysregulated by I/R. The functional enrichment analysis indicated that some genes differentially expressed in T3–treated rats were involved in several mitochondrial functions (cell death, organization and quality control, and mitochondrial solute/metabolite transport) and tissue remodeling (TGFβ and MAPK signaling, Smad pathway, ECM organization). In addition, most of the miRNAs up-regulated by T3 have been previously shown to increase myocardial resilience to noxious stimuli by targeting cell death, mitochondrial dynamics and pro–fibrotic processes.

Moreover, the emerging picture from the in silico analysis highlighted a complex network of cross talking nodes between mitochondrial cell death, pro–fibrotic pathways and adverse remodeling in the post–IR setting.

4.2 Molecular therapy

The discovery that during the second cardioprotection window observed following IPC or RIPC (24-72 h post-reperfusion) the expression of some genes was altered has sparked interest in identifying molecular therapies aimed at mimicking these late responses. **Cardioprotection by gene therapy.** Some preclinical studies showed that either pre– and post–conditioning ischemia triggered a cardioprotective genomic response in the heart. Although functional genomics studies with a network analysis approach would help in identifying and choosing the key players for cardioprotection, most of the studies in the literature in this regard have arbitrary selected single gene targets to test cardioprotection. According to the well known signaling pathways activated by IPC and RIPC, the interest was focused on transfer of genes such as the inducible isoform of nitric oxide synthase (iNOS), codifying antioxidant enzyme (SOD), heat shock proteins, anti–apoptotic proteins (Bcl–xL), tumor necrosis factor (TNF–α), the inducible form of heme oxygenase (HO–1). Gene transfer in the heart may allow myocardial cells to overexpress specific genetic information and therefore improving the resilience of the heart to I/R damage. To deliver the gene, a vector is needed and the adeno–associated virus (AAV) has proven particularly suitable, since it has shown specific tropism for cardiomyocytes (especially the serotype 9), good ability to transfect mammalian cells and drive the expression of the genes released in these cells for a long time. Unfortunately, the presence of AAV in the myocardial tissue triggers an immune response as a side effect. Moreover, the majority of preclinical studies used gene therapy to induce a permanently preconditioned phenotype (prophylactic cardioprotection) since gene delivery
preceded I/R event. This condition is far from the clinical reality, which allows treatment only after the infarct onset. However, it is noteworthy that Lin and coworkers showed that invasive injection of AAV bearing HO-1 gene into the border zone immediately after MI induction in mice promoted neovascularization in the ischemic region and limited LV fibrosis and dysfunction at 4 weeks.143 In addition, Kusmic and colleagues, in a proof of concept study on rats, showed that HO–1 up–regulation could be efficiently induced pharmacologically, by subcutaneous injection of cobalt protoporphyrin, several hours after infarct with positive outcome on mortality, infarct size and ventricular remodeling at 4 weeks.144 Cardioprotection by miRNA–based therapy. In recent decades, growing interest has been directed towards microRNAs (miRNAs), both as biomarkers and as a therapeutic tool in cardiovascular diseases, including I/R damage. MicroRNAs are a class of endogenous, small noncoding RNA that negatively regulate gene expression via degradation or translation inhibition of their specific target mRNAs. Yin and colleagues demonstrated that miRNAs induced by IPC played a crucial role in protection against myocardial I/R injury.145 The authors collected miRNAs induced by short bursts of global I/R in a Langendorff heart preparation in the mouse, and injected them directly into the myocardium of a recipient mouse 48 h before in vivo myocardial I/R injury. Results showed that miRNAs caused significant reduction in infarct size associated with the upregulation of protective proteins including eNOS, heat shock transcription factor-1 and HSP-70, suggesting their involvement in the delayed phase of IPC in the heart. Since then, several preclinical evidences have confirmed that miRNAs or extracellular vesicles containing miRNAs were able to reproduce the cellular effects of cardioprotection.146,147 A plethora of promising miRNAs (miRNA-1, miRNA-133, miRNA-21, miRNA-24, miRNA–145, miRNA-320, miRNA-29, miRNA-92a, miRNA-126, miRNA-199a, miRNA-208, and miRNA-195) have been proposed as key regulators in myocardial infarction, cardiac conduction, angiogenesis, cardiac hypertrophy, fibrosis, and cardiac protection.148,149 To date, on the one hand the use of miRNA (or mimetics) or their antagonists (antagomir) is envisioned as a therapeutic tool in cardioprotection from late I/R damage, on the other hand it is necessary to find a way for safe and effective delivery of this oligonucleotide therapy. A critical issue in targeting miRNA–based therapy to the heart is the degradation in the bloodstream of oligonucleotides. Several strategies of delivery have been approached to avoid this problem such as nanoparticle– or exosome/liposome–mediated, adhesive hydrogel patches and adenovirus or adeno–associated virus (AAV).150–152 Our knowledge of the safety and efficacy of miRNA therapy is still limited and clinical trials to treat cardiovascular disease are very few and under development yet. Moreover, further verifications of specific issues are required as the fact that a single miRNA can affect several mRNAs. This is commonly considered an advantage as it allows exerting wide effects on multiple pathological pathways, however it can also be seen as an important limitation, as capable of evoking unwanted responses in organs or tissues other
than the primary target. In addition, introduction of exogenous miRNA and its delivery system can have some serious side effects. In this context, it is also worth noting that most of the miRNAs mentioned are also involved in tumor biology.

5. Pitfalls to clinical translation

As seen so far, studies on animal models have highlighted many cardioprotective pathways helpful in reducing infarct size and cardiac dysfunction if activated before ischemia and, in some cases, even at the onset of reperfusion. Basic research has disclosed many cellular and molecular mechanisms involved and identified several putative targets to develop therapeutic strategies. However, despite the general consensus and enthusiasm that results from the experimental models have received – in terms of both endogenous and pharmacological cardioprotection – progress in capitalizing on this wealth of evidence and translating it into clinical practice has not been as rapid and in many occasions has proved disappointing. The claim that the observations obtained on animal models, most obtained in rodents, are not easily and directly translatable to humans and clinical practice is not acceptable as the only simplistic explanation. There are many potential explanations for the prevailing disappointment on the poor translation of cardioprotective strategies to patient benefit: 1) heterogeneity inherent in the patient population compared to that standardized in animal studies. In preclinical studies, in fact, homogeneous groups of single gender, uniform age, maintained on standard diets and carefully controlled environments are used. Furthermore, in most studies on animal models, comorbidities, that are very frequent in clinical reality, are not present or considered; 2) in preclinical studies, animals are not treated with chronic drug therapies, while the patient population often takes drugs that can influence ischemic damage or the mechanisms responsible for cardioprotection; 3) duration of ischemia is standard and controlled in animal studies. This makes less variable the results in the experimental population, both in terms of damage and cardioprotection. Furthermore, ischemia generally lasts a shorter time in animal models than in clinical reality, with a potentially more salvageable amount of myocardium with respect to patients. However, given the pressing demand for therapeutic attempts to minimize I/R injury and to reduce the one-year mortality from AMI and the increasing incidence of post-infarction heart failure, it is a urgent need to successfully address the transition from laboratory to clinic. On the one hand, in clinical medicine, greater attention and homogeneity is required in identifying the appropriate patient (e.g., myocardial infarction of the large anterior wall, a comparable interval of duration of ischemia and standardized treatment protocol). The studies should be carried on a large population and with adequate control groups. On the other hand, preclinical studies should increasingly open up to system biology approach rather than taking into consideration the individual actors in the process. To date, most cardioprotective strategies have been targeted to inhibit the pathways leading to cell death in the early phase, to reduce the extent of the infarct area. However, it is becoming clear that, in
addition to cardiomyocytes, cardioprotection should also target other cell types (endothelial cells, smooth muscle, nerves, fibroblasts and resident stem cells). Likewise, the attenuation of coronary microvascular I/R injury appears a worthwhile target of protection. Furthermore, more experimental studies are needed, ranging from small to large animals, which increase our still-limited understanding of the biological pathways and kinetics that lead to ventricular remodeling and dysfunction in the medium and long term, and which persecute long-term cardioprotective endpoints.

6. Conclusion
Given the worldwide prevalence of coronary artery disease, the development and implementation of adjuvant strategies to provide cardioprotection against I/R-induced damage is of great importance and remains a major unmet clinical need. Currently, the treatment of reperfusion injury following ischemia is primarily supportive, since no specific target-oriented therapy has been validated thus far. In the last decades, animal models have been largely used to gain novel insights on mechanisms underlying I/R injury, contributing in some cases to conceive promising targets of therapeutic relevance. Unfortunately, so far, the path to clinical application has been perceived as largely disappointing. Some potential explanations for this failure have been discussed in this review. One of the main objectives that preclinical research must pursue is a deeper understanding of the mechanisms triggered by I/R damage which lead to the deterioration of cardiac function in the medium and long term. Target for the cardioprotective strategies should not only be the survival and health of cardiomyocytes, but also of other cell types, as well as the physiology of the coronary microcirculation, often compromised as a consequence of reperfusion damage. Studies using system biology approaches and long–term endpoints are highly desirable to get a more comprehensive idea of the many processes involved and their complex network in the evolving I/R injury.
References

6. Reimer KA, Jennings RB. The “wavefront phenomenon” of myocardial ischemic cell death. II. Transmural progression of necrosis within the framework of ischemic bed size (myocardium at risk) and collateral flow. Lab Invest. 1979;40(6):633–44.


30. Schott RJ, Rohmann S, Braun ER, Schaper W. Ischemic preconditioning reduces infarct size in swine


72. Chien GL, Wolff RA, Davis RF, van Winkle DM. ‘Normothermic range’ temperature affects myocardial infarct...


97. Esposito ML, Zhang Y, Qiao X, Reyelt L, Paruchuri V, Schnitzler GR, Morine


119. Redington KL, Disenhouse T, Li J, Wei C, Dai X, Gladstone R, Manlhiot C, Redington AN. Electroacupuncture reduces myocardial infarct size and improves post-ischemic recovery by


