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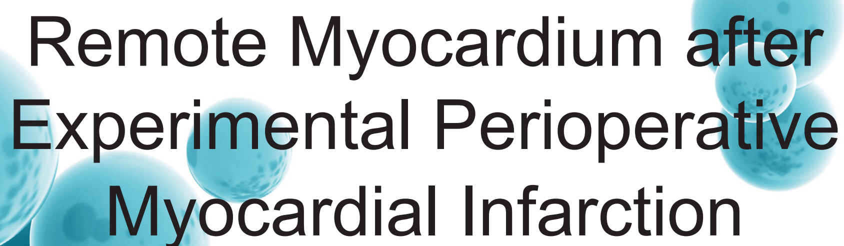
VILMA VUOHELAINEN



Remote Myocardium after Experimental Perioperative Myocardial Infarction

VILMA VUOHELAINEN

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ACADEMIC DISSERTATION
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the Board of the School of Medicine of the University of Tampere,
for public discussion in the small auditorium of building M,
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on 11 March 2016, at 12 o'clock.

UNIVERSITY OF TAMPERE

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Abstract

Perioperative myocardial infarction (PMI) may occur during cardiac surgery and lead to poor patient outcome. PMI is associated with myocardial ischemia-reperfusion injury (IRI) and may be associated with remote myocardial changes impairing recovery after cardiac surgery.

The aim of this study was to investigate, through the use of an experimental rat cardiac transplantation model, whether PMI impacts remote myocardium. We studied whether such remote myocardial changes were reversible by inhibiting the phosphodiesterase 5 and monoamine oxidase pathways by sildenafil and moclobemide, respectively.

We modified the widely used heterotopic rat cardiac transplantation model to simulate PMI. After transplantation and prior to IRI, the left anterior descending coronary artery (LAD) was permanently ligated, thus inducing a local myocardial infarction. Some rats were treated with either sildenafil 1mg/kg/day or moclobemide 10mg/kg/day. The rats were followed up to one hour, one day, two days or five days. Interstitial myocardial metabolism was studied using microdialysis. Histology, immunohistochemistry and gene expression analysis were studied from the myocardium and the intramyocardial arteries.

Distinctive remote myocardial changes were found after PMI. Histologically, remote intramyocardial artery nuclei in *tunica media* were edematous and inflammatory cells increased in the periadventitial space. Initiation of intimal bulging of remote intramyocardial arteries occurred after five days. Some inflammatory and hypoxic markers were elevated in the remote myocardium after PMI. The complement C4d and A Disintegrin And Metalloprotease 8 (ADAM8) revealed PMI. Moclobemide decreased periadventitial inflammation and sildenafil edema of remote myocardial arteries, adding to myocardial protection after PMI. Moclobemide decreased the expression of inflammatory (CD68, HMGB1) and hypoxia markers (HO-1), indicating myocardial recovery after PMI.

A rat cardiac model was established to study experimental PMI that causes remote myocardial changes. Despite the irreversibility of a local myocardial infarction area due to experimental PMI, remote myocardial changes may be attenuated by sildenafil and moclobemide.

Tiivistelmä

Sydänleikkauksen aikana mahdollisesti kehittyvä sydäninfarkti on merkittävä potilaan ennustetta huonontava riskitekijä. Siihen vaikuttaa lisäksi sydänlihaksen iskemia-reperfuusio -vaurio, joka voi aiheuttaa sydänlihakseen muutoksia edelleen heikentäen sydämen toipumista sydänleikkauksen jälkeen.

Tämän tutkimuksen tarkoituksena oli selvittää kokeellisen sydänleikkauksen aikana tapahtuvan sydäninfarktin vaikutuksia infarktialueen ulkopuoliseen sydänlihakseen. Muutosten tutkimiseksi käytimme modifioitua rotan heterotooppista sydänsiirtomallia. Selvitimme olivatko muutokset palautuvia vaikuttamalla sildenafiilin ja moklobemidin avulla fosfodiesteraasi 5- ja monoamiinioksidaasi -molekyylireitteihin.

Siirrännäisiin aiheutettiin paikallinen sydäninfarkti sulkemalla vasen etummainen laskeva sepelvaltimo. Osa rotista sai lääkehoitona sildenafiilia 1mg/kg/vrk ja osa rotista moklobemidia 10mg/kg/vrk. Tutkimusasetelmasta riippuen rottia seurattiin joko tunti, vuorokausi, kaksi vuorokautta tai viisi vuorokautta. Sydänlihassolujen aineenvaihduntaa tutkittiin soluvälinesteestä mikrodialyysin avulla. Lisäksi sydämen poikkileikkeistä tutkittiin sydänlihaskudoksen sekä sydänlihaksen sisäisten valtimoiden histologiaa, immunohistologiaa ja geenien ilmentymistä.

Kokeellisen sydänleikkauksen aikana tapahtuvan sydäninfarktin yhteydessä löysimme merkittäviä sydänlihaksen muutoksia infarktin ulkopuolisella alueella. Histologiassa havaitsimme sydänlihaksen valtimoiden seinämän sileälihassolujen tumien turvotusta ja periadventitia -tilan tulehdussoluja. Viisi vuorokautta infarktin jälkeen havaitsimme lisäksi viitteitä infarktialueen ulkopuolisten sydänlihasvaltimoiden intiman paksuuntumisesta. Tulehdusta sekä hapenpuutetta kuvaavat merkkiaineet olivat kohonneet infarktialueen ulkopuolisessa sydänlihaksessa. Komplementtijärjestelmään kuuluva C4d sekä disintegriini ja metalloproteaasi 8 -molekyyli voivat auttaa sydänleikkauksen aikana tapahtuvan sydäninfarktin diagnosoimisessa. Sildenafiilin ja moklobemidin sydänlihasta suojaava vaikutus perioperatiivisen sydäninfarktin

jälkeen voi liittyä sydänlihaksen infarktin ulkopuolisen alueen tulehduksen vähenemiseen. Moklobemidi vähensi tulehdusta (CD68, HMGB1) sekä hapenpuutetta (HO-1) kuvaavien merkkiaineiden esiintymistä, viitaten sydänlihaksen toipumiseen perioperatiivisen sydäninfarktin jälkeen.

Modifioitua rotan sydänsiirtomallia käytettiin kokeellisen sydänleikkauksen aikaisen sydäninfarktin aiheuttamien infarktialueen ulkopuolisten sydänlihasmuutosten tutkimiseen. Huolimatta siitä, että kokeellisen perioperatiivisen sydäninfarktin aiheuttamat vauriot ovat paikallisesti peruuttamattomia, voidaan infarktialueen ulkopuolisen sydänlihasalueen muutoksiin vaikuttaa sildenafiililla ja moklobemidillä.

List of original communications

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals [I-IV].

I **Vuohelainen V**, Raitoharju E, Levula M, Lehtimäki T, Pelto-Huikko M, Honkanen T, Huovila A, Paavonen T, Tarkka M, Mennander A. Myocardial infarction induces early increased remote ADAM8 expression of rat hearts after cardiac arrest. Scand J Clin Lab Invest. 2011;71:553-62.

II Mennander AA, **Vuohelainen V**, Äänismaa RS, Narkilahti S, Paavonen T, Tarkka M. Sildenafil after cardiac arrest and infarction; an experimental rat model. Scand Cardiovasc J Suppl. 2013;47:58-64.

III **Vuohelainen V**, Paavonen T, Hämäläinen M, Moilanen E, Mennander A. C4d deposition reveals myocardial infarction after cardiac arrest; an experimental study. Adv Clin Exp Med 2015;24:393-9.

IV **Vuohelainen V**, Hämäläinen M, Paavonen T, Karlsson S, Moilanen E, Mennander A. Inhibition of monoamine oxidase A increases recovery after experimental cardiac arrest. Interact Cardiovasc Thorac Surg. 2015;21:441-9.

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Abbreviations

ADAM8	A Disintegrin And Metalloprotease 8
ADAMs	A Disintegrin And Metalloproteases
AQP7	Aquaporin 7
AT	Antithrombin III
ATP	Adenosine triphosphate
Ca^{2+}	Calcium-ion
CABG	Coronary artery bypass grafting
C4d	Complement C4d
CD68	CD68 antigen
cDNA	Complementary DNA
cGMP	Cyclic guanocine monophosphate
DNA	Deoxyribonucleic acid
ECG	Electrocardiogram
eNOS	Endothelial nitric oxide synthase
FC	Fold changes
H^{+}	Proton
HIF1α	Hypoxia inducible factor 1 alpha
HIF1β	Hypoxia inducible factor 1 beta

HMGB1	High mobility group box 1
HO-1	Heme oxygenase-1
ICAM-1	Intracellular adhesion molecule-1
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
IRI	Ischemia-reperfusion injury
K^+	Potassium-ion
LAD	Left anterior descending coronary artery
M1	Proinflammatory M1 macrophage
M2	Anti-inflammatory M2 macrophage
MAO	Monoamine oxidase
MI	Myocardial infarction
mPTP	Mitochondrial permeability transition pore
mRNA	Messenger ribonucleic acid
Na^+	Sodium-ion
NaCl	Physiologic saline fluid
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
PCI	Percutaneous coronary interventions
PDE-5	Phosphodiesterase type 5
PMI	Perioperative myocardial infarction

PSU	Point score units
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SMC	Smooth muscle cell
SR	Sarcoplasmic reticulum
TGF-β	Transforming growth factor beta
TLR	Toll-like receptors
TNF-α	Tumor necrosis factor alpha
VCAM-1	Vascular cell adhesion molecule-1

1 Introduction

Perioperative myocardial infarction (PMI) is still an important cause of morbidity and mortality after cardiac surgery (Force 1990; Mackey 2006; Mangano 2006). Immediate intervention is mandatory and often necessitates emergency surgery (Laflamme 2012). Despite extensive research and treatment strategies, PMI worsens the postoperative outcome and occasionally leads to fatal complications (Chen 2007). Myocardial stunning and hibernation may ensue (Chen 1996; Haas 2000). A considerable number of patients develop left ventricular dysfunction and cardiac failure associated with myocardial remodeling (Anzai 2013; Steuer 2005).

Previous studies suggest that ongoing cardiac ischemia-reperfusion injury (IRI) impacts on dysfunction of the myocardium and leads to reperfusion arrhythmias, myocardial stunning and lethal reperfusion injury (Zhu 2013). The ongoing presence of myocardial ischemia during PMI may add to the myocardial inflammatory response and inflammatory cells infiltrate to the myocardium (Nahrendorf 2007; Yang 2002). Inflammation activates left-ventricular remodeling, leading to the expansion of myocardial infarction (MI), left ventricular dilatation and heart failure (Maekawa 2002; Tsujioka 2009).

Even during acute coronary syndrome, inflammation is not confined to the culprit vessel, but is widespread via the entire coronary circulation (Buffon 2002). After MI, inflammatory changes have been shown postmortem in the remote myocardium and the coronary arteries (Abbate 2004; Abbate 2008; Lee 2012). Remote myocardial inflammation has been found *in vivo* in a mouse model after MI (Nahrendorf 2007).

We hypothesized that IRI is an important component of PMI in regard to the remote myocardium. Experimentally, IRI may be achieved by transplanting the ischemic cardiac graft intra-abdominally back to the rat circulation. This thesis describes remote myocardial changes in an experimental setting using the modified heterotopic rat cardiac transplantation model, together with permanently ligating the left anterior descending coronary artery (LAD) to

simulate PMI. The presence of A Disintegrin And Metalloprotease 8 (ADAM8) and complement C4d (C4d) at the remote myocardium strongly suggests that PMI is not solely restricted to the local myocardium, thus explaining the dismal outcome. We also studied whether these remote myocardial changes are reversible by inhibiting phosphodiesterase-5 and monoamine oxidase (MAO) -pathways by sildenafil and moclobemide.

2 Review of the literature

2.1 Perioperative myocardial infarction – a clinical challenge

Preventive measures during cardiac surgery aim at minimizing the plausible and detrimental combined effect of cardiac arrest and IRI in order to attain a beneficial cardiac outcome. PMI is one of the leading causes of morbidity and mortality after cardiac surgery (Mackey 2006; Mangano 2006; Steuer 2005).

After coronary artery bypass graft surgery (CABG), PMI occurs in 2.8–9.8% of patients (Thielmann 2005; Yau 2008). There is no significant difference in the rate of PMI between off- and on-pump CABG (Lamy 2012). PMI is associated with increased hospitalization time and overall costs (Chen 2007; Mackey 2006). Patients with PMI have increased 30-day mortality after CABG (Järvinen 2014).

Early diagnosis of PMI is challenging; relying on traditional means, such as evaluation of ECG, creatine kinase MB and troponin release, may cause delay in diagnosis and long-term high mortality from 28% to 39% may ensue (Järvinen 2014; Thielmann 2005), including risk of heart failure and late death (Steuer 2005).

PMI occurs in 5-20% of patients undergoing percutaneous coronary interventions (PCI), mainly due to several procedural complications, such as dissection, loss of side branches, distal embolization, or distal microvascular thrombosis and platelet hyperreactivity (Hermann 2005; Lo 2014). PCI prior to CABG increases the risk for PMI and postoperative morbidity (Niclauss 2015).

2.2 Concept of perioperative myocardial infarction

After cardiac arrest and surgery, the intention is that the whole heart should recover. By definition, PMI includes MI during IRI; a local irreversible MI ensues, while the rest of the heart is susceptible to reversible IRI. PMI may

involve the destabilization of a vulnerable atherosclerotic plaque, followed by acute coronary artery thrombosis causing myocardial ischemia and infarction (Dawood 1996). PMI includes a prolonged imbalance between myocardial oxygen supply and demand (Landesberg 2001). The coronary bypass graft-related reasons for PMI are graft occlusion, kinking or overstretching, anastomotic stenosis and spasm (Bassiri 2011; Rasmussen 1997). Non-graft-related PMI includes inadequate protection of the myocardium with cardioplegia, incomplete revascularization, and distal coronary microembolization due to surgical manipulation (Yau 2008). PMI is associated with increased incidence of post-operative atrial fibrillation (Koletsis 2011). Myocardial stunning and hibernation may postoperatively worsen the prognosis after PMI (Chen 1996; Haas 2000; Yau 2008).

2.2.1 Some pathogenic mechanisms characterizing perioperative myocardial infarction

During PMI, ischemic myocardial cells suffer from nutrient and oxygen deprivation, metabolic acidosis, hyperkalemia and hypercalcemia (Kim 2006). Hypoxia causes excessive production of reactive oxygen species (ROS) (Kawaguchi 2011), complement activation (De Hoog 2014), chemokine (Kakio 2000) and cytokine release (van der Pouw Kraan 2014), and leads to myocyte and endothelial injury. Subsequent inflammatory mediators recruit neutrophils, monocytes and macrophages to the site of the damaged myocardium (Nahrendorf 2007). Infiltration of leukocytes to the ischemic myocardium includes integrin and cell adhesion molecule changes (Postadzhiyan 2008; Radecke 2015). Neutrophils cause cardiomyocyte damage by secreting substances, such as collagenase, elastase and other proteases (Zamilpa 2013), as well as the induction of intercellular adhesion molecules and ROS (Schaefer 2014).

2.3 Ischemia-reperfusion injury

Histology of cardiac IRI includes signs of cell injury, such as cell swelling, contraction of myofibrils and distruption of sarcolemma. Studies in animal

models of acute MI with IRI have shown that IRI may further increase the infarct size up to 50% of the initial size (Thiemermann 1989).

During ischemia, accumulation of mitochondrial calcium (Ca^{2+}) decreases antioxidant reserves and increases ROS, and may lead to the collapse of mitochondrial membrane potential and subsequent cell death (Kim 2006). Mitochondria may buffer small increases in intracellular Ca^{2+} via a Ca^{2+} exchanger (Kim 2006). The absence of oxygen switches cell metabolism to anaerobic respiration, resulting in the production of lactate and a drop in intracellular pH (Bond 1993).

Lactate is an end-product of anaerobic metabolism and tissue hypoxia, and several studies have shown a positive correlation between lactate levels and the risk of mortality and morbidity (Lee 2015). Pyruvate is the intermediate product between aerobic and anaerobic metabolism. During aerobic conditions, pyruvate is produced via glycolysis, largely bypassing the production of lactate (Polet 2013).

Acute myocardial ischemia increases interstitial glutamate levels after coronary artery occlusion (Liu 2010). Glutamate plays a vital role in keeping nitrogen balance in cells (Kawada 2005). Glycerol is released from damaged cells due to IRI and indicates degradation of the glycerophospholipids of the cell membranes (Metzsch 2006). Increased levels of glycerol suggest destruction of the cell membrane (Marklund 1997).

Reperfusion, in turn, induces rapid alterations in ion flux and causes rapid renormalization of pH (Bond 1993). Reperfusion after ischemia generates oxidative stress, which itself can mediate myocardial injury (Zhu 2013). Oxidative stress reduces the bioavailability of intracellular nitric oxide (NO), leading to increased neutrophil accumulation, superoxide radicals and the decrease of coronary blood flow (L`Abbate 2007; Nahrendorf 2007). Oxidative stress induces dysfunction of the sarcoplasmic reticulum, causing intracellular and mitochondrial Ca^{2+} overload (Kumar 2009). The increase in Ca^{2+} activates Ca^{2+} dependent protein kinase and proteases, such as calpain and endonucleases (French 2006). Calpain activation and subsequent action on contractile proteins have been implicated in the reduction of myofilament Ca^{2+} sensitivity observed in stunned myocardium (Kumar 2009; Urthaler 1997). Reperfusion injures vascular endothelial permeability, leading to the

recruitment of inflammatory cells. Activation of the complement cascade is complicit to the pathogenesis of reperfusion injury (Walsh 2005). NO interacts with ROS and generates various reactive nitrogen species capable of both contributing to and reducing injury (Brunner 2003; Wang 1996).

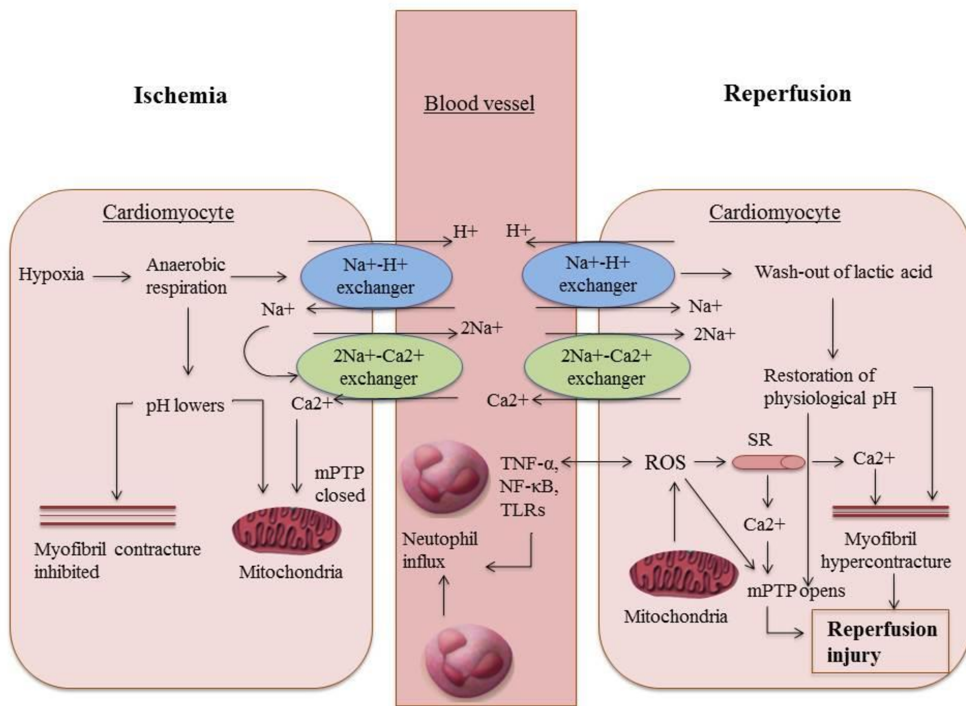


Figure 1. Schematic illustration of acute myocardial ischemia reperfusion injury (modified from Hausenloy 2013). Ca2+: calcium-ion; H+: proton; mPTP: mitochondrial permeability transition pore; Na+: sodium-ion; ROS: reactive oxygen species. SR: sarcoplasmic reticulum. TNF-α: tumor necrosis factor alpha; NF-κB: nuclear factor kappa B; TLRs: toll-like receptors.

2.4 Myocardial stunning and hibernation

Myocardial stunning and hibernation may be the consequence of distal embolization after PCI, coronary artery disease and global ischemia during CABG (Chen 1996; Haas 2000; Romero 2013). Stunning and hibernation include the generation of ROS, alteration in Ca^{2+} homeostasis, and possibly alteration in contractile protein structure (Eberhardt 2000; Kumar 2009).

Hibernation refers to decreased myocardial function during reduced myocardial perfusion, including the potential for functional recovery upon restoration of myocardial blood flow (Chen 1996). The diagnosis and restoration of coronary blood flow, with CABG or PCI, is essential, whereas untreated condition leads to heart failure (Romero 2013).

Stunning refers to the loss of myocardial contractility during reperfusion after ischemia and blood flow is usually normal to the stunned area (Eberhardt 2000). Stunned myocardium has potential to recover within hours but if unnoticed or untreated can lead to left ventricular dysfunction and heart failure (Hoole 2010).

2.5 Myocardial remodeling after myocardial infarction

Cardiac repair after MI includes three overlapping phases: the inflammatory, proliferative and maturation phase. During the inflammatory phase, free radicals are increased due to hypoxia and cardiomyocyte death. Cardiomyocyte death includes apoptosis (Baldi 2002) and necrosis of the infarct, peri-infarct and remote areas (Abbate 2008). Apoptosis represent the major independent form of myocyte death during the first hours after MI but necrosis may be a prominent mechanism during the first 24 hours (Kajstura 1996). Cellular stress such as IRI and MI induce apoptosis throught the intrinsic and extrinsic pathways (Figure 2.) and leads to release of cytochrome c from mitochondria and activation of caspases (Hori 2009). Caspases lead to cleavage of structural and regulatory cellular proteins, thus creating the apoptotic phenotype (Hori 2009). Cardiomyocyte apoptosis occurs in the infarct and peri-infarct areas during MI (Rafatian 2014). Cardiomyocyte necrosis in the infarcted heart generates damage-associated molecular patterns (Figure 2.), release of inflammatory mediators and infiltration of neutrophils and macrophages

(Lambert 2008). This inflammatory phase clears dead myocytes and matrix debris, which is crucial for cardiac wound healing (Huebener 2008).

Dying myocytes release intracellular proteins into the circulation and trigger inflammation (Fang 2015). The complement cascade, transforming growth factor beta (TGF-β), high-mobility group box -1 (HMGB1) and nuclear factor kappa B (NF-κB) lead to activation of Toll-like receptors (TLR)-mediated signaling pathways (Fang 2015; Frantz 2007) (Figure 2.). Generation of reactive oxygen species (ROS) in the ischemic myocardium induces directly pro-inflammatory cascades (Kawaguchi 2011). These events induce the synthesis of chemokines and cytokines, and upregulate the expression of adhesion molecules in endothelial cells and leukocytes, resulting in the infiltration of neutrophils into the infarction area (Deten 2002; Frangogiannis 1998; Yan 2013). Recruitment of neutrophils is followed by infilitration of monocytes and lymphocytes to the myocardium (Nahrendorf 2007).

Neutrophils and macrophages phagocytose dead cells and matrix debris and produce a variety of cytokines and chemokines, proteases and growth factors, mediating remodeling (Lambert 2008). During the proliferative phase, the expression of inflammatory mediators is suppressed, and most inflammatory cells undergo apoptotic death, activated myofibroblasts produce extracellular matrix proteins, and an extensive microvascular network evolves (Cleutjens 1995). During maturation of the scar, fibroblasts and vascular cells undergo apoptosis and scar-containing cross-linked collagen bundles are formed (Ruiz-Villalba 2015). The cellular and molecular events associated with the healing of MI influence left-ventricular remodeling. Remodeling may lead to expansion of MI, left ventricular dilatation and non-infarct hypertrophy. Macrophages in the infarction site impair cardiac repair, causing cardiac dilatation and heart failure (Maekawa 2002; Tsujioka 2009).

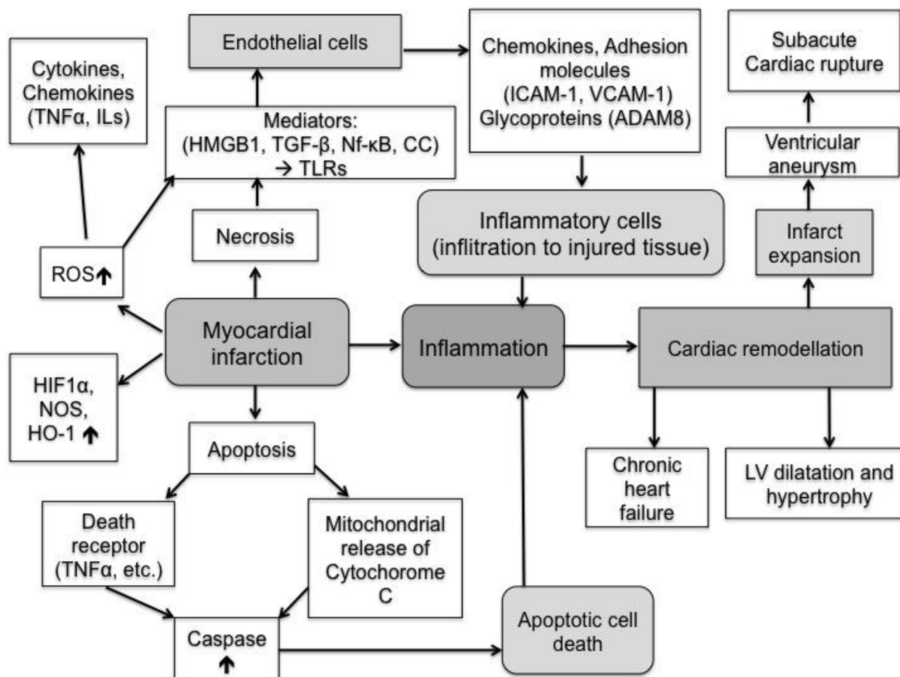


Figure 2. Schematic illustration representing increased inflammation after myocardial infarction and the association with left-ventricular remodeling (modified from Anzai 2013 and Fang 2015). ADAM8: A Disintegrin And Metalloprotease 8; CC: Complement cascade; HIF1α: Hypoxia inducible factor 1 alpha; HMGB1: High Mobility Group Box -1; HO-1: Heme oxygenase-1; ICAM-1: intracellular adhesion molecule-1; ILs: Interleukins; LV: Left ventricle; NF-κB: Nuclear factor kappa B; NOS: Nitric oxide synthases; ROS: Reactive oxygen species; TGF- β: Transforming growth factor beta; TLRs: Toll-like receptors; TNFα: Tumor necrosis factor alpha; VCAM-1: Vascular cell adhesion molecule-1.

2.6 Remote myocardium

Acute MI induces remote myocardial inflammation (Abbate 2004). The inflammation is not confined to the infarction site only (Buffon 2002). Remote myocardial inflammation including T-lymphocytes (Abbate 2004) and macrophages (Lee 2012) was found postmortem in patients with a history of MI (Abbate 2008). An increased number of macrophages was observed in the noninfarcted myocardium in an experimental model of MI (Nahrendorf 2007)

as well as in a clinical study (Carrick 2015). Cellular autoimmunity to cardiac myosin is present in patients with a recent history of MI (Moraru 2006). Subsequently, the remodeling of small intramyocardial arteries distal to the flow-limiting epicardial coronary stenosis occurs and restricts blood flow, leading to the incomplete recovery of myocardial function (Hong 2002). The inflammatory response to acute MI may act as a trigger to the remote neointimal hyperplasia of the intramyocardial arteries (Takaoka 2006).

2.7 Experimental surgery and perioperative myocardial infarction

The rat heterotopic cardiac transplantation model was orginally described by Ono and Lindsey in 1969 as a means to study transplantation rejection (Ono 1969). After introducing the model, it has been widely used for functional studies of ventricular unloading (Navaratnarajah 2013) and rejection (Ricci 2010). The model is also suitable for other species such as murine (Doenst 2001), rabbit (Eich 1993) and dog (Demers 2001). The model was later modified to study IRI (Perna 1996), the effect of a left ventricle assist device (Oriyanhan 2007), xenotransplantation (Adams 1999; Dedja 2005), and remodeling after MI (Suzuki 2002).

2.8 Selected markers of ischemia and inflammation

2.8.1 ADAM8

ADAM8 is a member of A Disintegrin And Metalloproteases (ADAMs). These are membrane-bound glycoproteins that exert potential impact on the inflammatory response associated with cell-cell and cell-matrix regulations during tissue remodeling (Li 2009). Briefly, ADAMs contain pro- and metalloprotease, disintegrin and cystein-rich domains, as well as epidermal growth factor-like, transmembrane and a sytoplasmic domains (Krätzschmar 1996). ADAM9, -10, -12, -15, -17 and -19 have been studied in the myocardium, and increased ADAM15 expression has been demonstrated in a rat model of MI (Li 2009).

ADAM8 is mainly expressed in the cells of the immune system, particulary monocytes and granulocytes (Yoshiyama 1997). Lipopolysaccharide, γ-interferon and tumor necrosis factor α (TNF-α) induce the expression of ADAM8 (Kataoka 1997). ADAM8 is increased during experimental spinal cord injury (Mahoney 2009). Reduced mRNA expression of ADAM8 is associated with decreased transplant arteriosclerosis in mice after allograft transplantation (Luckow 2004). An allelic variant of ADAM8 (rs2995300) is associated with the risk of MI (Levula 2009). ADAM8 promotes tumor dissemination in invasive breast cancer (Romagnoli 2014) and is an indicator of poor prognosis in colorectal cancer (Yang 2014).

2.8.2 iNOS and eNOS

NO is synthesized by nitric oxide synthases (NOS), which have three different isoforms: endothelial nitric oxide (eNOS), neuronal nitric oxide and inducible nitric oxide (iNOS) (Tsutsui 2010). All of the NOS isoforms are expressed in the cardiovascular system and contribute to the formation of NO (Tsutsui 2010).

NO is a chemical messenger which has a crucial protective role against cardiac IRI (Heinzel 2008; Merx 2014). It regulates mitochondrial respiration by improving myocardial oxygenation; inactivates caspases, thereby decreasing myocyte apoptosis (Heusch 2000); and contributes to myocardial hibernation by reducing oxygen consumption as well as preserving Ca^{2+} sensitivity and contractile function (Kobara 2003). NO regulates cardiac coronary blood flow and improves myocardial relaxation, thus optimizing cardiac performance (Rassaf 2006).

Decreased circulating eNOS is associated with left ventricular dysfunction and increased infarct size (Merx 2014). During ongoing IRI, NO decreases mainly due to dysfunctional endothelium and eNOS inactivity, causing decreased vasodilatation (Jones 1999). eNOS upregulation by cardioprotective drugs, such as statins, increase bioavailable NO and reduce myocardial necrosis (Cho 2014; Jones 2002).

Cytokines and endotoxins, together with hypoxia, increase iNOS expression (Tsutsui 2010). During MI, myocardial iNOS expression is upregulated and it exerts negative effects on cardiac function (Saito 2002). Increased iNOS may

reflect an alternative mechanism for NO production during ischemic heart failure and cardiomyopathy (Cho 2014; Haywood 1996). Selective iNOS inhibition improves myocyte contractility and protects from cardiomyocyte death during IRI (Ramasamy 2004).

2.8.3 ICAM-1, VCAM-1 and NF-κB

Endothelial cells and cardiomyocytes express intracellular adhesion molecule-1 (ICAM-1) in response to cytokine stimulation. ICAM-1 serves as an adhesive molecule for neutrophils (Schaefer 2014). ICAM-1 participates in neutrophil-mediated myocardial inflammation and damage (Postadzhiyan 2008).

Vascular cell adhesion molecule-1 (VCAM-1) is expressed on the endothelial cell surface (Cook-Mills 2002) and mediates inflammatory cell adhesion to the vascular endothelium (Tudor 2001). Elevated VCAM-1 serum levels predict an increased risk for cardiovascular events (Postadzhiyan 2008). VCAM-1 expression is increased during MI (Radecke 2015).

NF-κB is a protein complex that controls the transcription of DNA and is activated by various intra- and extra-cellular stimuli, such as cytokines, oxidized low-density lipoprotein and intercellular adhesion molecules (Beg 1996; Mathur 2011). NF-κB plays a central role in regulating the expression of various inflammatory genes and apoptosis in many cell types (Beg 1996). NF-κB is an important mediator of early inflammation after MI (Frantz 2007). NF-κB mediated activation of monocyte chemoattractant protein-1 during IRI may be responsible for increased monocyte infiltration in the injured rat kidney (Sung 2002). NF-κB inhibition decreases the risk of myocardial injury (Kim 2010) and reduces fibrosis in the left ventricle (Torina 2015) in a rat IRI+MI model.

2.8.4 Aquaporin-7

Aquaporin-7 (AQP7) belongs to the aquaglyceroporin subgroup of the aquaporin family. Other aquaglyceroporins are aquaporin -1, -3, -4, -5, -9 and -10 (Butler 2006). Aquaporins form cell transmembrane channels for water and glycerol (Ishibashi 1997). AQP7 acts as a glycerol and water facilitator in

cardiomyocytes (Hibuse 2009). Excessive AQP7 expression may be an early marker of tissue damage after IRI and edema (Liu 2010).

2.8.5 C4d

Complements are part of the innate immune system owing a crucial role in both innate and adaptive immunity, and in the elimination of circulating immune complexes. The complement cascade includes three molecular pathways: the classical, the lectin and the alternative pathways (Regele 2002; Roos 2001). Complements have several activators in ischemic myocardium, such as endogenous ligands, HMGB1 and mitochondrial components (De Hoog 2014; Kagiyama 1989).

C4d is a relatively stable end-product molecule of the classical complement pathway (Regele 2002). However, some argue that C4d is also generated via the lectin pathway (Roos 2001).

The complement system is involved in the pathogenesis of several inflammatory and immunological diseases, such as sepsis, acute respiratory distress syndrome, multiple organ failure and IRI (Ganter 2007; Walsh 2005). Complement activation is an early marker of tissue destruction and correlates with the severity of the injury in trauma patients (Ganter 2007). C4d deposition is associated with poor graft survival in kidney transplantation (Böhmig 2002). Complements are activated during IRI and MI (Walsh 2005) and are associated with the size of MI (De Hoog 2014). The expression of C4d reveals necrotic myocytes (Jenkins 2010).

2.8.6 TGF-β

TGF-β includes three structurally similar isoforms (TGF-β1, β2 and β3) (Cheifetz 1990). These isoforms have the same cell surface receptors as well as similar cellular targets, and are involved in a variety of biological processes (Cheifetz 1990). TGF-β1 is the predominant isoform, and it is found in almost every cell (Kapur 2011). TGF-β has a variety of activating molecules, such as plasmin and ROS (Barcellos-Hoff 1996; Lyons 1990).

High levels of TGF-β are expressed in the heart and rapidly activated during MI (Ellmers 2008). TGF-β plays a key role in hypertrophic and fibrotic remodeling of the heart by regulating cardiomyocyte growth, fibroplast activation and extracellular matrix deposition (Ellmers 2008; Okada 2005). The TGF-β pathway may be important in the expression of pro-inflammatory cytokines and chemokines during MI (Feinberg 2004). TGF-β has been shown to induce cardiomyocyte apoptosis (Schneiders 2005). Association with increased TGF-β and reduced ejection fractions after MI has been found (Talasaz 2013).

2.8.7 HMGB1

High Mobility Group Box-1 (HMGB1) is a non-histone protein residing in the nuclei of a variety of cells. It usually stabilizes the nucleosome structure by binding to DNA and may act as a transcriptional regulator through local distortion of the DNA structure (Goodwin 1973). HMGB1 is an inflammatory mediator with two distinctive release mechanisms: the passive release from inside the cell due to cell necrosis, and the active secretion from inflammatory cells (Gardella 2002; Scaffidi 2002). HMGB1 stimulates cytokine release and infiltration of inflammatory cells, such as macrophages and dendritic cells (Xu 2011). It also activates NF-κB to promote expression of inflammatory cytokines (Mathur 2011).

HMGB1 mediates lung injury, systemic inflammatory response syndrome and acute respiratory distress syndrome (Ueno 2004). HMGB1 increases atherosclerotic changes (Kalinina 2004). Decreased expression of HMGB1 may decrease smooth muscle cell (SMC) migration (Chen 2012; Yang 2012). Increased HMGB1 levels are associated with MI, cardiac failure, cardiac rupture and cardiac death (Andrassy 2008; Kohno 2009). To date, experimental studies are rather controversial in regard to the detrimental effect of HMGB1 after MI.

2.8.8 HIF1α

Hypoxia inducible factor 1 alpha (HIF1α) plays a crucial role during hypoxia (Iyer 1998). HIF1 is a heterodimer consisting of two subunits HIF1α and hypoxia inducible factor 1 beta (HIF1β). During hypoxia, HIF1α rapidly accumulates in

the cell nucleus, binds to HIF1β, and activates a host of genes involved in the cellular response to hypoxia (Iyer 1998).

HIF1α impacts on mitochondrial activity and ROS formation (Callapina 2005; Chandel 2000). HIF1α regulates the transcription of lactate dehydrogenase-5, which catalyses the reversible transformation of pyruvate to lactate. Increased level of HIF1α is an early response to MI (van der Pouw Kraan 2014).

2.8.9 IL-6

Expression and production of the proinflammatory cytokine interleukin 6 (IL-6) is upregulated during MI and inflammation (Deten 2002). IL-6 is secreted by macrophages, lymphocytes, fibroblasts, endothelial cells and SMC (Howells 1991). IL-6 expression is controlled by the cytokines interleukin-1, interleukin-4 and TNF-α (Gwechenberger 1999).

IL-6 has two separate intracellular signaling pathways. In the classical signaling pathway, IL-6 binds on the cell surface to its membrane- bound receptor and in the second signalling pathway, whereas IL-6 binds to a soluble form of the receptor (Anderson 2013). IL-6 may increase vasoconstriction and ROS production (Wassmann 2004), leading to macrophage growth and maturation (Mitani 2000).

IL-6 may exert a role in atherosclerosis (Schieffer 2000). IL-6 is involved in IRI, inflammation, myocardial remodeling and scar tissue formation after MI (Hilfiker-Kleiner 2010). Levels of IL-6 in the circulation have been shown to correlate with the size of MI (Karpinski 2008). In a rodent model of MI, the intramyocardial cytokines, such as TNF-α and IL-6, are upregulated, and the mRNA expression is elevated in the myocardium (Deten 2002).

2.8.10 HO-1

Heme oxygenase-1 (HO-1) is an enzyme that degrades heme to bilirubin (Chen 2005). HO-1 is induced under various oxidative stress conditions and expressed in the endothelium and macrophages during atherosclerosis (Chen 2005). HO-1 participates in the anti-inflammatory M2 phenotype transformation of macrophages (Weis 2009). HO-1 is induced by heavy metals, endotoxin, pro-

inflammatory cytokines, heme, NO and hypoxia (Otterbein 2000). HO-1 decreases SMC proliferation, endothelial apoptosis and angiogenesis (Duckles 2015). HO-1, together with the byproducts of HO-1, such as bilirubin, carbon monoxide and ferritin, protect the cell from oxidative damage (Otterbein 2000).

Elevated HO-1 expression increases eNOS activity (L´Abbate 2007). HO-1 induction lowers the release of lactate dehydrogenase and creatine phosphokinase and ameliorates mitochondrial membrane potential in cardiomyocytes (Issan 2014). Up-regulation of HO-1 after MI reduces the size of MI and mortality in an experimental MI model (Kusmic 2014).

2.8.11 CD68

CD68 antigen (CD68) is a transmembrane glycoprotein highly expressed in monocytes and macrophages (Holness 1993). CD68 is a widely used marker of macrophage phenotype (Sheu 2013).

In steady state, macrophages reside in many normal tissues, including the heart (Nahrendorf 2007). Circulating monocytes accumulate to inflammatory sites and may develop into macrophages (Swirski 2009). Macrophages may be categorized into the proinflammatory M1 and anti-inflammatory M2 fenotypes. M1 macrophages secrete primarily proinflammatory cytokines, whereas M2 macrophages secrete anti-inflammatory cytokines (Bourlier 2008; Hirata 2011).

During inflammation, M1 macrophages are mostly replaced by M2 macrophages (Arnold 2007). CD68 macrophages are expressed in atherosclerotic coronary artery plaques (Schieffer 2000). After acute MI, CD68 expression levels are upregulated in the circulation (Sheu 2013). Increased numbers of myocardial monocytes and macrophages are present during heart failure and in the penumbra area of MI in mice (Lee 2012).

2.9 Insight into perioperative myocardial protection and surgery

2.9.1 Cardioplegia

Cardioplegia affords myocardial protection during cardiac surgery. The optimal composition of cardioplegia is under debate (Scrascia 2011). Crystalloid cardioplegia causes intra-operative hemodilution and increases blood transfusion requirement as compared to blood cardioplegia (Günday 2014). Retrograde cardioplegia versus antegrade cardioplegia may lead to higher myocardial lactate consentration and higher myocardial oxygen uptake (Ahlsson 2012). Custodiol (Bretschneider´s histidine-tryptophan-ketoglutarate) is an intracellular crystalloid cardioplegic solution used for myocardial protection (Scrascia 2011). Some of the studies have shown that cold blood cardioplegia is equally cardioprotective than Custodiol (Braathen 2011; Viana 2013). Single antegrade dose of Custodiol offers safe and satisfactory myocardial protection (Matzelle 2014), but not optimal right ventricle protection as compared with intermittent warm blood cardioplegia (Gaudino 2013). Custodiol may afford relatively long myocardial protection (Matzelle 2014).

2.9.2 Some potential treatment strategies of perioperative myocardial infarction

Treatment of PMI includes CABG, PCI and pharmacological support (Head 2014). The ultimate treatment strategy is to confine the irreversible MI area while protecting the reversible remote myocardium. Traditional management of patients with MI includes limiting infarction size, reducing arrhythmias and attenuating progressive left ventricle remodeling (Table 1.).

Table 1. Some examples of pharmacological interventions aiming to treat myocardial infarction (MI) or perioperative myocardial infarction (PMI). * Experimental study

Pharmacological intervention	Effect after MI/PMI	Adverse effects	Reference
Cyclosporine A	Reduces MI size	No adverse effects	Piot 2008
Exenatide (GLP-1 analog)	Reduces MI size by 30%	No major adverse effects	Lønborg 2012
Atrial natriuretic peptide	Reduces MI size	Hypotension	Kitakaze 2007
Adenosine	Reduces MI size	Low incidence of adverse events	Ross 2005
Glucose-insulin-potassium infusion	Lower in-hospital mortality	Similar as in placebo	Selker 2012
Metoprolol	Reduces MI size	No excess adverse events	Ibanez 2013
Pexelizumab (an anti-C5a antibody)	6.7% reduction in PMI/death (nonsignificant)	Similar as in placebo	Smith 2011
Cariporide (sodium hydrogen transporterantagonist)	Reduces PMI	Increases overall mortality	Mentzer 2008
Acadesine (adenosine analogue)	Reduces PMI and mortality decreases	Similar as in placebo	Mangano 2006
Metformin	Reduces post-PCI MI	No reported adverse effects	Li 2014
Anakinra-1 (IL-1 receptor inhibitor)	Prevent post-MI heart failure	Similar as in placebo	Abbate 2010

Angiotensin converting enzyme inhibition	Reduces cardiovascular mortality No improvement in clinical outcome after CABG	Cough, well tolerated Cough, hypo/hypertensio	Bertrand 2009 Rouleau 2008
Aspirin	Reduces perioperative MI	Increase bleeding	Hastings 2015
Aldosterone antagonist	Lowers proBNP levels and mortality Reduces myocardial apoptosis	No difference compared to placebo	Montalescot 2014 Rafatian 2014*
Statins (3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors)	Reduces PMI	No excess adverse events	Schouten 2009
Non-steroidal anti-inflammatory drug	Increase mortality, left ventricular dysfunction Increased risk for MI	 Increased risk of bleeding	Timmers 2007* Olsen 2012

Potential therapeutic strategies to prevent or reverse left-ventricular remodeling include modulators of NO activity and signalling pathways (Das 2005), statins (Wang 2015), phosphodiesterase 5A inhibitors such as sildenafil (Korkmaz 2009), anti-oxidant strategies (Ertracht 2011; Kunduzova 2002), modulators of inflammation and pro-inflammatory cytokines (Deten 2002; Hilfiker-Kleiner 2010), metalloprotease inhibitors (Mani 2015) and stem cell transfer (Gao 2015). Ischemic pre- and postconditioning may reduce myocardial injury in patients with acute MI (Staat 2005; White 2015).

2.9.3 Sildenafil and myocardial protection

Sildenafil is a potent and selective inhibitor of cyclic guanosine monophosphate (cGMP) -specific phosphodiesterase type 5 (PDE-5). PDE-5 is responsible for the degradation of cGMP by hydrolysis to guanosine 5´ -monophosphate. The inhibition of PDE-5 by Sildenafil preserves the cGMP pool, potentiates downstream signaling, and causes smooth muscle relaxation and vasodilatation (Turko 1999). Decreased catabolism of cGMP causes the opening of mitochondrial ATP-sensitive K^+ -channels through the activation of protein kinase G (Han 2002). Orginally, sildenafil was developed to treat angina pectoris and subsequently became a drug for erectile dysfunction (Boolell 1996). Sildenafil has also been extensively studied to treat pulmonary hypertension (Wilkens 2001) and may have protective effects on the myocardium after resuscitation (Zhang 2014). Sildenafil mediates protection by iNOS and eNOS (Salloum 2003). The resulting increase in NO is thought to be one of the mediators of the subsequent protective signaling cascade (Das 2005; Ockaili 2002).

In an experimental kidney IRI model, sildenafil has antiapoptotic effects mediated by iNOS and eNOS (Choi 2009). Sildenafil administered before ischemia reduces the size of MI in an *in vivo* rabbit model (Ockaili 2002) through inhibition of iNOS (Salloum 2003). Sildenafil protects mouse cardiomyocytes against necrosis and apoptosis in an iNOS- and eNOS-dependent, NO signaling pathway (Das 2005). Pretreatment with sildenafil attenuates apoptosis and left ventricular dysfunction in a mice model of doxorubicin-induced cardiomyopathy (Fisher 2005). Sildenafil provides a pharmacological preconditioning effect on the endothelium (McLaughlin 2014).

2.9.4 Moclobemide and myocardial protection

The cardiac muscle is closely dependent on oxidative energy supplied by mitchondrial fatty acid β-oxidation, the electron transport chain, and oxidative phosphorylation (Sack 1996). Cardiomyocytes are dependent on mitochondrial bioenergetics and metabolism, and the cardiac muscle is especially vulnerable to mitochondrial dysfunction (Lou 2013). During IRI, the mitochondria are considered to be the main ROS source of the cell (Carpi 2009). ROS produced

by MAO triggers the signaling pathways, leading to cell proliferation and hypertrophy or apoptosis (Bianchi 2005; Pchejetski 2007).

MAOs A and B (MAO-A and MAO-B) are flavoenzymes closely associated with the outer membrane of mitochondria (Hauptmann 1996). The enzymes catalyze the oxidative deamination of amines (Pizzinat 1999). MAOs are involved in the degration of neutrotransmitters within the central nervous system and are also implicated in the metabolism of biogenic amines in vascular cells and other peripherial tissues (Sturza 2013; Zhao 2013). MAO-A and MAO-B have similar substrate specificy, oxidaze dopamine (Sader-Mazbar 2013), serotonine (Nishi 2006), norepinephrine (Heinonen 1993) and tyramine (Hauptmann 1996). ROS, hydrogen peroxide and other oxidation products generated during the oxidation of amines may trigger cell signaling and lead to oxidative stress (Pizzinat 1999). MAO- inhibitors are in clinical use for the treatment of depression and several neurodegenerative conditions, including Parkinson’s disease and Alzheimer´s disease (Sano 1997; Zhao 2013). The systemic MAO inhibition leads to the accumulation of catecholiamines, which increase the sympathetic activity and cause hypertension (van Haelst 2012).

Moclobemide is a reversible MAO-A inhibitor (van Haelst 2012). MAO-A catalyzes the degradation of biogenic amines, such as tyramine and serotonin, thus generating ROS (Bianchi 2005) and SMC proliferation (Day 2006).

TVP1022, an S-enantiomer of rasagiline and MAO inhibitor, reduces the infarction size in a rat model of MI, which appears to involve the inhibition of the mitocondrial permeability transition pore (Ertracht 2011). Serotonin has been shown to stimulate SMC migration and proliferation in the pulmonary artery (Day 2006; Lee 2001), and the signaling may be mediated via the sphingolipid pathway (Coatrieux 2007). Generation of hydrogen peroxide by MAO attenuates the release of endothelial NO (Sturza 2013).

2.9.5 Heparin and outcome

Heparin is a highly sulfated glycosaminoglycan with an antithrombin binding site. It acts as an anticoagulant and prevents the formation of clots (Bagheri 2014). Heparin binds to the enzyme inhibitor antithrombin III (AT), causing a conformational change and activation of AT. Activated AT inactivates thrombin

and proteases, such as factor Xa and IXa (Bagheri 2014). Heparin and its low molecular-weight derivatives are effective at preventing deep-vein thromboses, pulmonary emboli and blood clotting after surgery (Bergqvist 2002). Heparin is wildly used in cardiothoracic surgery to counteract the coagulant effects of thrombin (Bagheri 2014). During cardiopulmonary bypass, heparin is administered in a weight-based manner according to measured clotting times (Radulovic 2015).

Heparin-coated cannulas reduce thrombosis (Borowiec 1992) and are widely used in the clinics (Lindholm 2004). Cardiopulmonary bypass together with heparin cause activation of the complement system and white blood cells (Kutay 2006; Shastri 1997). Low molecular-weight heparin improves the survival of cardiac allografts (Shapira 1999). Heparin has been shown to reduce neointimal proliferation in experimental studies (Li 2015) as well as human coronary artery SMC migration (Kohno 1998).

3 Aim of the study

The aim of the study was to investigate whether experimental PMI and IRI are associated with remote myocardial changes. We hypothesized that experimental PMI includes the interaction of local MI area with the remote myocardium after IRI. Using a modified syngeneic and heterotopic rat cardiac transplantation model, we investigated whether early pharmacological intervention may impact these changes.

More specifically, the intent was to investigate:

- Early remote myocardial and intramyocardial histopathological changes after IRI and PMI with ADAM8 [I] and whether C4d positivity is associated with PMI after cardiac arrest [III]
- Some potential markers of hypoxia and inflammation associated with PMI and IRI [I-IV] and the impact of sildenafil [II] and moclobemide [IV] on these early remote myocardial changes

4 Materials and methods

4.1 Experimental animals

Fischer 344 rats were obtained from Harlan Laboratories, Netherlands. Male rats weighing 175-270g, served both as donors and recipients, underwent a heterotopic cardiac transplantation. Normal hearts from non-operated rats served as controls. The rats were kept in a Tampere University vivarium and received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1996). The experimental work was approved by the Finnish State Provincial Office.

4.2 Surgical procedure

Rats were anesthetized with Sevofluran inhalation and with Barbiturate (Mebunat vet®; 5mg/100g) intraperitoneally [IV] or with mixture of Ketamine (Ketalar®; 7.5 mg/100g) and Metedomidine (Domitor®; 0.05 mg/100g) intraperitoneally [I-III]. After anesthesia, the thoracic cavity was surgically opened and the donor heart exposed. The ascending aorta of the cardiac graft was infused with antegrade infiltration of 2 ml of cold (4°C) temperature Custodiol (Custodiol®) without heparinization [III], or with heparin [IV] or with cold (4°C) temperature physiologic saline fluid without heparinization [I-II] to arrest the heart. The graft was preserved in a cold Custodiol/physiologic saline-solution while the recipient rat was prepared. Heterotopic cardiac transplantation was performed intra-abdominally by joining the graft aorta to the abdominal aorta, and the graft pulmonary artery to the inferior vena cava of the recipient (Figure 3.). Total warm ischemia time before total graft reperfusion was 20-30 min after cardiac arrest. In some of the cardiac grafts, the LAD was also ligated permanently at its proximal part with a single 7-0

suture to yield PMI (Figure 4.). After the procedure, Buprenorfin (Vetergesic®; 0,3mg/ml) 0,01mg/100g and Carprofen (Norocarp®/Rimadyl®; 50mg/ml) 0,5mg/100g were given subcutaneously for pain relief.

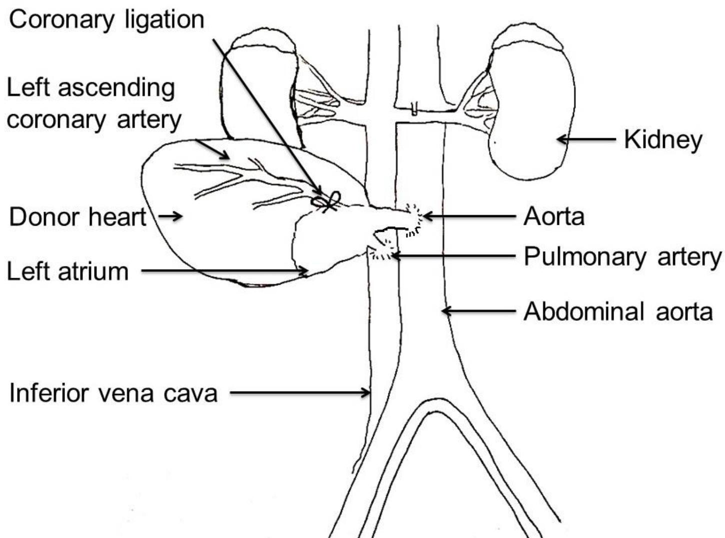
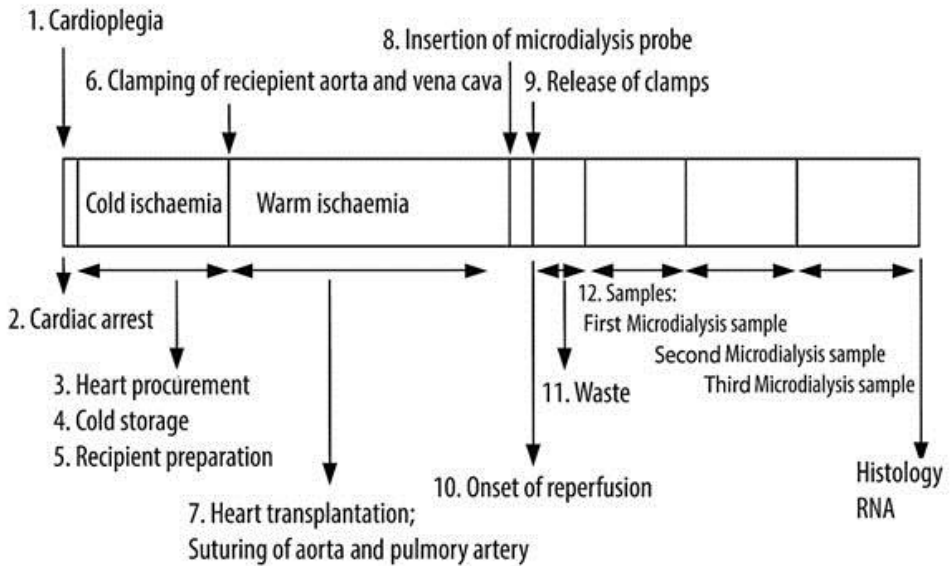
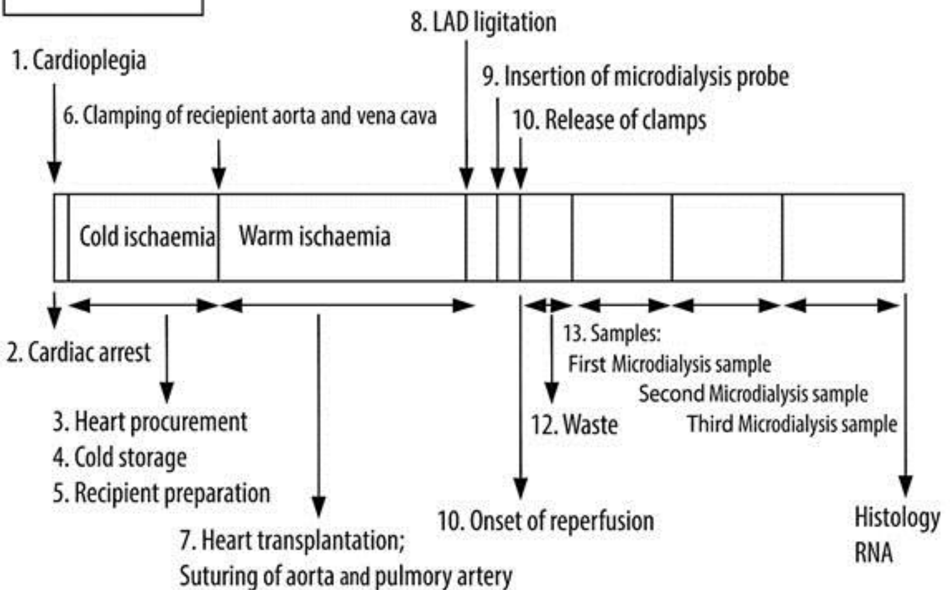
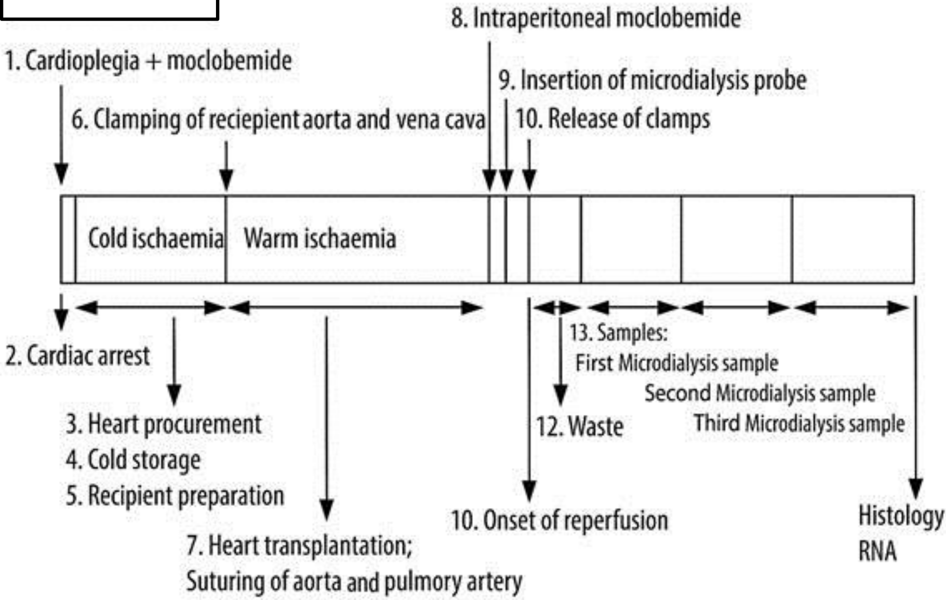
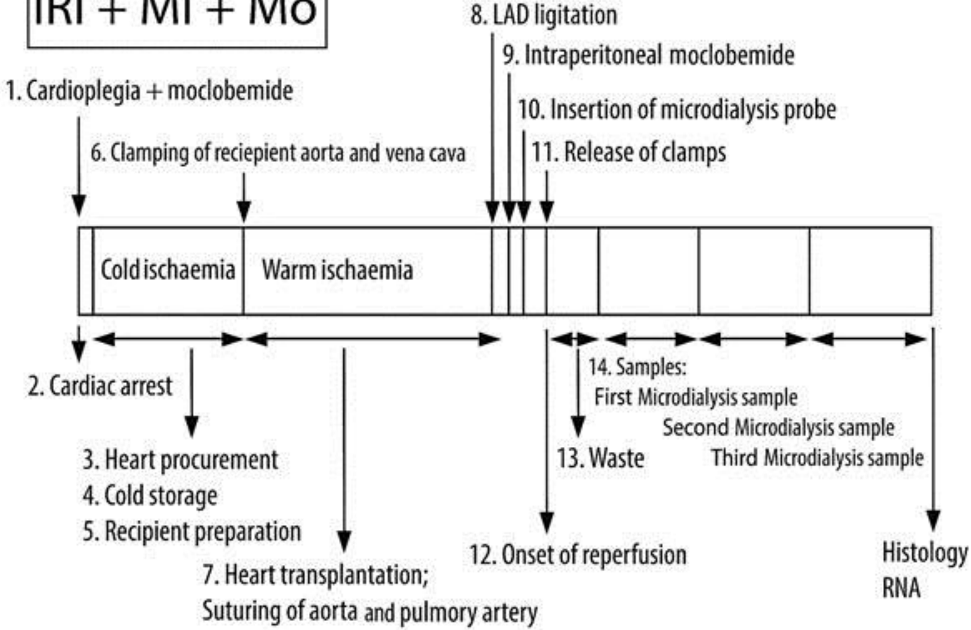


Figure 3. Schematic illustration of the experimental procedure.


IRI
1. Cardioplegia
8. Insertion of microdialysis probe
6. Clamping of reciepient aorta and vena cava
9. Release of clamps
Cold ischaemia
Warm ischaemia
2. Cardiac arrest
12. Samples:
First Microdialysis sample
Second Microdialysis sample
Third Microdialysis sample
3. Heart procurement
4. Cold storage
5. Recipient preparation
11. Waste
10. Onset of reperfusion
Histology
RNA
7. Heart transplantation;
Suturing of aorta and pulmory artery


IRI + MI
8. LAD ligitation
1. Cardioplegia
9. Insertion of microdialysis probe
6. Clamping of reciepient aorta and vena cava
10. Release of clamps
Cold ischaemia
Warm ischaemia
2. Cardiac arrest
13. Samples:
First Microdialysis sample
Second Microdialysis sample
Third Microdialysis sample
3. Heart procurement
4. Cold storage
5. Recipient preparation
12. Waste
10. Onset of reperfusion
Histology
RNA
7. Heart transplantation;
Suturing of aorta and pulmory artery


IRI + Mo
1. Cardioplegia + moclobemide
6. Clamping of reciepient aorta and vena cava
8. Intraperitoneal moclobemide
9. Insertion of microdialysis probe
10. Release of clamps
Cold ischaemia
Warm ischaemia
2. Cardiac arrest
3. Heart procurement
4. Cold storage
5. Recipient preparation
7. Heart transplantation;
Suturing of aorta and pulmory artery
10. Onset of reperfusion
12. Waste
13. Samples:
First Microdialysis sample
Second Microdialysis sample
Third Microdialysis sample
Histology
RNA


IRI + MI + Mo
1. Cardioplegia + moclobemide
6. Clamping of reciepient aorta and vena cava
8. LAD ligitation
9. Intraperitoneal moclobemide
10. Insertion of microdialysis probe
11. Release of clamps
Cold ischaemia
Warm ischaemia
2. Cardiac arrest
3. Heart procurement
4. Cold storage
5. Recipient preparation
7. Heart transplantation;
Suturing of aorta and pulmory artery
12. Onset of reperfusion
13. Waste
14. Samples:
First Microdialysis sample
Second Microdialysis sample
Third Microdialysis sample
Histology
RNA

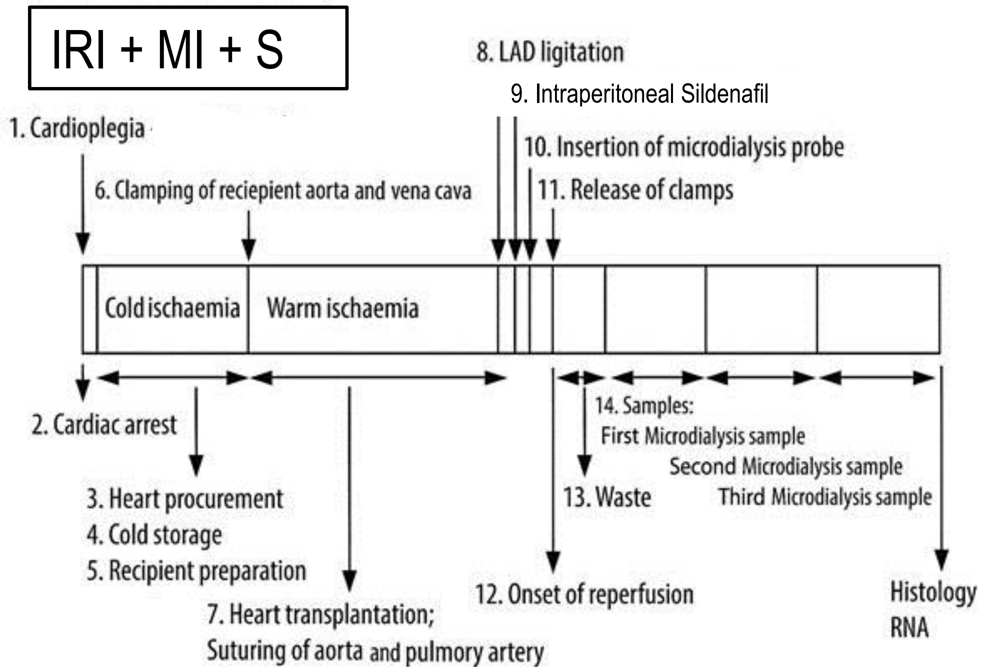


Figure 4. Surgical procedure illustrated as a time line. IRI: hearts undergoing transplantation with ischemia-reperfusion injury only; IRI+Mo: transplanted cardiac grafts with moclobemide -treatment; IRI+MI: transplanted grafts with left anterior descending coronary artery (LAD) ligation; IRI+MI+Mo: transplanted cardiac grafts with LAD occlusion and moclobemide; IRI+MI+S: transplanted grafts with LAD occlusion and sildenafil -treatment. RNA: ribonucleic acid (modified from [IV]).

4.3 Medication

Twenty-two rats were treated with sildenafil 1 mg/kg/day s.c. [II]. Twenty-eight rats were treated with moclobemide 10 mg/kg/day s.c. [IV].

Table 2. Study protocol. IRI (ischemia-reperfusion injury), IRI+MI (ischemia-reperfusion injury with myocardial infarction). The numbers indicate the amount of rats in every study group. NaCl: physiologic saline fluid.

Article	IRI	IRI+MI	Sildenafil+ IRI/IRI+MI	Moclobemide+ IRI/IRI+MI	PLEGIA	ANESTHESIA
I	21	27	-/-	-/-	NaCl	Sevoflurane + Ketamine/ Medetomidine
II	23	39	-/22(IRI+MI)	-/-	NaCl	Sevoflurane + Ketamine/ Medetomidine
III	7	9	-/-	-/-	Custodial	Sevoflurane + Ketamine/ Medetomidine
IV	14	14	-/-	14(IRI)/ 14(IRI+MI)	Custodial + Heparin	Sevoflurane + Barbiturate

4.4 Graft patency

Graft patency was achieved by means of palpation using a score from 0 to 6; 0 indicated no pulse, 2 indicated weak pulsation, and 6 meant normal contractility and strong pulsation [I-IV].

4.5 Microdialysis [IV]

Immediately after reperfusion, a thin microdialysis catheter (CMA 70; 20,000 dalton cutoff, 0.6 mm diameter; CMA Microdialysis AB) was introduced in the myocardium adjacent to the LAD [IV]. The catheter was perfused using a CMA 107 microinfusion pump with saline (0,2 μl/min). After a tissue stabilization period of 15 min, three samples were collected in cycles of 10 min up to 45 min. The collection vials with the microdialysis perfusate were analyzed with a CMA 600 Microdialysis Analyzer (CMA Microdialysis AB) using a spectrophotometric assay. Glucose, pyruvate, lactate, glycerol and glutamate were measured.

4.6 Tissue samples

The recipient rats were sacrificed according to the study protocol. 0.5-1 hour after reperfusion [I, IV], one day after reperfusion [I], two days after reperfusion [I-III] and five days after reperfusion [IV]. The basal part of the heart graft was frozen [II-III] or stored in a RNA stabilization solution (RNAlater, Applied Biosystems, CA, USA) [I, IV] for quantitative RT-PCR analysis. The apex part of the heart was fixed in formalin and embedded in paraffin.

4.7 Histology

Five- µm thick sections were stained with Hematoxylin and Eosin. Morphological changes were evaluated from the septum, the right ventricular wall, the left anterior ventricular wall and the left posterior ventricular wall.

Presence of hemorrhage, myocardial ischemia and intramyocardial arterial changes were investigated and graded according to an arbitrary scale from 0 to 2 and expressed as point score units (PSU). A representative cross-sectional intramyocardial artery was chosen randomly from the left posterior ventricular wall, representing remote myocardium. Sections were chosen in order to visualize *tunica media* and smooth muscle cells. Round, clear nuclei represented normal nuclei. Sharp-edged, dark nuclei were defined as non-preserved nuclei. Vacuolized cells with squeezed, shrink nuclei represented edematous and non-preserved nuclei. Normal, vacuolated and sharp-edged media cell nuclei were manually counted separately. Recovered nuclei were estimated as the number of clear smooth-edged media cell nuclei, divided by the total number of media cell nuclei including vacuolated and sharp-edged, dark media cell nuclei [II]. To obtain the relative number of vacuolated nuclei, the number of vacuolated nuclei was divided by the number of round smooth-edged media cell nuclei [III]. Periadventitial inflammation and intimal thickness were graded from 0-2.

4.8 Immunohistochemistry [I-III]

The paraffin-embedded slides were deparaffinized with three changes of xylene and then rehydrated in a series of graded ethanol and rinsed well under

running distilled water. Thereafter, the slides were placed in a preheated retrieval buffer for 30 minutes and cooled for 5 minutes, followed by a five-minute rinse under running distilled water. After heat-induced epitope retrieval, the slides were placed on an autostainer (DAKO Corp, Carpinteria, California, USA). Staining was achieved by using the immuniperoxidase technique: the slides were incubated with 3% hydrogen peroxide in ethanol for five minutes to inactivate the endogenous peroxides, and then by using various monoclonal antibodies (Table 3.). The samples were incubated with the properly diluted monoclonal antibody, washed and incubated consecutively with peroxidase, and incubated in 3,3-diaminobenzidine. Finally, the sections were counterstained with Hematoxylin and Eosin and subsequently mounted.

Table 3. Different antibodies. eNOS: endothelial nitric oxide synthase; iNOS: inducible nitric oxide synthase; ADAM8: A Disintegrin And Metalloprotease 8; C4d: complement C4d.

Variable	Antibody
eNOS	eNOS (1:100), DAKO Corp
iNOS	iNOS (1:100), DAKO Corp
ADAM8	AF1031 (1:40), R&D Systems, Minneapolis, MN, USA
C4d	C4d (1:50), Biomedica Gruppe

Staining for eNOS, iNOS and ADAM8 was scored according to an arbitrary scale from 0 to 3 and expressed as PSU, where 0 means no staining visualized; 1, individual positively-stained nuclei; 2, groups of positively-stained nuclei; 3, intensive and global positively-stained area. Positively stained C4d deposition was counted from a representative cross-sectional intra-myocardial artery chosen randomly from the left ventricular wall. The total number of myocardial C4d deposition was calculated accordingly and expressed as PSU [I-III].

4.9 Quantitative reverse transcription-PCR analysis

The frozen [II-III] or RNAlater-stored [I, IV] tissue from the base of the heart was homogenized, and the ribonucleic acid (RNA) extracted. 50-100 ng of total RNA was reverse-transcribed into cDNA by using reverse transcription reagents. The quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed with standard protocol.

The cDNA obtained from the reaction (amount corresponding to approximately 1 ng of total RNA) was subjected to qRT-PCR for AQP7, eNOS, iNOS, TGF-β, HMGB1, HIF1α, IL6, HO-1, ADAM8, ICAM-1, VCAM-1 and Nfkb-1.

The expression levels of different variables were evaluated and the data were normalized to the expression of the housekeeping gene.

4.10 Statistics

Data is presented as individual values and shown as mean ± standard error of the mean [II-IV] or standard deviation and the median [I, III]. Kruskall-Wallis, non-parametric statistics, were utilized for comparison between independent groups [I-II, IV]. Two-way ANOVA was utilized to analyze among groups [I-II]. Non-parametric data between two groups were analyzed with the Mann-Whitney U test [I-IV]. Pearson correlation was performed to investigate for the relation between continuous variables [III]. Statistical significance was attributed to p values lower than 0.05 [I-IV]. Statistical analyses were performed with commercial statistical software (SPSS Inc, Chicago, IL). Power calculation was set to display the 95% confidence interval and performed with statistical software (PowerAndPrecision 4.0, Biostat, Englewood, NJ) [I].

5 Results

5.1 Surgical outcome

There were no statistical differences between the study groups in terms of surgical outcome. The transplanted grafts had palpation score of 5-6 out of 6 after 1 hour of reperfusion [I-IV].

A third of the hearts (33%) with IRI+MI and only 18% with IRI+MI+sildenafil had a palpation score less than 2 out of 6 at two days of reperfusion, whereas 82% of the hearts with IRI and IRI+MI+sildenafil remained patent for two days ($p < 0.05$) [II].

One heart with IRI+MI and IRI was non-palpable one day after reperfusion. All other hearts with IRI+MI and with IRI had a palpation score less than 2 out of 6 after two days of reperfusion [III].

All of the transplanted hearts remained patent and had a palpation score of 5-6 out of 6 for five days after reperfusion [IV].

5.2 Microdialysis

Interstitial myocardial metabolism was evaluated with microdialysis, which did not show significant changes in glutamate, glycerol, pyruvate or lactate in IRI as compared with IRI + MI. Moclobemide increased pyruvate after 15 minutes of reperfusion (29.19 ± 7.64 vs 13.86 ± 8.49, μM, $p = 0.028$) during the evolving MI, as compared with IRI+moclobemide. Glycerol increased after IRI with moclobemide as compared with IRI (38.70 ± 10.03 vs 21.70 ± 15.57, μM, $p = 0.047$). Concomitantly, after 15 minutes of reperfusion, IRI+moclobemide increased lactate as compared with IRI alone (2.40 ± 0.28 vs 1.02 ± 1.28, μM, $p = 0.047$). IRI+MI+moclobemide increased lactate as compared with IRI+MI (2.10 ± 0.39 vs 0.37 ± 0.29, μM, $p = 0.004$). Lactate remained increased 35 minutes after reperfusion in IRI+moclobemide as compared with IRI (2.25 ±

0.86 vs 1.10 ± 0.98, μM, $p = 0.040$). Glutamate increased after 35 minutes of reperfusion in IRI+moclobemide as compared with IRI (17.46 ± 11.86 vs 4.53 ± 4.80, μM, $p = 0.028$) [IV].

5.3 Histopathological changes

There were no hemorrhagic differences between the study groups [I-IV]. After two days of reperfusion, a global ischemic area was faintly observed in the left anterior ventricular wall of the myocardium; this represented mild myocardial edema in the area corresponding to the developing MI [I, III]. Inflammatory cells in the myocardium increased in hearts with IRI+MI as compared with IRI (0.71 vs 0.14, PSU, respectively, $p = 0.04$) [III]. After five days of reperfusion, a well-developed granulation area was found in the left anterior ventricular wall corresponding to the evolving MI (Figure 5.) [IV].

IRI+MI caused edema of the remote intramyocardial artery wall nuclei early after reperfusion (57.0 (IRI+MI) vs 40.0 (IRI) and 5.0 (Control), respectively, $p < 0.05$) [I].

One hour after reperfusion, periadventitial inflammation of the intramyocardial arteries of the left anterior ventricular wall and ventricular septum was observed in IRI+MI but not in IRI (0.80 ± 0.56 vs 0, PSU, respectively, $p = 0.003$) [IV]. The periadventitial inflammation was also seen in the remote left posterior ventricular wall area in IRI+MI but not in IRI (0.80 ± 0.56 vs 0.13 ± 0.36, PSU, respectively, $p = 0.045$) [IV].

After two days, edematous arterial wall nuclei decreased in number in IRI+MI as compared with IRI and Controls (32.0 vs 45.5 and 2.0, respectively, $p < 0.05$) [I].

Global myocardial edema and inflammation tended to decrease after IRI+MI+sildenafil as compared with IRI+MI (2.00 ± 0.27 vs 2.45 ± 0.21 and 0.60 ± 0.17 vs 1.00 ± 0.12, PSU, respectively) [II]. After two days, the relative number of recovered remote intramyocardial artery wall nuclei was significantly increased in the IRI+MI+sildenafil as compared with IRI+MI (72 ± 3.60 vs 56 ± 4.46, PSU, respectively, $p < 0.05$) [II]. After five days, periadventitial inflammation of the remote intramyocardial arteries was decreased in IRI+MI+moclobemide as compared with IRI+MI (0.80 ± 0.83 vs

2.00 ± 0.70, PSU, respectively, p = 0.049) [IV]. Intimal proliferation as depicted by the onset of intimal thickness was decreased in IRI+MI+moclobemide compared with IRI+MI (0.80 ± 0.83 vs 2.40 ± 0.54, PSU, respectively, p = 0.017) (Figure 6.) [IV].

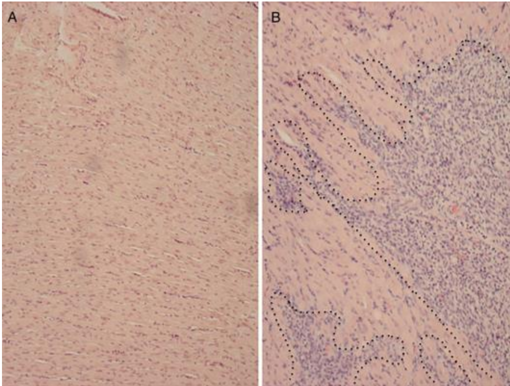


Figure 5. Representative histology of the heart after 5 days of reperfusion. A: Graft without LAD ligation (IRI). B: Graft with LAD ligation (IRI+MI). Hematoxylin-eosin staining X10. Note the outer border of myocardial infarction in B (black dotted line) [IV].

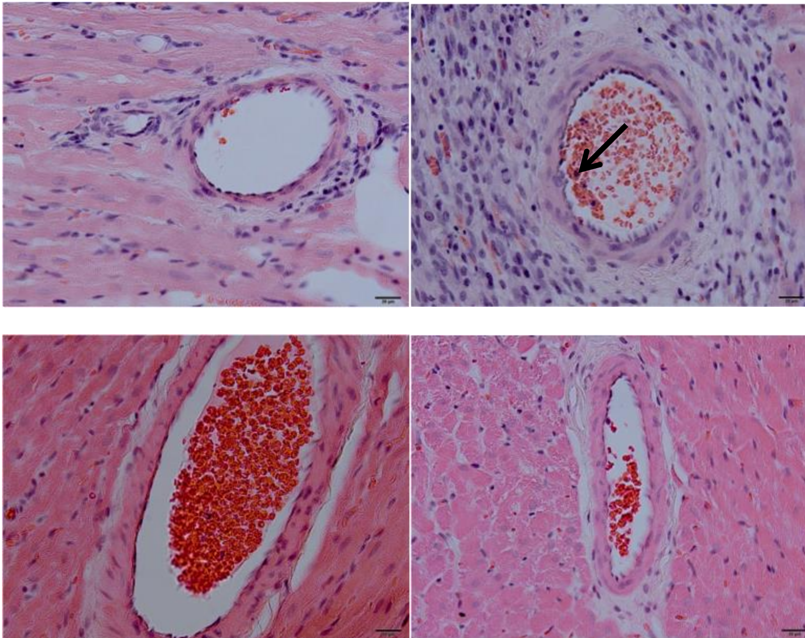


Figure 6. Histopathological changes after five days. Top left: cross section of intramyocardial artery after ischemia reperfusion injury. Top right: intramyocardial artery after ischemia reperfusion injury with left anterior descending coronary artery ligation. Bottom left: ischemia reperfusion injury with moclobemide. Bottom right: ischemia reperfusion injury with left anterior descending coronary artery ligation and moclobemide. Note bulging of the intimal layer (black arrow) and periadventitial inflammation at the top right. Hematoxylin and Eosin, X40 [IV].

5.4 Immunohistochemistry

After two days, ligation of LAD and the developing MI decreased staining of eNOS in IRI+MI as compared with IRI (1.0 vs 2.6, PSU, respectively, $p < 0.005$) [I]. ADAM8 was observed to stain intramyocardial arteries in MI grafts [I]. Statistically, increased myocardial staining for C4d was observed in IRI+MI as compared with IRI (80.13 vs 20.29, PSU, respectively, $p = 0.02$). An increased number of C4d deposition was also specifically observed in the media of the intramyocardial arteries situated along the left anterior wall corresponding to

the infarction area in IRI+MI in contrast IRI (2.75 vs 0.86, PSU, respectively, $p = 0.01$) [III].

Decreased staining of eNOS was observed in IRI+MI+sildenafil and IRI+MI as compared with IRI after two days (0.75± 0.29 and 0.60± 0.40 vs 2.50± 0.28, PSU, respectively, $p < 0.05$) [II].

5.5 qRT-PCR

After two days, the expression of ADAM8 increased in IRI+MI as compared with IRI (1.9 vs 1.0, FC, $p < 0.05$). In line with increased ADAM8 expression, a tendency for increased ICAM-1, VCAM-1 and Nfkb-1 expressions of remote myocardium in IRI+MI were observed at 48 h [I]. Instead, in transplanted hearts sacrificed when the palpation score was less than 2 out of 6, the expression of eNOS decreased in IRI+MI as compared with IRI (0.51 vs 1.58, PSU, respectively, $p = 0.05$); there were no differences in the expressions of iNOS and TGF-β between the two study groups (2.06 vs 5.98, PSU, respectively, $p = 0.64$ and 1.03 vs 2.69, PSU, respectively, $p = 0.23$) [III].

After two days, the expression of iNOS decreased in IRI+MI+sildenafil as compared with IRI+MI (0.02 ± 0.01 vs 1.01 ± 0.23, FC, respectively, $p < 0.05$), whereas no major difference was observed in expression of AQP7 [II]. After five days, there were no differences in the expressions of IL6 and HIF1α, but the expressions of HMGB1, CD68 and HO-1 decreased in IRI+MI+moclobemide as compared with IRI+MI (1.33 ± 0.20 vs 1.75 ± 0.24, FC, $P = 0.028$; 5.15 ± 1.10 vs 9.59 ± 2.75, FC, $p = 0.050$; 10.41 ± 4.17 vs 21.28 ± 10.01, FC, $p = 0.047$) [IV].

6 Discussion

Developing MI during cardiac surgery or PCI is an irreversible process that may interact IRI, leading to the concept of PMI. PMI is a major clinical challenge. Little is known as to whether the dismal outcome after PMI is reflected by the presence of global myocardial changes. A few studies show that remote inflammatory areas are present postmortem in patients with coronary artery occlusion disease (Abbate 2008). A local MI has a global inflammatory effect on the myocardium (Abbate 2008; Li 2009). Inflammation after MI is associated with cardiac remodeling as well as left ventricular dilatation and dysfunction (Maekawa 2002; Tsujioka 2009).

Revascularization of an acutely obstructed coronary artery causing PMI may be insufficient to prevent global heart dysfunction leading to acute stunning and subsequent cardiac remodeling. At least to some extent, IRI may also occur during cardiac surgery without cardiopulmonary bypass (Karu 2009). The heart often needs immediate rest such as the infusion of cardioplegia and the insertion of a left ventricular assist device that decreases cardiac overload (Kapur 2013). We did not observe differences in treating the cardiac graft with either antegrade cold Custodial or NaCl-solution prior to IRI. These maneuvers, however, do not abolish the ongoing effect of PMI that induces remote myocardial inflammation. In contrast to MI per se, it may be possible to interact with the remote myocardial inflammation (Zhang 2008).

6.1 The heterotopic rat cardiac transplantation to study perioperative myocardial infarction

Studies on PMI are scarce due to the lack of histology and experimental model. The heterotopic rat cardiac transplantation model is widely used to study rejection mechanisms after xeno- and allotransplantation (Adams 1999; Dedja 2005). The model has been introduced previously to study MI per se (Suzuki 2002). The model enables to study the effect of a left ventricular assist device.

The benefits of this transplantation model include the relatively low mortality after surgery (Wang 2006). Administration of heparin together with antegrade perfusion at the onset of graft ischemia [IV] may enhance cardiac survival.

We simulated the clinical concept of cardiac arrest and PMI by using a modified heterotopic rat cardiac transplantation model. We experimentally studied PMI by inducing MI during IRI; we specifically investigated the remote myocardial histopathology during the early phase of experimental PMI. To verify the presence of MI, we analyzed myocardial histology and expressions of eNOS, iNOS, ADAM8 and C4d.

6.2 Remote myocardium

A persistent regional coronary flow reduction, related to a sustained regional left ventricular dysfunction, is responsible for early changes occurring during local myocardial ischemia. Ischemic myocytes are associated with intramyocardial edema and inflammation at the site of MI (Chen 2000). Local myocardial ischemia has impact on the remote myocardium as well. Remote inflammation has been shown in an *in vivo* mouse model of MI (Nahrendorf 2007) and postmortem in patients with history of MI (Abbate 2008). Remote intramyocardial arterial edema seems to be a hallmark of subsequent remote periadventitial inflammation associated with global IRI in grafts with local MI.

This study demonstrates that MI may induce remote myocardial inflammation after IRI. MI is associated with global myocardial inflammation (Abbate 2004) that may enhance the risk for arteriosclerosis (Takaoka 2006). It is unlikely that postmortem ischemia alone would be responsible for the remote myocardial changes after MI (Abbate 2004). Consistent with the postmortem findings, periadventitial inflammation and early arteriosclerotic-like changes of the remote intramyocardial arteries are observed in rats´ hearts with IRI and MI, confirming the concept that PMI induces remote myocardial changes.

Remote intramyocardial arteries of cardiac grafts with PMI expressed ADAM8. The expression of other inflammatory molecules, such as ICAM-1, VCAM-1 and Nfkb-1, tended to increase after PMI. All of these molecules participate in inflammation (Frantz 2007; Postadzhiyan 2008; Radecke 2015; Tudor 2001). The marker of complement cascade activation, namely C4d (Regele 2002), was

present in cardiac grafts with PMI postmortem. ADAM8 is also considered as a mediator of ischemia (Li 2009). It may again be stated that remote myocardial changes after PMI impact the heart as a whole.

6.3 Sildenafil enhances myocardial recovery after perioperative myocardial infarction

It is important to acknowledge the limitations to interact with MI. On the other hand, intervening with remote myocardium may prevent from detrimental outcome after PMI. In our study, sildenafil ameliorates recovery after PMI.

Sildenafil tends to decrease myocardial edema and inflammation, and enhances recovery of remote intramyocardial artery wall nuclei. This may be due to the cardioprotective role of NO; the protective effect of sildenafil may be based on eNOS and iNOS (Choi 2009), though other molecules are also involved (Elrod 2007). NO regulates mitochondrial respiration, myocardial oxygenation, inactivates caspases and decreases myocyte apoptosis (Heusch 2000). Sildenafil increases the NO reserves of the heart and enhances survival after IRI (Das 2005).

During MI, endothelium is dysfunctional and eNOS is downregulated (Jones 1999). iNOS reflects myocardial damage and during MI the iNOS expression is upregulated (Berges 2005; Saito 2002). Sildenafil induces iNOS inhibition (Salloum 2003), and the induction of myocardial protection is mediated by iNOS- and eNOS- dependent, NO signaling pathway (Das 2005). The decrease of eNOS may reflect myocardial ischemia, whereas the increase of iNOS reflects myocardial response to IRI (Zhang 2008). In our study, iNOS and eNOS expressions decreased in hearts with PMI and sildenafil treatment. Decreased iNOS expression may also suggest for a temporary loss of NO reserves of the heart with PMI (Desrois 2004), and long-term myocardial protection may be due to delayed iNOS increase.

6.4 Moclobemide preserves remote myocardium

Periadventitial inflammation, the expression of myocardial CD68 and the onset of arteriosclerotic-like changes of remote intramyocardial arteries may decrease with moclobemide. The generation of ROS by MAO may be associated with the sphingolipid metabolism, leading to cell proliferation and apoptosis (Bianchi 2005; Pchejetski 2007). Vascular SMC proliferation is dependent on MAO-catalyzed tyramine and serotonin metabolism (Coatrieux 2007; Day 2006). In our study the intimal proliferation and SMC migration decreased with the moclobemide treatment.

Moclobemide has beneficial effect on myocardial metabolism and remote myocardium during PMI. Moclobemide increases cellular energy production by increasing pyruvate.

Increased HMGB1 expression is associated with MI and adverse outcomes, such as cardiac failure and cardiac death (Andrassy 2008; Kohno 2009). After MI increased HO-1 reduces the size of MI (Kusmic 2014). Moclobemide decreases expressions of HMGB1 and HO-1 after PMI. HMGB1 increases inflammation and atherosclerotic-like changes (Kalinina 2004; Umahara 2014). The decreased HMGB1 expression may inhibit SMC migration (Yang 2012). Decreased HO-1 expression may indicate that no further protection from oxidative stress is needed after MI.

6.5 Limitations of the study

All limitations including the experimental approach of our studies must be acknowledged. The results must be confirmed in a clinical setting prior to their extrapolation in the treatment of patients. It was beyond our scope to demonstrate the specific molecular mechanisms behind the inflammatory response. We did not evaluate cardiac function. The modified heterotopic transplantation model including syngeneic rats and ligation of LAD is most suitable for initial studies of a plausible complication after cardiac surgery; further studies on molecular mechanisms are warranted after PMI.

7 Conclusions

This study presents a modified experimental heterotopic cardiac transplantation model, which simulates clinical heart surgery including cardiac arrest and PMI. PMI, including MI associated with IRI, cause global changes. These changes are inflammatory and can be seen distantly from the myocardial infarction area, in the remote myocardium and intramyocardial arteries. The remote myocardial changes are revealed by ADAM8 and postmortem by C4d expression. The primary changes can be seen as early as one hour after IRI and MI. Remote inflammation may be observed two days after IRI and MI. Arteriopathy of the remote intramyocardial arteries may be observed 5 days after IRI and MI. It is possible to target pharmacological intervention, as in our study with sildenafil and moclobemide, towards these inflammatory changes. Biomarkers for early diagnostics of PMI need to be studied clinically. In future, the reversibility of remote myocardial changes after PMI may offer exciting novel possibilities to impact an otherwise often dismal clinical entity.

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Vilma Vuohelainen

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ORIGINAL ARTICLE

Myocardial infarction induces early increased remote ADAM8 expression of rat hearts after cardiac arrest

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Abstract

Background. A disintegrin and metalloproteinase-8 (ADAM8) is a potential surrogate of inflammation which has recently been associated with myocardial infarction. We evaluated in a rat cardiac transplantation model whether ischemia-reperfusion injury alone (IRI) or with early regional myocardial infarction (MI) would suffice to induce inflammatory myocardial remodeling and ADAM8 expression. ***Material and methods.*** Isogenic heterotopic cardiac transplantation after cardiac arrest was performed to 48 Fischer 344 rats to induce ischemia-reperfusion injury (IRI), of which 27 rats also underwent ligation of the left anterior coronary artery (LAD) of the heart to yield MI. Histology was performed at 0.5, 24 and 48 h after transplantation. ADAM8 was evaluated by qRT-PCR after graft harvesting. ***Results.*** After 0.5 and 48 h respectively, edematous intramyocardial artery nuclei and periadventitial inflammation were more prominent in MI after transplantation, as compared with IRI alone and Controls (57.0 vs 40.0 and 5.0; 1.9 vs 1.1 and 0.9, point score units, $p < 0.05$, respectively). The expression of ADAM-8 was increased in MI as compared with Controls (1.9 vs 1.0, 1.9 fold increase) at 48 h. In grafts with MI, ADAM8 was localized using immunohistochemistry to the vicinity of the area corresponding to the developing infarction as well as in intramyocardial arteries remote to the infarction area. ***Conclusions.*** Remote histopathological changes of ischemic cardiac grafts are associated with increased expression of ADAM8 thus emphasizing a global myocardial impact of MI.

Key Words: *Infarction, ADAM8, rat, cardiac transplantation, myocardial arteries, cardiac arrest*

Introduction

Myocardial infarction (MI) leading to early death is well documented [1]. Though extensive research and treatment modalities are mainly aimed at treating acute myocardial infarction in order to prevent early complications, a considerable number of patients develop cardiac failure associated with myocardial remodeling [2–4].

Recent evidence suggests that MI may develop spreading inflammation which interferes with heart function and subsequent formation of remote myocardial changes [5]. Adaptive changes occur in the microvascular bed distal to epicardial coronary arteries [6]. Though local ongoing ischemia leading to MI may be surpassed by coronary artery bypass grafting, clinical evidence suggests that inflammation after MI may not necessarily be dampened and may also have an impact on the development of remote intramyocardial arteriopathy further aggravating ischemia [6,7]. On the other hand, the ischemic heart undergoes ischemia-reperfusion injury (IRI) due to cardiac arrest and cardiopulmonary bypass; the aorta is cross-clamped, cardioplegia inserted, and after weaning from cardiopulmonary bypass, the cross-clamp is released while the heart undergoes IRI, potentially aggravating the myocardial inflammatory status. Currently, it is not ascertained whether IRI alone with or without MI may have an impact on the early myocardial inflammatory profile leading to remote histopathological changes.

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A disintegrin and metalloproteinases (ADAM) are a family of membrane-bound glycoproteins that have potential impact on inflammatory response associated with cell-cell and cell-matrix regulations and tissue remodeling [8,9]. ADAM9, -10, -12, -15, -17 and -19 have been studied in the myocardium, and ADAM15 has recently been associated in an experimental setting with MI [9]. ADAMs contain structurally a prodomain, a disintegrin, a metalloprotease, a cystein-rich, a cytoplasmic, a transmembrane and epidermal growth factor-like domains [10]. Structurally similar to ADAM15 [11], ADAM8 has been shown to be increased in endothelial cells after experimental spinal cord injury [12]. On the other hand, reduced intragraft mRNA expression of ADAM8 has been associated with decreased transplant arteriosclerosis in mice after allograft transplantation [13]. An allelic variant of ADAM8 gene (rs2995300) has recently been associated with the risk of myocardial infarct [14]. ADAM8 may be a most attractive mediator of ischemia and inflammation associated with cancer [15] and asthma [16].

At present, there is sparse information on myocardial ADAM8 during inflammatory processes such as IRI and MI in the context of cardiac resuscitation of the unloaded heart. In this study, we investigated whether IRI would suffice to induce expression of ADAM8 with or without a local well-documented MI, together with the early spreading of remote histopathological changes. We utilized our previously established rat transplantation model with IRI and MI [17] to indicate whether remote changes would occur during MI. We speculate that inflammation secondary to MI may harness globally the myocardium thus challenging for early intervention.

Methods

Rats

The study was approved by the Finnish State Provincial Office. Ninety-six inbred Fischer 344 rats (F344/ NHsd, Harlan Laboratories, The Netherlands) weighing 175–200 g, served as donors ($n = 48$) and recipients ($n = 48$). Three Normal hearts from non-operated rats and 3 Controls (recipient hearts with sham operation) in each time point (0.5 h, 24 h and 48 h, nine rats altogether) are included. The rats were kept in Tampere University vivarium and received humane care in compliance with the 'Principles of Laboratory Animal Care' formulated by the National Society for Medical Research and the 'Guide for the Care and Use of Laboratory Animals' prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1996).

Surgical procedure

After harvesting, the ascending aorta of the cardiac graft was infused with antegrade infiltration of 2 ml cold (4°C) temperature physiologic saline fluid without heparinization. All 48 grafts underwent heterotopic cardiac transplantation intra-abdominally by joining the graft aorta to the aorta and the graft pulmonary artery to the inferior vena cava of the recipient, as previously described [17,18]. From the recipient aorta, the transplanted heart received oxygenated blood that was introduced into the coronary arteries of the graft. Via the coronary sinus, this blood circulated into the right atrium and eventually the right ventricle, from where deoxygenated blood was repulsed to the recipient rat throughout the pulmonary vein [19,20]. Therefore the nutritional flow of the myocardium consisted of oxygenated blood, and the transplanted heart was not ischemic after reperfusion upon transplantation. Since the aortic valve was competent, oxygenated blood was not allowed to fill the left ventricle, and therefore the transplanted heart simulated a non-working resting state of the left side of the graft. This heterogenous transplantation model allows one to study IR *in vivo* without interferences of myocardial stress factors. The model thus simulated the clinical concept of acute cardiac arrest resuscitated with initiation of cardiopulmonary bypass and left ventricle assist device [21]. Total ischemia time before total graft reperfusion was 20–30 min after cardiac arrest. In 27 grafts, the LAD was also ligated permanently at its proximal part with a single 7-0 suture to yield MI. The ligation knot for LAD obstruction was placed at the bifurcation of the first diagonal branch due to coronary vessel distribution, since the apex is most vulnerable to ischemic changes leading to MI. Twenty-one other grafts with IRI underwent transplantation only.

Tissue samples

The recipient rats were sacrificed after 0.5 ($n = 16$), 24 ($n = 12$) and 48 ($n = 20$) hours of reperfusion. For 30 rats, the basal part of the cardiac graft was procured and stored in RNA stabilization solution (RNAlater, Applied Biosystems, CA, USA) for quantitative RT-PCR analysis. The apex part of the graft was embedded in paraffin and 5-μm sections were cut.

Histology

For histology, the 5-μm sections were stained with haematoxylin and eosin. Evaluation was performed blinded to the study protocol, treatment allocation and time sequence by two investigators (VV and AM) and technically unclear slides were rejected. The following variables were evaluated: presence of myocardial edema, hemorrhage and ischemia. As the vacuolization of nuclei of the media layer of intramyocardial arteries

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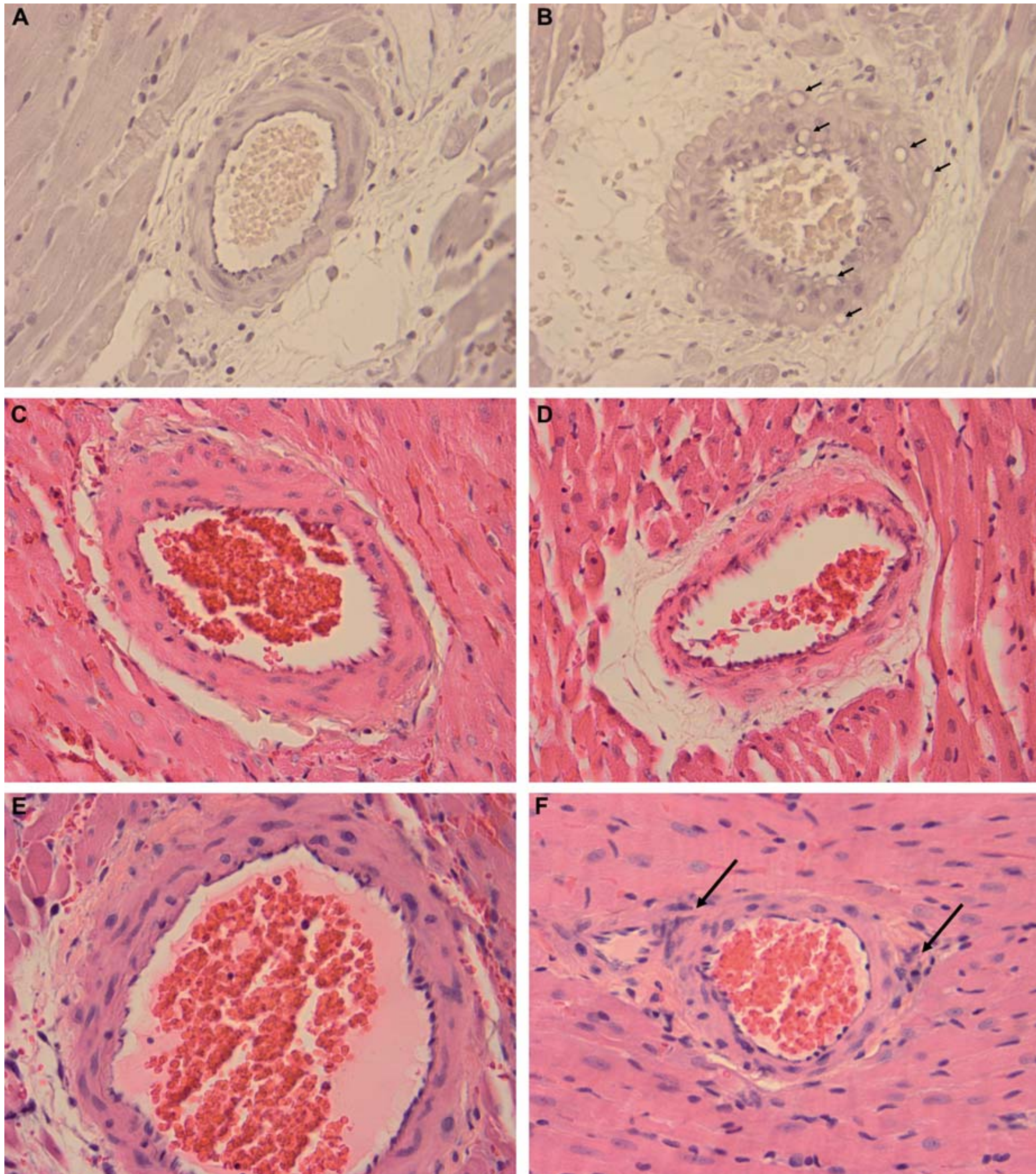

Figure 1. Representative histology of a remote intramyocardial artery of grafts with ischemia-reperfusion injury only (IRI, right column; A, C, E) and grafts with myocardial infarction (MI, left column; B, D, F). A and B, C and D, E and F show grafts 0.5, 24, and 48 h after transplantation, respectively (×23). Note edema of intramyocardial artery shown as vacuolization of vessel wall in B (small arrows) and periadventitial inflammation in F (long arrows).

reflected edema, a representative cross-sectional intramyocardial artery was chosen randomly from the left anterior ventricular wall and the left posterior ventricular wall representing remote myocardium. As the minority of the arterial wall nuclei was round-shaped representing normal nuclei, sharp-edged blue nuclei of the media cells were defined as non-preserved and manually counted together with vacuolated and edematous nuclei. Periadventitial inflammation was graded according to an arbitrary scale from 0 to 2 and expressed as point score units (PSU): 0, no inflammation; 1, presence of occasional inflammatory cells; 2, groups of inflammatory or proliferating cells.

Immunohistochemistry for ADAM8 and eNOS

Fifteen grafts were randomly selected 48 h after transplantation for immunohistochemistry. Paraffin-embedded slides were deparaffinized with three changes of xylene, and rehydrated in a series of graded ethanol, and rinsed well under running distilled water.

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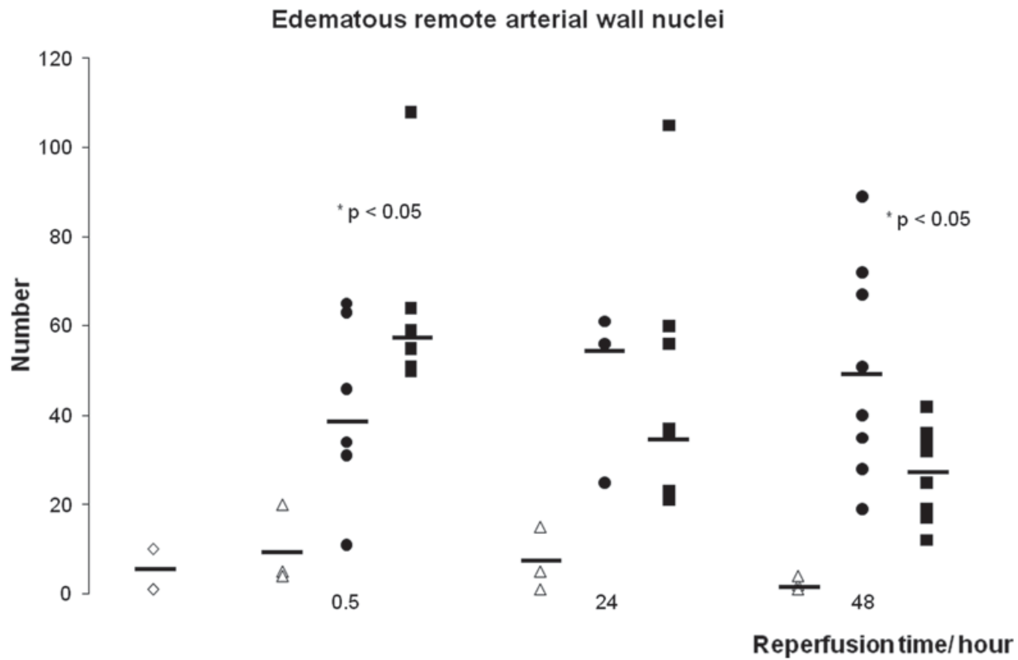

Figure 2. Number of edematous remote intramyocardial artery wall nuclei in grafts with ichemia-reperfusion injury only (IRI, open circles) and grafts with myocardial infarction (MI, black boxes). Open triangles show recipient hearts representing Controls, and open diamonds Normal hearts. As compared with IRI, grafts with MI have increased and decreased the number of edematous and vacuolized intramyocardial artery nuclei 0.5 and 48 h after reperfusion, respectively. $*p < 0.05$, Kruskal-Wallis. Horizontal bars indicate the medians.

Slides were placed in a preheated retrieval buffer, 0.1 mmol EDTA, pH 8.0, for 30 min, then cooled in the buffer for 5 min, followed by a 5-min rinse under running distilled water. After heat-induced epitope retrieval, slides were placed on an autostainer (DAKO Corp, Carpinteria, California, USA). Sections were incubated with 3% hydrogen peroxide in ethanol for 5 min to inactivate the endogenous peroxides, incubated either in 1:100 eNOS (eNOS, DAKO Corp) for 30 min, followed by rinsing with Tris-buffered saline solution with Tween 20 (TBST) wash buffer. Secondary incubation was with DUAL-labeled polymerhorseradish peroxidase (K4061; DAKO Corp) for 15 min. The slides were rinsed with TBST wash buffer. Sections were then incubated in 3,3-diaminobenzidine (K3467, DAKO Corp) for 5 min,

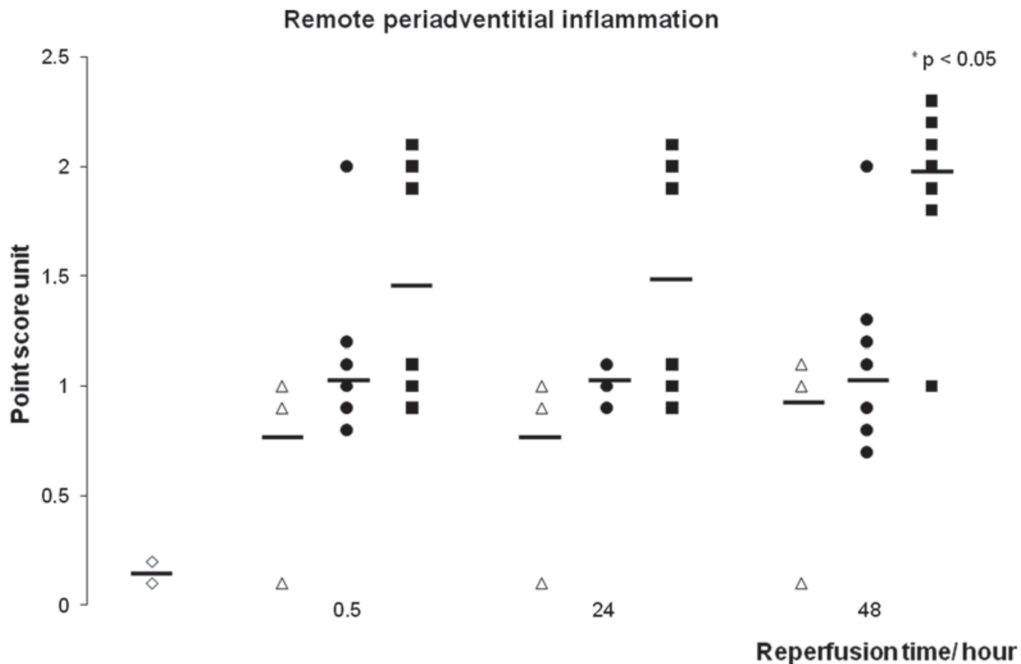

Figure 3. Intensity of periadventitial inflammation of remote intramyocardial arteries in grafts with ichemia-reperfusion injury only (IRI, open circles) and grafts with myocardial infarction (MI, black boxes). Open triangles show recipient hearts representing Controls, and open diamonds Normal hearts. As compared with IRI, grafts with MI have increased remote inflammation at the vicinity of intramyocardial arteries 48 h after reperfusion. $*p < 0.05$, Kruskal-Wallis. Horizontal bars indicate the medians.

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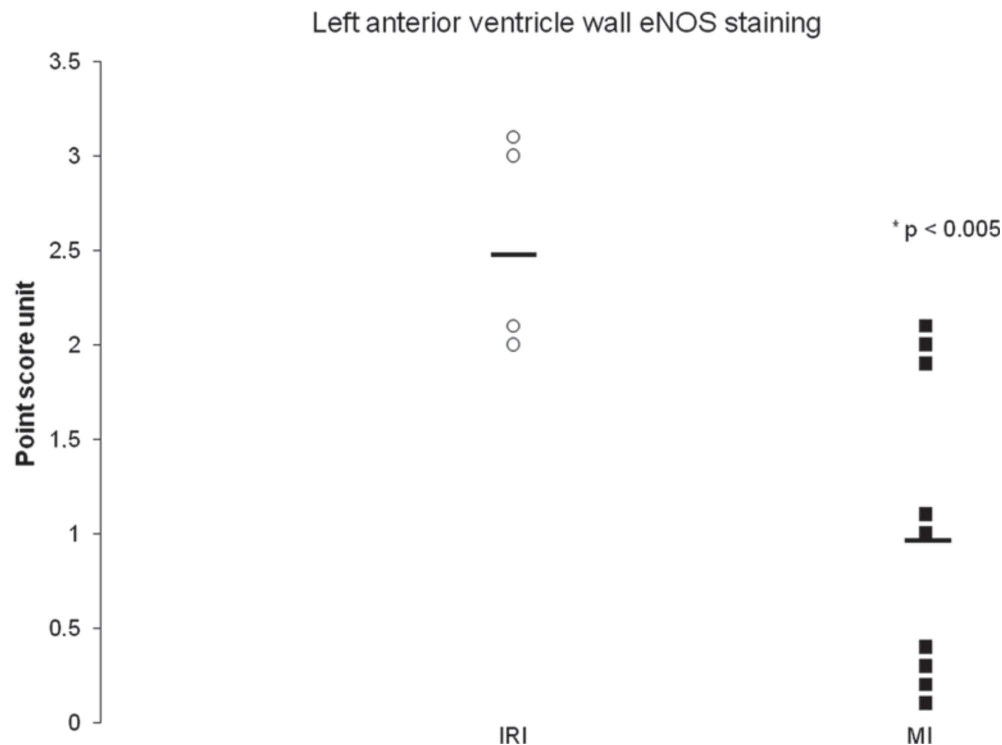

Figure 4. Grade of staining of immunohistochemistry for endothelial nitric oxide synthase (eNOS) 48 h after transplantation in the left anterior ventricle wall in grafts with myocardial infarction (MI, black boxes) as compared with grafts with ichemia-reperfusion injury only (IRI, open circles). Note decreased grade of staining expressed as point score units (PSU) in MI demonstrating the presence of developing infarction. $^{*}p < 0.05$, Mann-Whitney test. Horizontal bars indicate the medians.

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counterstained with modified Schmidt hematoxylin for 5 min, and rinsed for 3 min in tap water to blue sections, dehydrated with graded alcohols, and cleared in three changes of xylene before mounting. Staining for eNOS was scored according to an arbitrary scale from 0 to 3 and expressed as PSU, where 0 means, no staining visualized; 1, individual positively-stained nuclei; 2, groups of positively-stained nuclei; 3, intensive and global positively-stained area. For Adam-8, immunohistochemistry was performed using the the N-Histofine® Simple Stain MAX PO staining method (Nichirei Biosciences Inc., Tokyo, Japan) and paraffin-embedded vascular samples without any counterstain as described in detail previously [22]. A goat anti-human ADAM8 antibody (AF1031, R&D Systems, Minneapolis, MN, USA) was used at 1:40 dilution. Two grafts were rejected due to technical failure.

Quantitative RT-PCR analysis

For quantitative RT-PCR analysis, 18 grafts, nine Controls and three Normal hearts were randomly selected. RNAlater-stored tissues were homogenized using Ultra-Turrax® T80 homogenizer (IKA, Staufen, Germany). The RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) and RNEasy® Mini-Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality of RNA samples was confirmed with RNA 6000 Nano Chip Kit (Agilent, Santa Clara, CA, USA). 100 ng of total RNA was reverse-transcribed into cDNA in reaction volume of 20 μl. The quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed with standard protocols on Abi Prism 7300 instrument (Applied Biosystems, CA, USA). The PCR reaction was performed with TaqMan® Gene Expression assays for ADAM-8 (ID Rn01454348_m1) and GAPDH (ID Rn01462662_g1) (both from Applied Biosystems) according to the manufacturer's instructions with TaqMan® Universal PCR Master Mix. The expression levels of ADAM8, and GAPDH as an internal Control/housekeeping gene were evaluated. Ct values were determined for every reaction and the relative quantification was calculated using the 2-ΔΔCt method [23]. Briefly, the data was normalized to the expression of housekeeping gene GAPDH, and values of Control samples were used as a calibrator. As evaluation for each sample was repeated three times, the mean and standard error of mean for the ADAM8 expression levels were calculated for each group.

For evaluation of the Intercellular Adhesion Molecule-1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and Nfkb-1, a gene taking part in the encoding of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in order to confirm the presence of remote inflammatory reaction during MI, TaqMan Rat inflammatory array (Applied Biosystems, Foister City, CA, USA, ID 4378708) was utilized for eight randomly selected hearts. Then, 100 ng of total RNA was reverse-transcribed into

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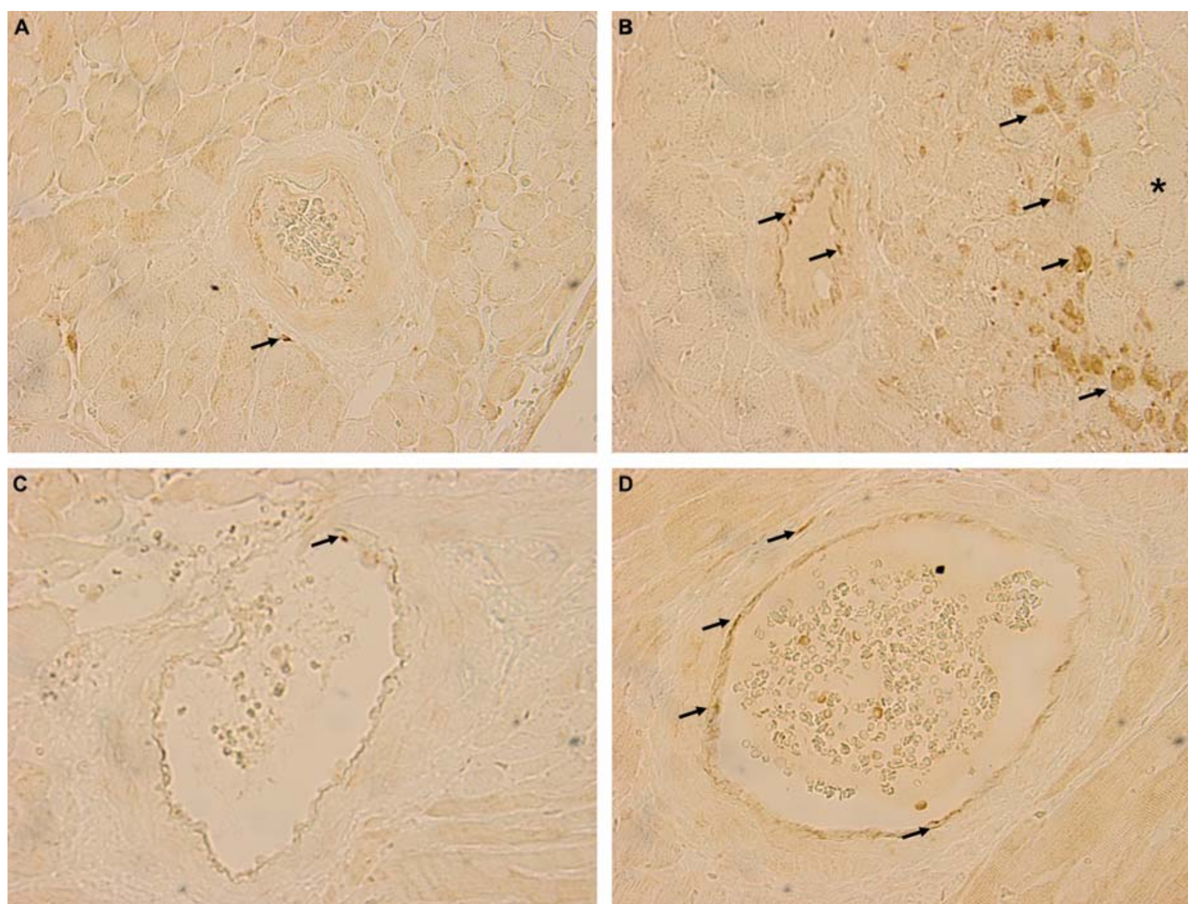

Figure 5. Representative immunohistochemistry 48 h after transplantation for ADAM-8 demonstrating the location of positive staining (arrow) in grafts with myocardial infarction (MI) as compared with grafts with ichemia-reperfusion injury only (IRI). (A) and (B) show the area of the left anterior ventricle in IRI and MI, respectively. (C) and (D) show the area with a remote intramyocardial artery in IRI and MI, respectively. Note increased staining at the border of the developing infarction and endothelial cells in remote intramyocardial artery in MI as compared with IRI. The asterisk (*) shows the core of developing infarction in the graft with MI.

cDNA in a reaction volume of 20 µl using the High Capacity cDNA Kit (Applied Biosystems) according to manufacturer's instructions. LDA cards were loaded with 15 µl of undiluted cDNA, 35 µl of nuclease free water and 50 µl of PCR Universal Master Mix (Applied Biosystems) and run according to the manufacturer's instructions. Samples were run and analyzed as duplicates, and both cDNA synthesis and PCR reactions were validated for inhibition. Again, GAPDH was used as a house-keeping gene. The results were analyzed using SDS 2.2 Software (Applied Biosystems).

Statistics

Data is presented as individual values and medians. Kruskal-Wallis non-parametric statistics were included for comparison between independent groups. Two-way ANOVA was utilized to analyze among groups. Nonparametric data between the two groups were analyzed with the Mann-Whitney U test. Statistical analyses were performed with commercial statistical software (SPSS 17.0, SPSS Inc, Chicago, IL). Power calculation was set to display the 95% confidence interval and performed with statistical software (PowerAndPrecision 4.0, Biostat, Englewood, NJ).

Results

Histology

There were neither hemorrhagic nor major ischemic histopathological differences between the grafts 0.5 h after operation. However, myocardial edema and the presence of vacuolated non-clear nuclei of intramyocardial artery cells were recorded in the left anterior ventricular wall and ventricular septum in grafts with MI, whereas the nuclei of IRI group were mainly normal throughout the myocardium (Figure 1). Opposite to the myocardial area nourished by the LAD, the number of edematous intramyocardial artery wall nuclei, that is the number of non-preserved media cell nuclei, was significantly higher in MI grafts as compared with the IRI group early after reperfusion and Controls (57.0 vs 40.0 and 5.0, respectively, $p < 0.05$, Figure 2). After 2 days, these edematous arterial wall nuclei decreased in number in MI as compared with IRI and Controls (32.0 vs 45.5 and 2.0, respectively, $p < 0.05$, Figure 2). The index of periadventitial inflammation of the remote intramyocardial arteries was significantly elevated in MI after 1 and 2 days as compared with IRI vs Controls (1.5 and 1.9 vs 1.0, and 1.1 vs 0.9 and 1.0, respectively, PSU, $p < 0.05$, Figure 3).

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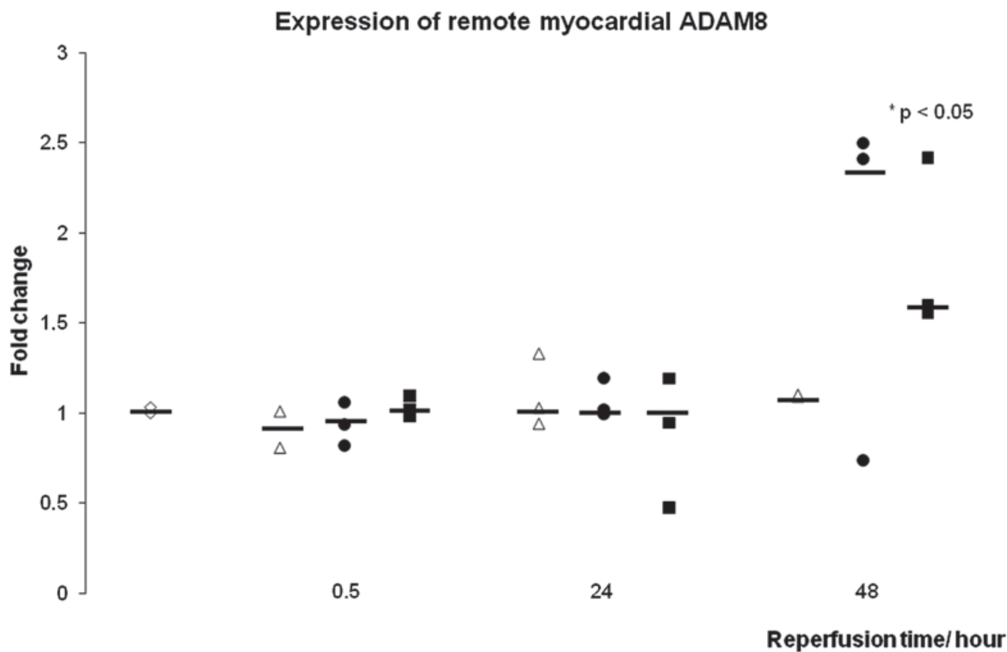

Figure 6. ADAM-8 mRNA expression of rat heart grafts with myocardial infarction (MI, black boxes) and in grafts with ichemia-reperfusion injury only (IRI, open circles) 48 h after transplantation. Open triangles show recipient hearts representing Controls, and open diamonds Normal hearts. Expression shown as fold change compared with normal rat hearts without intervention (Controls). Horizontal bars indicate the medians. Note the significant fold change 2 days after transplantation in OI ($^{*}p < 0.05$), as compared with Controls.

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Immunohistochemistry

Decreased staining for eNOS was detected in intramyocardial arterial walls in MI as compared with IRI, indicating developing MI of the left anterior ventricle in grafts with ligation of LAD after 48 h (1.0 vs 2.6, PSU, respectively, $p < 0.005$, Figure 4). ADAM8 was observed to stain also at the vicinity of intramyocardial arteries of the left anterior ventricular wall in MI grafts. At the remote area, the intima of remote intramyocardial arteries were stained with ADAM8 in MI (Figure 5).

ADAM8 expression (Figure 6)

At 48 h, ADAM8 expression increased in MI as compared with Controls (1.9 vs 1.0, 1.9 fold increase, $p < 0.05$). For the effect size (group means of 1.9 vs 1.0), SD (0.5 and 0.2), sample sizes (3 and 9), and alpha (0.050, 2-tailed), power is 0.988. Statistically, this increase was not significant in grafts with IR alone. At 0.5 and 24 h, ADAM8 expression was as high in grafts with MI, IR and Controls vs Normal hearts without transplantation. In line with increased ADAM8 expression, a tendency for increased ICAM-1, VCAM-1 and Nfkb-1 expressions of remote myocardium in hearts with IR and MI were observed at 48 h (Table I).

Discussion

This study shows that an occluded coronary artery after transplantation, by developing infarction to the anterior wall of the left ventricle, has an early remote histopathological impact on the myocardium in areas not primarily nourished by the occluded artery in question. Remote myocardial remodeling due to IRI and MI, is represented by increased expression of ADAM8 together with concomitant periadventitial inflammation and edema of intramyocardial arteries, while decreased eNOS staining confirms the presence of the local developing infarction in MI grafts only. MI seems to have an early increment on remote histopathological changes after IR.

Abbate et al. have shown that distant inflammatory areas are found postmortem in patients devoid of multivessel coronary artery disease [5]. It has been suggested that local myocardial infarction, besides

Table I. Remote expressions of the basis of eight rat hearts.

Group	Reperfusion time/hour	ICAM-1/ fold change	VCAM-1/ fold change	Nfkb-1/ fold change
Normal		1	1	1
Normal		0.70	0.81	0.73
Control	0.5	0.54	0.51	0.76
IR	0.5	1.24	0.85	0.88
IR	48	5.94	5.02	2.28
IR	48	2.97	1.71	1.57
MI	48	3.16	5.42	1.85
MI	48	5.61	2.38	1.53

Nfkb-1 is a gene taking part in the encoding of the protein NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells); ICAM-1, Intercellular Adhesion Molecule-1; VCAM-1, Vascular Cell Adhesion Molecule-1; Normal, normal rat heart; Control, rat heart with sham operation; IR, isogenic rat heart with ischemia-reperfusion injury; MI, isogenic rat heart with ischemia-reperfusion injury and myocardial infarction of the heart apex.

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inducing local inflammatory reaction and subsequent scarring, has a global inflammatory effect on the myocardium [5,9,24]. Remodeling is dependent on the complex biochemical cascades initiated by an inflammatory trigger such as coronary artery occlusion. It is also known that deliberating a single anatomically occluded culprit coronary artery occlusion does not necessarily suffice to overcome phenomena such as stunning, which may persist due to IRI, MI and global inflammation. A persistent regional coronary flow reduction, related to a sustained regional left ventricular dysfunction, is responsible for early changes occurring during local myocardial ischemia such as increasing myocardial interstitial edema [3]. This is also the case in our model, where ischemic myocytes are intermingled with intramyocardial edema and later inflammation. Remote intramyocardial artery edema seems to be a hall-mark of subsequent remote periadventitial inflammation associated with global ischemia-reperfusion injury in grafts with local MI.

Using the rat transplantation model with persistent LAD occlusion, we have demonstrated earlier that MI increases the gene expression of aquaporin-7, a marker for early edema in the heart graft [17]. Here, we show that grafts with MI have decreased staining for eNOS, a marker of viable endothelium in the area nourished by LAD. Likewise, in a previous study [25], it was shown that persistent heart ischemia induced a marked loss of eNOS staining. This demonstrates that grafts with MI differ from grafts with IR only, by having altered endothelial function [26,27] in the area of infarction induced by permanent ligation of LAD. Comparing grafts with IRI only and MI with Normal hearts and Controls with sham operations, we also demonstrate the effect of IR upon transplantation by excluding the impact of the surgical trauma, stress response and systemic inflammatory perturbations on myocardial function and histology. In our model, the global effect of local ischemia and infarction is therefore confined solely to the heart graft that simulates the clinical concept of cardiac arrest and bypass. Indeed, plasma parameters of cytokines or growth factors such as TNFalpha may not clearly differ in this model at these early time-points after transplantation with or without IR, least of all with MI [28]. To further demonstrate remote myocardial inflammatory reaction during IR and MI, we therefore evaluated ICAM-1, VCAM-1 and Nfkb-1, all well-established markers of ischemia and inflammation [29]. ICAM-1 and VCAM-1 are vascular adhesion molecules present during activation of inflammatory cascades. Nfkb-1 is a gene taking part in the encoding of the protein NF-kB, which is a protein complex that controls the transcription of DNA and is activated by various intra- and extra-cellular stimuli such as cytokines and intercellular adhesion molecules. Interestingly, the upregulation of NF-kB has recently been shown to be associated with eNOS [30]. After 2 days, ICAM-1, VCAM-1 and Nfkb-1 have a tendency to show increased expression in remote myocardium with IRI and MI of the apex (Table I). This finding is still to remain descriptive as only eight hearts fitted into the simultaneous inflammatory kit and evaluated according to the manufacturer's instructions.

After 48 hours post transplantation, the presence of a local developing infarction in grafts with MI was also confirmed with increased expression of ADAM8, as compared with Controls. Transplantation alone also induced elevated ADAM8 in grafts with IRI, though statistically the expression was not significant. It is, however, pertinent to acknowledge the role of type II error, which may mask a true significant difference in hearts with IR but without MI as compared with Controls. As ADAM8 is stained in the intima of remote intramyocardial arteries besides the myocardium at the border of the left anterior ventricle corresponding to the developing infarction in MI, it is interesting to realize that these intramyocardial arteries are also vulnerable to inflammation. While edema is an initial event after immediate ischemia, the messenger of inflammation may in our model be represented by ADAM8 leading to subsequent activation of intramyocardial arteries. Global myocardial inflammation after edema may be triggered by the initial global myocardial IRI upon transplantation in our model, but a local developing infarction may sustain remote intramyocardial and periadventitial inflammation together with elevated ADAM8 staining and expression.

The basis of the heart was utilized for PCR, and the apex for histology and immunohistochemistry. The apex is most vulnerable to ischemic changes after LAD obstruction distally to the bifurcation of the first diagonal branch due to coronary vessel distribution. Tissue procured for PCR was obtained from the basis of the heart, proximal to the ligation knot of LAD obstruction, whereas the distal part of the heart including the apex of the heart encompassed myocardium distal to the ligation knot with MI. It was our intention to study unviable myocardial tissue devoid of infarction for PCR in order to examine plain remote tissue after IR in hearts with or without MI. We did not want to compare PCR with histology, since these findings would not have been from comparable myocardial tissue. In contrast, we wanted to demonstrate that PCR changes do occur in non-infarcted myocardium after IR and at the presence of a developing left anterior infarction area of the left ventricle. In addition, we wanted to demonstrate that LAD obstruction is responsible for even early histological and immunohistological changes at the vicinity of the developing infarction, though at this early stage (2 days), clear demarcation and organizational infarction area has yet to be developed after 4–7 days [28]. Remote histological area, from where intramyocardial arteries were depicted and evaluated, encompassed only the contralateral

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area devoid of the developing infarction area. Clearly, our study is not to relate infarction per se with PCR changes. Instead, we were only capable of investigating remote myocardial PCR changes with or without an infarction area at the apex of the heart.

It remains to be shown whether these changes may be reversible or attenuated with or without treatment, and whether they may be involved in subsequent functional deterioration of the heart leading to cardiac failure. We unfortunately did not measure left ventricle ejection fraction as we did not study the hearts after their induction of left ventricle remodeling phase at day 7 after transplantation [28]. This study did not relate heart function with histopathology, and we did not evaluate the long-term ADAM8 expression. However, transplantation of the heart made it amenable to stabilize an acute infarction in a low-pressurized environment mimicking left ventricle assist devices [17,18,21,28]. We perceive that instead of studying the effect of MI only, we induced IRI as well upon transplantation, thus mimicking the clinical setting with cardiac arrest during surgery. At an early stage of developing infarction in grafts with MI, it is anticipated that ADAM8 reflects the angiogenesis and inflammatory capacity of myocardium, instead of being associated with cell death alone [12]. Indeed, ADAM8 immunostaining was positive only at the border of the developing infarction together with the remote intramyocardial arteries, and negative at the core of infarction. It is tempting to speculate that endothelial cells rendered vulnerable after ischemia in the developing infarction express decreased eNOS and, via induced ADAM8 glycoprotein expression, attract enhanced amounts of circulating leukocytes in intramyocardial arteries to infiltrate the periadventitial area [12]. Increased remote expression of ADAM8 suggests that IRI and MI with developing local myocardial infarction have an impact on a global remote myocardial process. To our best of knowledge, our study is the first to suggest that heart IR and MI has a remote myocardial ADAM8 increase thus emphasizing the pervasive effect of MI on the vulnerable myocardium with IR after cardiac arrest.

Taken together, persistent MI confirmed with decreased eNOS staining, induces distant myocardial changes such as vacuolization and inflammation of the intramyocardial arterial wall, together with increased ADAM8 expression. Local MI and IRI induce increased remote ADAM8 expression representing global cardiac remodeling. Subsequent experimental studies are warranted to seek for possible reversibility and molecular mechanisms associated with remote cardiac changes.

Acknowledgements

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ORIGINAL ARTICLE

Sildenafil after cardiac arrest and infarction; an experimental rat model

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Abstract
Objectives. Resuscitation after cardiac arrest may lead to ischemia-reperfusion injury and infarction. We evaluated whether Sildenafil, a phosphodiesterase-5 inhibitor, has an impact on recovery after cardiac arrest in a rat cardiac transplantation model. *Design.* Sixty-one Fischer344 rats underwent syngeneic heterotopic cardiac transplantation after ischemia and ligation of the left anterior coronary artery of the heart to yield myocardial infarction (IRI + MI). Of these, 22 rats received subcutaneously injected Sildenafil (1 mg/kg/day) (IRI + MI + S). Twenty-three additional grafted animals with transplantation only served as controls with ischemia reperfusion injury (IRI). After 2 days, immunohistochemistry for eNOS, and RT-PCR for iNOS and Aquaporin-7 were performed after graft harvesting and histology. *Results.* Two days after transplantation, remote intramyocardial arteries were more preserved in IRI + MI + S as compared with IRI + MI and IRI (0.74 ± 0.14, 0.56 ± 0.23 and 0.55 ± 0.22, PSU, $p < 0.05$, respectively). Decreased eNOS staining confirmed the presence of developing infarction in IRI + MI and IRI + MI + S. The expression of iNOS was significantly lower during IRI + MI + S as compared with IRI + MI (0.02 ± 0.01 and 1.02 ± 0.02, FC, $p < 0.05$). *Conclusions.* Administered at the onset of reperfusion and developing infarction, Sildenafil has an impact on myocardial recovery after cardiac arrest and ischemia.

Key words: *cardiac arrest, infarction, ischemia-reperfusion, rat, sildenafil*

Introduction

Uncontrolled ischemia-reperfusion injury (IRI) after cardiac arrest may lead to permanent ongoing ischemia eventually developing myocardial infarction (MI). Traditionally, resuscitation aims at stabilizing the heart after cardiac arrest in order to avoid global and permanent heart damage. When feasible, the heart can be temporarily assisted by inserting a left ventricular assist device to reduce the myocardial work load. However, even though prompt revascularization is performed to stenosed culprit coronary arteries, irreversible myocardial damage may occur and prevent myocardial recovery (1).

IRI and local ongoing ischemia has an early global effect on myocardial remodeling. IRI and MI may be investigated by the widely used heterotopic rat cardiac transplantation model (2–4). This model provides the possibility to investigate the cardiac graft in a non-working state simulating the presence of a left ventricle assist device, while the recipient heart keeps the animal alive. Instead of endangering the recipient heart, in order to achieve MI, it is practical to ligate permanently the left anterior descending coronary artery (LAD) of the heterotopically transplanted cardiac graft. This animal model mimics the clinical concept of having an area of local and persisting MI after complete revascularization of the globally ischemic heart.

There is growing interest towards nitric oxide (NO) metabolism in terms of attenuating the consequences of IRI after resuscitation (5). The ubiquitous involvement of NO in cardiac pathophysiology suggests that adjunct therapeutic manipulation aimed at

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interfering with NO metabolism may prove successful after myocardial damage (6). Sildenafil, a potent phosphodiesterase-5 inhibitor, inhibits the breakdown of cGMP and enhances NO-driven cGMP accumulation. It has been shown that preoperatively administered Sildenafil decreases renal IRI (7), enhances cardioprotection after experimental transplantation (8,9) and attenuates myocardial infarction area after reversible IRI (6). These beneficial effects of Sildenafil are related to NO metabolism, though other possible mechanisms may also be involved (10). However, previous studies have concentrated on the preconditioning effect of Sildenafil (11,12), and the treatment has been initiated well before the onset of induced IRI. In this study, we investigate whether Sildenafil has an impact on remote myocardial changes during IRI after MI.

Materials and methods

Rats

The study was approved by the Finnish State Provincial Office. Eighty-four inbred Fischer 344 rats (F344/NHsd, Harlan Laboratories, The Netherlands) weighing 175–200 g, underwent heterotopic cardiac transplantation. The rats were kept in Tampere University vivarium and received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86-23, revised 1996).

Surgery

During harvesting of the donor heart, the ascending aorta of the cardiac graft was infused with antegrade infiltration of 2 ml cold (4°C) temperature physiologic saline fluid without heparinization. Eighty-four heterotopic cardiac transplantations were performed intra-abdominally by joining the graft aorta to the aorta and the graft pulmonary artery to the inferior vena cava of the recipient, as previously described (3). From the recipient aorta, the transplanted heart received oxygenated blood that was introduced into the coronary arteries of the graft. Through the coronary sinus, this blood circulated into the right atrium and eventually the right ventricle, from where deoxygenated blood was repulsed to the recipient rat throughout the pulmonary vein. Therefore the nutritional flow of the myocardium consisted of oxygenated blood, and the transplanted heart was not ischemic after reperfusion upon transplantation. Since the aortic valve was competent, oxygenated blood was not allowed to fill the left ventricle, and therefore the transplanted heart simulated a non-working resting state of the left side of the graft. This heterogenous transplantation model allowed one to study IRI in-vivo without interferences of myocardial stress factors. The model thus simulated the clinical concept of acute cardiac arrest resuscitated with initiation of cardiopulmonary bypass and left ventricle assist device (1). Total warm ischemia time before total graft reperfusion was 20–30 minutes after cardiac arrest. In 61 grafts, the LAD was also ligated permanently at its proximal part with a single 7-0 suture to yield MI at the apex of the heart graft. The ligation knot for LAD obstruction was placed at the bifurcation of the 1rst diagonal branch due to coronary vessel distribution, since the apex is most vulnerable to ischemic changes leading to MI. Of these grafts, 22 rats were treated with Sildenafil administered 1 mg/kg/day subcutaneously initiated after reperfusion of the graft (IRI + MI + S), hence 39 grafts with IRI underwent transplantation and LAD ligation without treatment (IRI + MI). Twenty-three grafts underwent transplantation only (IRI).

Graft patency

Graft patency was achieved by means of palpation using a score from 0 to 6; 0 indicated no pulse, 2 indicated weak pulsation, and 6 meant normal contractility and strong pulsation. The palpation score, as a direct measure of cardiac vitality and effective contractility, proved to be a reliable and convenient test for definition of the end point for graft survival, with no variability or bias in the evaluations of independent observers (13).

Tissue samples

The recipient rats were followed and sacrificed upon termination of graft palpation (palpation score 0 to 2) yielding 63 hearts after 2 days of reperfusion. The basal part of the cardiac graft that did not include the infarction was procured for quantitative RT-PCR analysis to investigate for the remote myocardium. In contrast, the apex part of the graft, including MI in cardiac grafts with LAD ligation, was embedded in paraffin and 5-μm sections were cut.

Histology

For histology, the 5-μm sections were stained with Hematoxylin and Eosin. Evaluation was performed

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blinded to the study protocol, treatment allocation and time sequence by 2 investigators (AM and VV) and technically unclear slides were rejected. The following variables were evaluated: presence of myocardial edema, hemorrhage and ischemia. As the vacuolization of nuclei of the media layer of intramyocardial arteries reflected edema, a representative cross-sectional intramyocardial artery was chosen randomly from the left anterior ventricular wall and the left posterior ventricular wall representing remote myocardium. As the minority of the arterial wall nuclei was round-shaped representing normal nuclei, sharp-edged blue nuclei of the media cells were defined as non-preserved and manually counted together with vacuolated and edematous nuclei. Periadventitial inflammation was graded according to an arbitrary scale from 0 to 2 and expressed as point score units (PSU): 0, no inflammation; 1, presence of occasional inflammatory cells; 2, groups of inflammatory or proliferating cells.

Immunohistochemistry for eNOS and iNOS

Twenty hearts were randomly selected 2 days after transplantation for immunohistochemistry. Paraffin-embedded slides were deparaffinized with 3 changes of xylene, and rehydrated in a series of graded ethanol, and rinsed well under running distilled water. Slides were placed in a preheated retrieval buffer, 0.1 mmol EDTA, pH 8.0, for 30 minutes, then cooled in the buffer for 5 minutes, followed by a 5-minute rinse under running distilled water. After heat-induced epitope retrieval, slides were placed on an autostainer (DAKO Corp, Carpinteria, California, USA). Sections were incubated with 3% hydrogen peroxide in ethanol for 5 minutes to inactivate the endogenous peroxides, incubated either in 1:100 eNOS (eNOS, DAKO Corp) or iNOS (iNOS, DAKO Corp) for 30 minutes, followed by rinsing with Tris-buffered saline solution with Tween 20 (TBST) wash buffer. Secondary incubation was with DUAL-labeled polymer-horseradish peroxidase (K4061; DAKO Corp) for 15 minutes. The slides were rinsed with TBST wash buffer. Sections were then incubated in 3,3-diaminobenzidine (K3467, DAKO Corp) for 5 minutes, counterstained with modified Schmidt hematoxylin for 5 minutes, and rinsed for 3 minutes in tap water to blue sections, dehydrated with graded alcohols, and cleared in 3 changes of xylene before mounting. Stainings for eNOS and iNOS were scored according to an arbitrary scale from 0 to 3 and expressed as PSU, where 0 means, no staining visualized; 1, individual positively stained nuclei; 2, groups of positively stained nuclei; 3, intensive and global positively stained area. Two hearts were rejected due to technical failure.

Quantitative RT-PCR analysis

The frozen tissue of the base of the heart was homogenized and RNA was extracted using a rotor-stator homogenizer and NucleoSpin® RNA II kit (Machery-Nagel GmbH & Co, Düren, Germany) according to the manufacturer's instructions. 50 ng of total RNA was reverse-transcribed into cDNA in reaction volume of 20 μl. The quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed with standard protocols on Abi Prism 7300 instrument (Applied Biosystems, CA, USA). The PCR reaction was performed with TaqMan® Gene Expression assays for aquaporin-7 (ID Rn00569727_m1) and GAPDH (ID Rn01462662_g1) (both from Applied Biosystems) according to the manufacturer's instructions with TaqMan® Universal PCR Master Mix. All samples were performed as three replicates.

The expression levels of Aquaporin-7, iNOS and GAPDH as an internal control/house keeping gene were evaluated. Ct values were determined for every reaction and the relative quantification was calculated using the $2^{-\Delta\Delta Ct}$ method (14). Briefly, the data were normalized to the expression of house keeping gene GAPDH, and values of Control samples were used as a calibrator. The media and standard error of mean for the Aquaporin-7 and iNOS expression levels were calculated between IRI + MI and IRI + MI + S groups to demonstrate the effect of Sildenafil on remote myocardium during MI.

Statistical analysis

Data were presented as the mean and standard error of the mean. Kruskall-Wallis non-parametric statistics was included for comparison between independent groups. Two-way ANOVA was utilized to analyze among groups. Nonparametric data between 2 groups were analyzed with Mann-Whitney U test. Statistical analyses were performed with commercial statistical software (SPSS 19.0, SPSS Inc, Chicago, IL).

Results

Heart graft patency

A third of the hearts (33%) with IRI + MI and only 18% with IRI + MI + S had a palpation score less than 2 out of 6 at 2 days of reperfusion, whereas 82% of the hearts with IRI and IRI + MI + S remained

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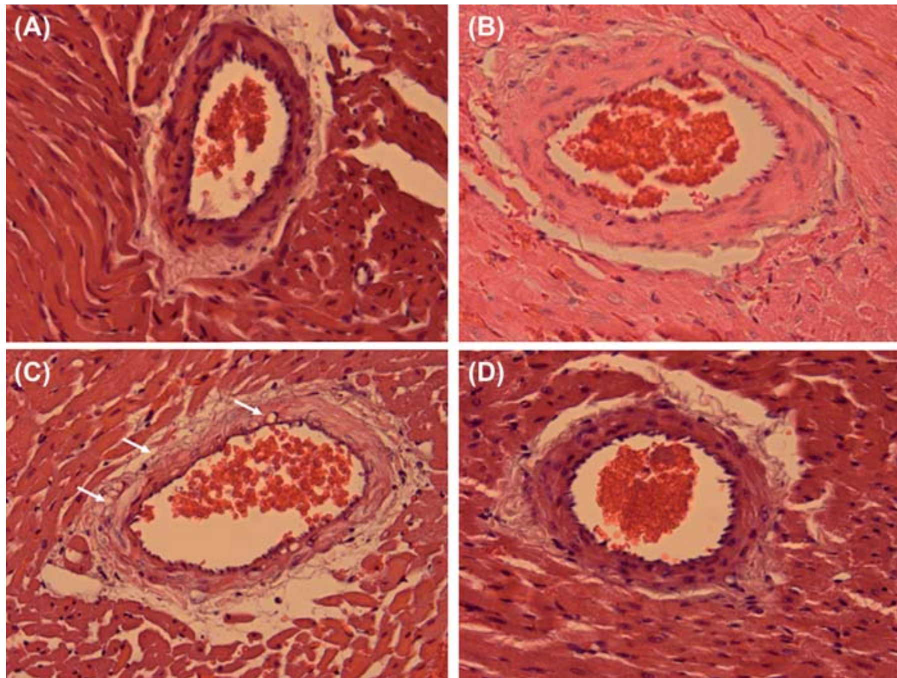

Figure 1. Representative histology of a remote intramyocardial artery of a normal heart (A), a graft with ischemia-reperfusion injury only (IRI; B), a graft with ischemia-reperfusion injury and myocardial infarction (IRI + MI; C), and a graft with ischemia-reperfusion injury and myocardial infarction treated with Sildenafil (IRI + MI + S; D) 2 days after reperfusion simulating resuscitation. X40. Note edema of intramyocardial artery shown as vacuolization of vessel wall in C (small arrows).

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patent for 2 days in comparison with only 67% hearts with IRI + MI ($p < 0.05$).

Histology

There were no hemorrhagic or major ischemic myocardial differences between the hearts with IRI + MI + S and IRI + MI at 2 days after operation (1.90 ± 0.25 vs 1.80 ± 0.22 and 2.20 ± 0.19 vs 1.85 ± 0.16, PSU, respectively. Global myocardial edema and inflammation tended to decrease in IRI + MI + S as compared with IRI + MI (2.00 ± 0.27 vs 2.45 ± 0.21 and 0.60 ± 0.17 vs 1.00 ± 0.12, PSU, respectively), but no significant changes were observed among groups. After 2 days, the relative number of recovered remote intramyocardial artery wall nuclei, that is the number of clear smooth-edged media cell nuclei divided by the total number of media cell nuclei including vacuolated and sharp-edged dark media cell nuclei, was significantly increased in IRI + MI + S as compared with IRI + MI (72 ± 3.60 vs 56 ± 4.46, PSU, respectively, $p < 0.05$, Figures 1 and 2).

Immunohistochemistry for eNOS and iNOS

Statistically, decreased staining of eNOS was observed in hearts with IRI + MI + S and IRI + MI as compared with IRI after 2 days (0.75 ± 0.29 and 0.60 ± 0.40 vs 2.50 ± 0.28, PSU, respectively, $p < 0.05$, Figure 3). There was a tendency for decrease staining in iNOS in hearts with IRI + MI + S as compared with IRI + MI, though statistical significance was not reached (1.87 ± 0.33 vs 2.50 ± 0.25, PSU, respectively, Figure 4).

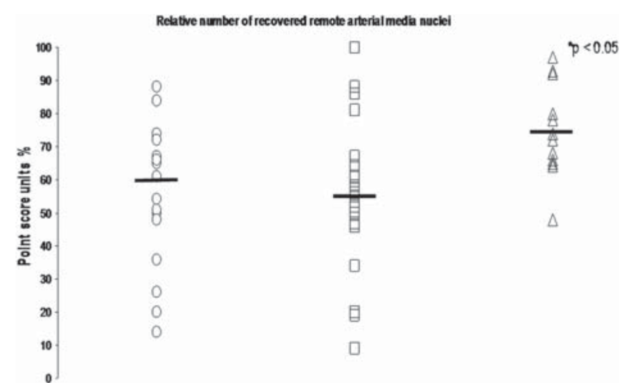

Figure 2. Relative number of recovered remote intramyocardial artery wall nuclei in grafts with ischemia-reperfusion injury only (IRI, circles), grafts with ischemia-reperfusion injury and myocardial infarction (IRI + MI, boxes), and grafts with ischemia-reperfusion injury and myocardial infarction treated with Sildenafil (IRI + MI + S, triangles). As compared with IRI and IRI + MI, IRI + MI + S have increased relative number of recovered remote intramyocardial artery nuclei 2 days after reperfusion. $^{*}p < 0.05$, Kruskal-Wallis. Horizontal bars indicate median.

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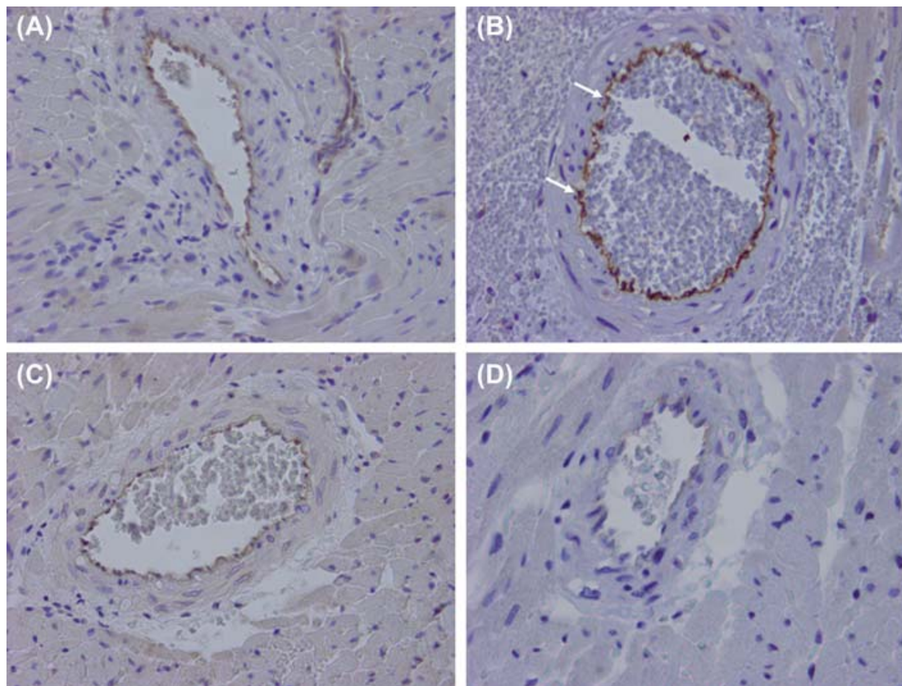

Figure 3. Representative immunohistochemistry for eNOS of a remote intramyocardial artery of a normal heart (A), a graft with ischemia-reperfusion injury only (IRI; B), a graft with ischemia-reperfusion injury and myocardial infarction (IRI + MI; C), and a graft with ischemia-reperfusion injury and myocardial infarction treated with Sildenafil (IRI + MI + S; D) 2 days after reperfusion simulating resuscitation. X40. Note intensive positive endothelial staining (arrows) in a remote intramyocardial artery of a graft with ischemia-reperfusion injury only (IRI; B) as compared with grafts with infarction without (IRI + MI; C) or with Sildenafil (IRI + MI + S; D).

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iNOS and Aquaporin-7 expressions

iNOS expression was significantly lower in IRI + MI + S as compared with IRI + MI after 2 days (0.02 ± 0.01 vs 1.01 ± 0.23, FC, respectively, $p < 0.05$), whereas no major difference was observed in Aquaporin-7 expression (0.47 ± 0.05 vs 0.78 ± 0.23, FC, respectively).

Discussion

It is important to acknowledge the limited possibility in treating the ischemic myocardium. An infarction represents an irreversible process, which itself is no longer amenable to treatment. This effect is well-known in clinics, where revascularization of an acutely obstructed target coronary artery causing MI is insufficient to prevent global heart dysfunction leading to acute stunning. The heart needs immediate rest such as infusion of cardioplegia and insertion of a left ventricular assist device that decreases cardiac overload. These maneuvers, however, do not abolish the ongoing effect of MI that induces remote histopathological changes.

The presence of a developing local infarction, often the cause of acute cardiac dysfunction during cardiac arrest, has also been advocated as enhancing the poor cardiac outcome after resuscitation (1). After cardiac arrest, resuscitation aims at preventing the heart from further damage due to MI (1). The coexistence of myocardial edema and inflammation remote to the infarction area may be reversible (15). Salvation and protection of the viable myocardium are of uttermost importance, particularly as infarction has the remote ongoing myocardial effects (15). This study demonstrates that Sildenafil treatment initiated after IRI and MI can prolong cardiac graft patency in a rat transplantation model simulating resuscitation. This finding is confirmed by a preserved palpation score (2 or more out of 6) in IRI + MI + S compared with IRI + MI.

Experimental data suggest that preoperative Sildenafil augments the nitric oxide reserves of the heart, which in turn, enhances the cardiac capacity to survive after IRI (16). According to in-vitro preconditioning studies, Sildenafil increases myocardial iNOS (16). We attempted to simulate the clinical setting, were treatment is initiated after onset of resuscitation, that is after reperfusion from cardiac arrest and warm ischemia together with induction of myocardial infarction. In this study, Sildenafil has an impact even when treatment was initiated after induction of warm ischemia and infarction. Infarction itself was irreversible, since LAD was permanently ligated, therefore eNOS was down-regulated in hearts with MI (3). The remote intramyocardial changes, in contrast, were reversible with Sildenafil (Figures 1 and 2).

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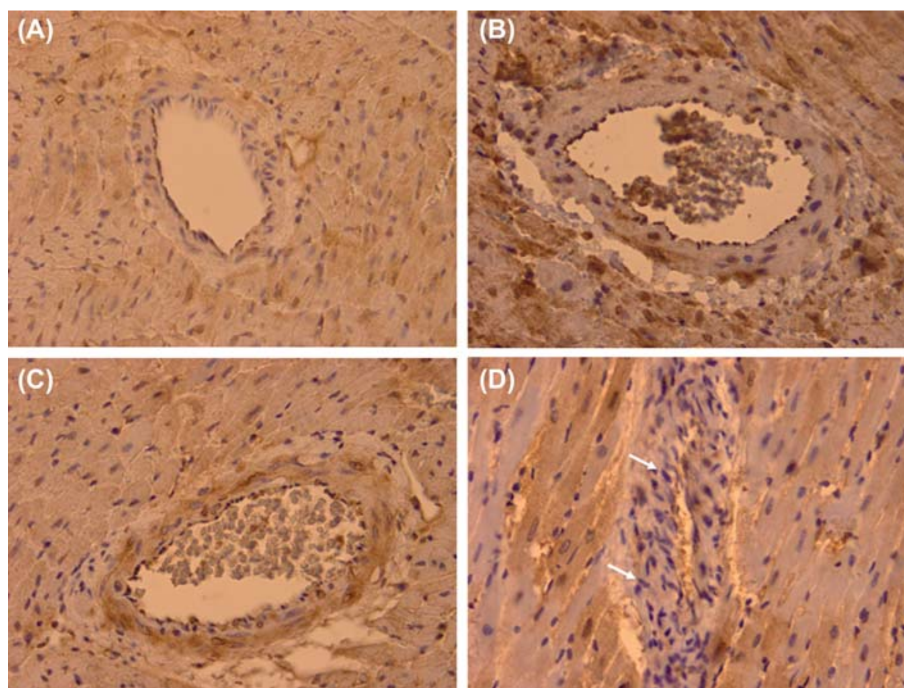

Figure 4. Representative immunohistochemistry for iNOS of a remote intramyocardial artery of a normal heart (A), a graft with ischemia-reperfusion injury only (IRI; B), a graft with ischemia-reperfusion injury and myocardial infarction (IRI + MI; C), and a graft with ischemia-reperfusion injury and myocardial infarction treated with Sildenafil (IRI + MI + S; D) 2 days after reperfusion simulating resuscitation. X40. Note tendency for decreased positive staining (arrows) in a remote intramyocardial artery of a graft with ischemia-reperfusion injury and myocardial infarction treated with Sildenafil (IRI + MI + S) as compared with grafts without (IRI; B) or with infarction (IRI + MI; C).

Controversial data exist on whether eNOS and/or iNOS are elevated after myocardial ischemia (6), but most interpret iNOS elevation as a surrogate for myocardial damage (6). On the other hand, some state that eNOS is increased after induction of myocardial protection. As a consensus, eNOS decrease may reflect the state of heart ischemia, whereas iNOS increase reflects response to IRI (15,17). It was beyond our scope to demonstrate the specific mechanisms underlying the effectiveness of Sildenafil per se in terms of NO metabolism, and therefore we concentrated on defining the outcome of remote myocardium susceptible to reversible ischemia. We selected evaluation of iNOS expression of the remote myocardium to indicate the severity of myocardial damage after IRI and MI. After 2 days, Sildenafil decreased remote myocardial iNOS expression after IRI and MI, suggesting for salvation of the remote myocardium.

We may thus speculate that in our model iNOS expression, obtained from the basal heart tissue far away from the infarction area, may mirror the state of the remote myocardium itself instead of revealing the mechanism of action of the medication. Decreased iNOS expression may also suggest for a temporary loss of NO reserves of the heart with IR and MI (18), and long-term myocardial protection may be due to delayed iNOS increase. Sildenafil may also include other mechanisms as speculated in a renal study (10). We therefore wanted to confirm the permanent effect of LAD ligation on remote myocardium by evaluating Aquaporin-7, a mediator of water balance associated with glycerol metabolism considered as a surrogate of MI (2,19). Thus, Aquaporin-7 expression was observed in both untreated and Sildenafil-treated hearts with MI due to LAD ligation at the apex of the heart.

In summary, Sildenafil, administered at the onset of IRI and MI, maintained patent remote intramyocardial arteries while altering remote iNOS expression profile of the cardiac graft. Though still speculative at this point, Sildenafil may be a possible adjunct during resuscitation of the ischemic heart.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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ORIGINAL PAPERS

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C4d Deposition Reveals Myocardial Infarction After Cardiac Arrest – Experimental Study*

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. The diagnosis of regional myocardial infarction (MI) after cardiac arrest and ischemia-reperfusion injury (IRI) is a major clinical challenge.
Objectives. We evaluated in a rat cardiac transplantation model whether IRI alone or with MI would induce complement C4d deposition.
Material and Methods. Isogenic heterotopic cardiac transplantation was performed in 16 Fischer 344 rats to induce IRI, of which 9 rats also underwent ligation of the left anterior coronary artery (LAD) of the heart to yield MI. Histology and qRT-PCR for endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) and transforming growth factor β (TGFβ) were performed after cessation of heart beat. C4d was evaluated by immunohistochemistry.
Results. Myocardial inflammation and C4d deposition was increased in grafts with IRI+MI as compared with IRI (0.71 vs. 0.14, PSU, respectively, $p < 0.04$ and 80.13 vs. 20.29, PSU, respectively, $p < 0.02$). The expression of eNOS decreased in grafts with IRI + MI as compared with IRI ($p < 0.05$). Receiver operating characteristic (ROC) curve analysis showed that IRI + MI was associated with C4d deposition (AUC 0.837; S.E. 0.116; $p = 0.035$; 95% C.I. 0.610–1.000).
Conclusions. Increased C4d deposition may be amenable to identify early MI after cardiac arrest. Early treatment aimed towards complement activation may provide a novel means for induced MI after cardiac arrest (**Adv Clin Exp Med 2015, 24, 3, 393–399**).

Key words: myocardial infarction, C4d, complement, heterotopic rat cardiac transplantation.

The definitive diagnosis of regional myocardial infarction (MI) after cardiac arrest and ischemia-reperfusion injury (IRI) is a major clinical and forensic challenge. Uncontrolled IRI after cardiac arrest may lead to permanent ongoing ischemia eventually developing MI. When feasible, the heart can be temporarily assisted by inserting a left ventricular assist device to reduce the myocardial workload. Even though prompt revascularization is performed to stenosed culprit coronary arteries, irreversible myocardial damage may ensue [1]. After cardiac dysfunction and surgery, interpretation of MI from the release of traditional markers- such as creatinine kinase, troponin T and pro-BNP may be blurred by the presence of inflammation and tissue destruction [2, 3].

IRI and MI may be investigated by the widely used heterotopic rat cardiac transplantation model [4]. This model provides the possibility to investigate the cardiac graft in a non-working state simulating

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the presence of a left ventricle assist device, while the recipient heart keeps the animal alive. Instead of endangering the recipient heart, in order to achieve MI, it is practical to ligate permanently the left anterior descending coronary artery (LAD) of the heterotopically transplanted cardiac graft. This animal model mimics the clinical concept of having an area of local and persisting MI after complete revascularization of the globally ischemic heart. Using this experimental model, we have recently demonstrated that MI after IRI has a remote myocardial impact after cardiac arrest [4].

Few *postmortem* evidence [1] and experimental studies [5, 6] suggest that complements are activated after MI. Complement activation is an early marker of tissue destruction [7–13]. The end-production of the complement activation cascade C4d represents a stable molecule suitable for evaluation. It is not known whether complement activation occurs after cardiac arrest, least to say whether C4d reveals the developing MI after IRI. We therefore tested the power of complement activation, as evidenced by C4d deposition, to detect MI after cardiac arrest and IRI in our heterotopic rat cardiac transplantation model. Histology and qRT-PCR for endothelial nitric oxide synthase (eNOS), inducible nitric oxide synthase (iNOS) and transforming growth factor β (TGFβ) were performed after cessation of heartbeat to verify the effect of IRI and MI on the remote myocardium by looking for delicate changes in gene expressions associated with the activation of C4d. Nitric oxide synthases and TGFβ mirror the molecular cascade activation related to recovery from IRI [14].

Material and Methods

Ethics

The study was approved by the State Provincial Office. Thirty two inbred Fischer 344 rats (F344/ /NHsd, Harlan Laboratories, The Netherlands) weighing 200–270 g, served as donors (n = 16) and recipients (n = 16). The rats were kept *in vivarium* and received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86–23, revised 1996).

Surgical Procedure

The rats were anesthetized with sevoflurane (Baxter, USA) for inhalation, and a mixture of ketamine (Ketalar®; Orion Pharma Oy, Espoo, Finland; 7.5 mg/100g) and medetomidine (Dormitor®; Pfizer Oy Animal Health, Espoo, Finland; 0.05 mg/100 g) intraperitoneally. A modified heterotopic transplantation of the heart was performed to all 16 grafts, as previously described [4]. Briefly, before harvesting, cold 4ºC infusion with cardioplegia fluid (Custodiol®; Bretschneider HTK solution for cardioplegia and miltiorgan protection, Germany) was infused into the donor aorta in order to arrest the heart and maximize myocardial protection. After harvesting, the cardiac graft was immersed into cold (4ºC) temperature physiologic saline fluid. The graft aorta was joined to the aorta and the graft pulmonary artery to the inferior *vena cava* of the recipient. From the recipient aorta, the transplanted heart received oxygenated blood that was introduced into the coronary arteries of the graft. *Via* the coronary sinus, this blood circulated into the right atrium and eventually the right ventricle, from where deoxygenated blood recirculated to the recipient rat throughout the pulmonary artery. Therefore, the nutritional flow of the myocardium consisted of oxygenated blood, and the transplanted heart was not ischemic after reperfusion upon transplantation. Since the aortic valve was competent, oxygenated blood was not allowed to fill the left ventricle, and therefore the transplanted heart simulated a non-working resting state of the left side of the graft. This heterogenous transplantation model allowed us to study IRI *in vivo* without interferences of myocardial stress factors. Total ischemia time before total graft reperfusion was 30 min after cardiac arrest. After the procedure, carprofen (Rimadyl®; Pfizer Oy Animal Health, Helsinki, Finland) 0.1~0.15 mL was given subcutaneously for pain relief.

Experimental Groups

The rats were randomized into 2 groups. Seven grafts underwent transplantation only to serve as controls with IRI. In 9 grafts, the LAD was also ligated permanently at its proximal part with a single 7–0 suture yielding a confined local MI (IRI + MI); the ligation knot for LAD obstruction was placed immediately before the bifurcation of the 1st diagonal artery branch of the LAD.

Graft Patency

Graft patency was achieved by means of palpation using a score from 0 to 6; 0 indicated no pulse, 2 indicated weak pulsation, and 6 meant normal contractility and strong pulsation. The palpation score, as a direct measure of cardiac vitality and effective contractility, proved to be a reliable and convenient test for definition of the end point

for graft survival, with no variability or bias in the evaluations of independent observers [15].

Tissue Samples

The recipient rats were sacrificed when palpation score of the cardiac graft decreased to less than 2 out of 6. The basal part of the cardiac graft was snap frozen in liquid nitrogen and stored at –70ºC for further analysis. The middle half of the graft was embedded in paraffin and 5 µm sections were cut and stained with hematoxylin-eosin.

Histology

Evaluation of histology was performed blinded to the study protocol. The following variables were evaluated: presence of myocardial edema, hemorrhage and inflammation. Periadventitial inflammation was graded according to an arbitrary scale from 0 to 2 and expressed as point score units (PSU): 0, no inflammation; 1, presence of occasional inflammatory cells; 2, groups of inflammatory or proliferating cells. Vacuolated nuclei of the media layer of intra-myocardial arteries reflected edema and were counted in a representative cross-sectional intra-myocardial artery chosen randomly from the left anterior ventricular wall. Round and smooth-edged blue nuclei of the media cells were defined as normal and expressed as PSU. The number of vacuolated nuclei was divided by the number of round smooth-edged media cell nuclei to obtain the relative number of vacuolated nuclei.

Immunohistochemistry for C4d

Immunohistochemistry was performed using Ventana Lifesciences Benchmark XT© Staining module. The paraffin-embedded slides were deparaffinized with 3 changes of xylene, and rehydrated in a series of graded ethanol, and rinsed well under running distilled water. Slides were placed in a preheated retrieval buffer, 0.1 mmol EDTA, pH 8.0, for 30 min, then cooled in the buffer for 5 min, followed by a 5-min rinse under running distilled water. After heat-induced epitope retrieval, slides were placed on an autostainer (DAKO Corp, Carpinteria, California, USA). Sections were incubated with 3% hydrogen peroxide in ethanol for 5 min to inactivate the endogenous peroxides and incubated in C4d (dilution 1:50) (Biomedica Gruppe) for 30 min, followed by rinsing with Tris-buffered saline solution with Tween 20 (TBST) wash buffer. Secondary incubation was with DUAL-labeled polymerhorseradish peroxidase (K4061; DAKO Corp) for 15 min. The slides were rinsed with TBST wash buffer. Sections were then incubated in 3,3-diaminobenzidine (K3467, DAKO Corp) for 5 min, counterstained with modified Schmidt hematoxylin for 5 min, and rinsed for 3 min in tap water to blue sections, dehydrated with graded alcohols, and cleared in 3 changes of xylene before mounting. Positively stained C4d deposition was counted in a representative cross-sectional intra-myocardial artery chosen randomly from the left anterior ventricular wall. The total number of myocardial C4d deposition was calculated accordingly and expressed as PSU.

Quantitative RT-PCR Analysis

The frozen tissue of the base of the heart was homogenized and RNA extraction was carried out with GenElute™ Mammalian Total RNA Miniprep kit (Sigma-Aldrich, St. Louis, MO, USA) with proteinase K treatment. Total RNA was then reverse-transcribed to cDNA using TaqMan® Reverse Transcription reagents and random hexamers (Applied Biosystems, Foster City, CA, USA). cDNA obtained from the RT reaction (amount corresponding to approximately 1 ng of total RNA) was subjected to quantitative PCR using QuantiTect® Primer Assays (Qiagen, Valencia, CA, USA) for eNOS, iNOS, TGFβ and GAPDH, Maxima® SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Waltham, MA, USA) and ABI PRISM 7000 Sequence detection system (Applied Biosystems, Foster City, CA, USA). PCR reaction parameters for SYBR® Green detection were as follows: incubation at 50ºC for 2 min, incubation at 95ºC for 10 min, and thereafter 40 cycles of denaturation at 95ºC for 15 s and annealing and extension at 60ºC for 1 min. Each sample was determinated in duplicate. Ct values were determined, and the relative quantification was calculated using the $2^{-\Delta\Delta Ct}$ method [16]. Values of control samples were used as a calibrator, and the expression levels of eNOS, iNOS and TGFβ were normalized against GAPDH.

Statistical Analysis

Statistical analysis was performed using SPSS for Windows (v. 20.0). Data is presented as median or mean ± standard error of the mean (SEM). Data was analyzed with Mann-Whitney *U* test when appropriate. Pearson correlation was performed to investigate for the relation between continuous variables. The predictive value of C4d deposition to identify remote immunological reactivity associated with myocardial infarction after IRI was assessed by Receiver operating characteristic (ROC) curve analysis. P-values < 0.05 were considered significant.

Results

Heart Graft Patency

One heart with IRI + MI and IRI were non-palpable one day after reperfusion. All other hearts with IRI + MI and with IRI had a palpation score less than 2 out of 6 after 2 days of reperfusion (Table 1).

Table 1. Number of dysfunctional hearts after reperfusion with palpation score < 2 out of 6

Group, number	1 day	2 day
IRI, n = 7 (%)	1 (14%)	6 (86%)
IRI + MI, n = 9 (%)	1 (11%)	8 (89%)

IRI = hearts with ischemia-reperfusion injury;
IRI + MI = hearts with ischemia-reperfusion injury and myocardial infarction.

Histology

Two days after operation, there were no hemorrhagic or major remote ischemic myocardial differences between the hearts with IRI and IRI + MI (1.29 vs. 0.86, PSU, respectively, p = 0.59 and 1.57 vs. 1.43, PSU, respectively, p = 0.83). Global myocardial edema and epicardial inflammation did not differ among IRI and IRI + MI (2.29 vs. 2.14, PSU, respectively, p = 0.79 and 0.57 vs. 0.43, PSU, respectively, p = 0.94). A global ischemic area was faintly observed in the left anterior ventricular wall of the myocardium in IRI + MI; this was recorded as mild myocardial edema in the area corresponding to the developing infarction. However, there was no statistical difference in the number of sharp-edged dark media cell nuclei of intramyocardial arteries in hearts with IRI and IRI + MI (10.86 vs. 13.40, PSU, respectively, p = 1). There was also no difference in the number of clear smooth-edged media cell nuclei of remote intramyocardial arteries in hearts with IRI as compared with IRI + MI (24.83 vs. 17.50, PSU, respectively, p = 0.688). Myocardial inflammation increased in hearts with IRI + MI as compared with IRI (0.71 vs. 0.14, PSU, respectively, p = 0.04).

Immunohistochemistry for C4d

Statistically, increased myocardial staining for C4d was observed in hearts with IRI + MI as compared with IRI (80.13 vs. 20.29, PSU, respectively, p = 0.02, Fig. 3A). Increased number of C4d deposition was also specifically observed in the media of intramyocardial arteries situated along the left anterior wall corresponding to the infarction

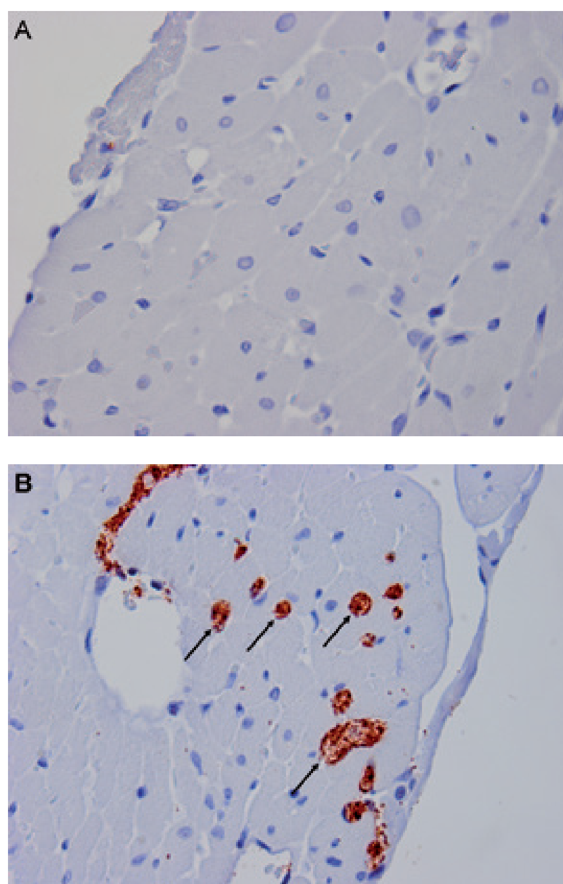

Fig. 1. Representative immunohistochemistry for myocardial C4d deposition of a heart with ischemia--reperfusion injury only (IRI; A), and a graft with ischemia-reperfusion injury and myocardial infarction (IRI + MI; B) 2 days after reperfusion. X40. Note intensive positive C4d staining (arrows) in remote myocardium of the heart in B

area in hearts with IRI + MI in contrast to only few staining in hearts with IRI (2.75 vs. 0.86, PSU, respectively, p = 0.01, Fig. 3B). The relative number of C4d deposition, that is the total number of C4d deposition divided by the number of observed intramyocardial arteries, remained elevated in hearts with IRI + MI as compared with hearts with IRI (1.73 vs. 1.25, PSU, respectively, p = 0.03).

eNOS, iNOS and TGFß Expressions

The expression of eNOS decreased in hearts with IRI + MI as compared with IRI (0.51 vs. 1.58, respectively, p = 0.05). There were no differences in the expressions of iNOS and TGFβ in hearts with IRI + MI as compared with IRI (2.06 vs. 5.98, respectively, p = 0.64 and 1.03 vs. 2.69, respectively, p = 0.23). The Pearson correlation between C4d staining of the myocardium and the expression of eNOS was –0.357 (p = 0.156), and –0.017 (p = 0.481)

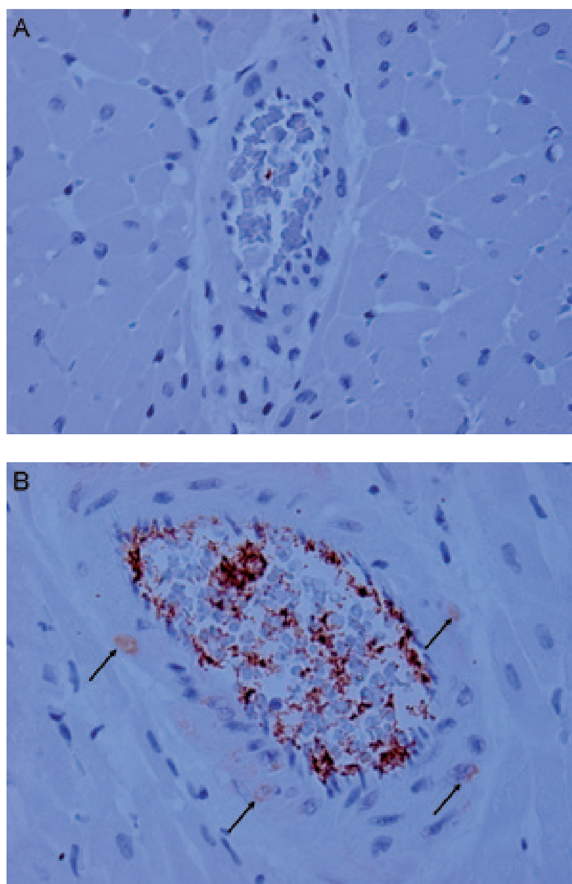

Fig. 2. Representative immunohistochemistry of an intramyocardial artery for C4d deposition of a heart with ischemia-reperfusion injury only (IRI; A), and a graft with ischemia-reperfusion injury and myocardial infarction (IRI + MI; B) 2 days after reperfusion. X40. Note positive C4d staining (arrows) in an intramyocardial artery of the heart in B

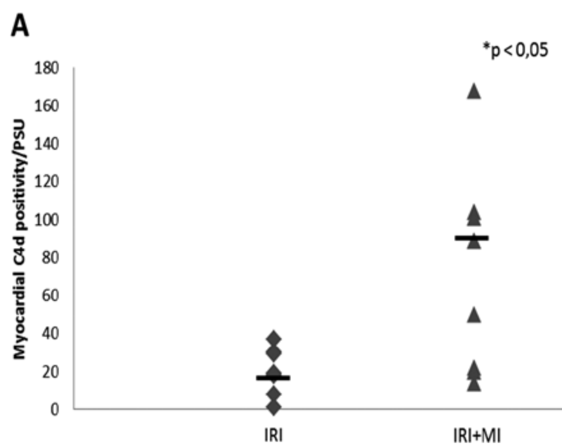

Fig. 3A. Number of myocardial C4d deposition in hearts with ischemia-reperfusion injury only (IRI, diamonds), and grafts with ischemia-reperfusion injury and myocardial infarction (IRI + MI, triangles). Note increased myocardial C4d deposition 2 days after reperfusion in hearts with IRI + MI as compared with IRI. *p < 0.05, Mann-Whitney. Horizontal bars indicate median

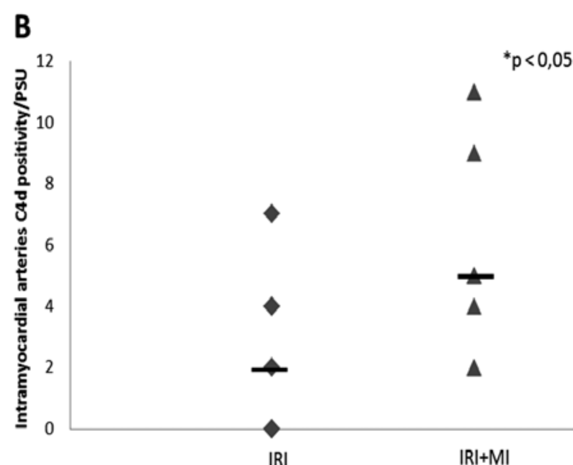

Fig. 3B. Number of intramyocardial artery wall C4d deposition in hearts with ischemia-reperfusion injury only (IRI, diamonds), and grafts with ischemia-reperfusion injury and myocardial infarction (IRI + MI, triangles). Note increased intramyocardial artery wall C4d deposition 2 days after reperfusion in hearts with IRI + MI as compared with IRI. *p < 0.05, Mann--Whitney. Horizontal bars indicate median

between C4d staining of intramyocardial arteries and the expression of eNOS. Statistically, these correlations were not significant.

ROC Curve Analysis

The predictive value of C4d positive staining to myocardial infarction after IRI was assessed by Receiver operating characteristic (ROC) curve analysis. ROC analysis showed that myocardial infarction was associated with C4d deposition (AUC 0.837; S.E. 0.116; p = 0.035; 95% C.I. 0.610–1.000), but not with presence of inflammation per se (AUC 0.786; SE 0.131; p = 0.074; 95 % CI 0.529–1.000).

Discussion

We demonstrate with this heterotopic rat cardiac transplantation model simulating the clinical concept of total cardiac arrest that C4d reveals MI in hearts that ceased to beat 2 days after IRI despite myocardial inflammation. In contrast, IRI alone did not suffice to induce increased C4d deposition.

A major challenge to the clinician is to define the presence of MI in hearts lost after IRI. It has previously been shown that complement activation occurs in the infarction area in otherwise healthy hearts [1, 5, 6, 17]. In our study, C4d was found in the myocardium remote to the infarction after IRI, and C4d was not only encompassed in the infarction area. C4d deposition was present

specifically in the wall of the intramyocardial arteries remote to the infarction. This is in accordance to previous observation stating that complement deposition was particularly prominent in the arterial wall indicating subsequent development of arteriopathy [18]. Importantly, complement C3d and C5b-9 depositions were significantly increased in larger areas of the hearts of patients with a reinfarction than in patients with single infarcts [1]. An immunological effect of MI in ischemic hearts undergoing IRI seems noteworthy and determines the outcome of the remote myocardium.

In contrast to some [1, 5, 6, 19], we did not observe increased C4d deposition in hearts with IRI per se. Reperfusion alone has not been demonstrated convincingly in humans to induce C4d deposition [1]. It is plausible that though C4d may be considered rather stable, a wash-out effect may occur after complete reperfusion in grafts with IRI *in vivo* [20]. On the other hand, it has been demonstrated that C4d deposition is a controversial indicator- at least of humoral rejection in ABO--compatible liver allografts. Diagnostic interpretation, based alone on the complement split product C4d, should be cautiously done [21]. The utility of C4d deposition to detect early myocardial infarction may help in the interpretation of perioperative ischemic injury [11, 22]. The intensity of microvascular C4d staining may differ during humoral rejection as compared with myocardial infarction alone [22].

In our study, we did not specifically investigate for the size of MI, since all grafts with IRI + MI had occlusion of the LAD exactly at the same anatomic site. As previously observed, complement activation together with C3d and C5b-9 depositions did not correlate with infarction size alone [1]. To verify the presence of MI during IRI, we analyzed eNOS expression of the myocardium to confirm the remote myocardial effect of MI. We also investigated the expressions of iNOS, TGFβ and studied intramyocardial artery histology. Nitric oxide synthases mirror the molecular cascade activation related to recovery from IRI [14], and these parameters were chosen to confirm the effect of IRI on the remote myocardium by seeking for delicate changes in gene expressions influencing the vascular endothelium. Decreased eNOS was observed in grafts with IRI + MI as compared with grafts with IRI alone. Importantly, myocardial inflammation was eminent during MI, but in contrast to C4d, it was not possible to identify the hearts with MI by the presence of inflammation per se since tissue destruction to some extension is also involved during IRI and cardiac dysfunction alone. Both iNOS and TGFβ were not capable to distinguish between IRI + MI and IRI as the presence of inflammation after cardiac dysfunction was imminent.

There is a strong need for specific markers of infarction independent of inflammation. Complement activation predicts concomitant inflammation. As an indicator of ongoing inflammation, activated complement deposition was increased in diseased aortic valves [23]. This study hints that myocardial C4d deposition due to MI after IRI and cardiac dysfunction occurs concomitantly with inflammation. It was beyond the scope of this study to investigate for the involvement of the alternative vs. classical complement pathway, and both may be present [6, 7]. We did not evaluate for gene expressions of C4d since the detection of C4d deposition by immunohistochemistry is relatively accurate and proved comparable at least to immunofluorescence methods [24].

In summary, C4d is a powerful indicator of the impact of MI to the remote myocardium after IRI. This observation may serve as an important adjunct in the diagnosis of MI after IRI. The inhibition of C4d deposition and the investigation of peripheral blood for complements may further elucidate the impact of complement activation during MI and IRI.

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