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CLINICAL AND EXPERIMENTAL STUDIES ON CARDIOMYOCYTE APOPTOSIS IN ISCHEMIA-REPERFUSION INJURY AND MYOCARDIAL PROTECTION DURING CARDIAC SURGERY

by

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To my dear wife, Noora

ABSTRACT

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Clinical and experimental studies on cardiomyocyte apoptosis in ischemia-reperfusion injury and myocardial protection during cardiac surgery

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Background Arresting the heart with aortic cross-clamp and cardioplegia solution (ischemia) during open heart surgery, and returning the coronary blood flow (reperfusion), cause injury to the myocardium. Cardioplegia solution, as well as preconditioning with ischemia or pharmacological agents, protects the myocardium from this injury. Cardiomyocyte apoptosis has shown to be induced during ischemia-reperfusion injury, but the meaning of this induction is not clear. The purpose of this thesis was to study the role of apoptosis in ischemia-reperfusion injury, in myocardial protection, and in postoperative ventricular dysfunction associated with open heart surgery.

Materials and methods Animal models of ischemia-reperfusion were used to compare different ischemia times, protected and unprotetected ischemia and different administrations of the cardioprotective drug levosimendan. Patients undergoing aortic valve replacement were randomized to receive either antegrade or retrograde cardioplegia. Apoptosis (TUNEL, caspase-3, Bcl-2, Bax, Bad) was assessed from myocardial biopsies, and the ventricular functions were measured by magnetic resonance imaging and by echocardiography.

Results In animal studies, the longer ischemia time induced more cardiomyocyte apoptosis; local, unprotected ischemia induced more apoptosis than global, protected ischemia but did not have an effect on ejection fraction; intracoronary levosimendan during ischemia prevented apoptosis but was associated with impaired left ventricular function when compared with preischemic intravenous infusion. With retrograde cardioplegia there was more cardiomyocyte apoptosis in the left ventricle than with antegrade cardioplegia. The systolic and diastolic ventricular functions immediately after surgery and the left ventricle remodeling in the long term were impaired with retrograde cardioplegia.

Conclusions These studies provided new evidence about cardiomyocyte apoptosis in myocardial protection. Longer ischemia time induced more myocardial apoptosis. Local, unprotected ischemia induced more cardiomyocyte apoptosis but was not associated with impaired ejection fraction. Intracoronary administered levosimendan during ischemia did not equally protect the myocardium when compared with preischemic intravenous infusion. Retrograde cardioplegia was associated with inferior myocardial protection during aortic valve replacement.

Keywords Cardiomyocyte apoptosis, ischemia-reperfusion injury, myocardial protection, cardioplegia, cardiopulmonary bypass, levosimendan.

TIIVISTELMÄ

Markus Malmberg

Tutkimus sydänlihassolujen ohjelmoidun solukuoleman vaikutuksesta sydänlihaksen suojaukseen avosydänleikkauksen aikana.
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Tausta Sydämen pysäyttäminen sydänleikkauksen aikana ja verenkierron palauttaminen sydämen käynnistämisen yhteydessä vaurioittavat sydänlihasta. Kardioplegialiuos, sydämen valmentaminen pysäytystä varten sekä useat lääkeaineet suojaavat sydäntä. Sydänlihassolujen ohjelmoidun solukuoleman (apoptoosin) on osoitettu lisääntyvän sydänleikkauksen aiheuttaman vaurion yhteydessä, mutta sen tarkkaa merkitystä ei tunneta. Tässä tutkimuksessa haluttiin selvittää apoptoosin merkitystä sydänlihasvauriossa, sydämen suojauksessa ja pumppaustoiminnassa sydänleikkauksen aikana.

Materiaali ja menetelmät Eläintöissä sydänlihasvauriomalleilla verrattiin sydämen eri pysäytysaikoja, suojattua ja suojaamatonta sydänlihaksen hapenpuutetta sekä sydäntä suojaavan lääkkeen (levosimendaanin) eri annostelumuotoja. Aorttaläppäleikkauksen aikana potilaille annettiin kardioplegialiuosta joko sepelvaltimoiden (antegradinen) tai sepellaskimoiden kautta (retrogradinen). Apoptoosi (TUNEL, kaspaasi-3, Bcl-2, Bax, Bad) määritettiin sydänkammiosta otetuista näytepaloista ja sydämen pumppaustoimintaa mitattiin magneettitutkimuksella ja ultraäänitutkimuksella.

Tulokset Eläinkokeissa todettiin, että pidempi aortan sulkuaika aiheutti enemmän sydänlihassolujen apoptoosia; paikallinen, suojaamaton hapenpuute aiheutti enemmän apoptoosia kuin yleinen, suojattu hapenpuute, mutta ei vaikuttanut vasemman kammion yleiseen pumppaustoimintaan; sepelvaltimoihin sydämen pysäytyksen aikana annosteltu levosimendaani esti apoptoosia mutta siihen liittyi heikentynyt vasemman kammion toiminta, kun sitä verrattiin suonen sisäiseen annosteluun ennen pysäytystä. Retrogradinen kardioplegia aiheutti vasemmassa kammiossa enemmän sydänlihassolujen apoptoosia kuin antegradinen. Lisäksi vasemman kammion systolinen ja diastolinen toiminta välittömästi toimenpiteen jälkeen ja vasemman kammion uudelleen muotoutuminen olivat heikentyneet retrogradista kardioplegiaa käytettäessä.

Johtopäätökset Väitöskirjatutkimuksessa saatiin uutta tietoa sydänlihassolujen apoptoosista sydämen suojauksessa. Paikallinen, suojaamaton hapenpuute aiheutti enemmän apoptoosia sydänlihakseen, mutta tämä ei vaikuttanut sydämen kokonaistoimintaan. Sydämen pysäytyksen aikana sepelvaltimoihin annosteltu levosimendaani ei suojannut sydäntä yhtä tehokkaasti kuin ennen pysäytystä laskimoon annosteltu levosimendaani. Retrogradinen kardioplegia osoittautui heikommaksi sydämen suojausmenetelmäksi vasemman kammion osalta kuin antegradinen.

Avainsanat Sydänlihassolujen apoptoosi, sydänlihasvaurio, sydämensuojaus, kardioplegia, sydänkeuhkokone, levosimendaani.

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ABBREVIATIONS

AMI	Acute myocardial infarct
ATP	Adenosine triphosphate
AVR	Aortic valve replacement
Bad	Bcl-2-associated death promoter protein
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma-2 protein (family of apoptosis regulator genes)
Bcl-X_L	B-cell lymphoma-extra large molecule
Bik	Bcl-2-interacting killer gene
Bnip3	Bcl-3/adenovirus E1B19 kd-interacting protein
CABG	Coronary artery bypass grafting
Caspase	Cysteine-dependent aspartate-directed proteases
CK-MBm	Creatine kinase MB isoenzyme
CPB	Cardiopulmonary bypass
cTnC	Cardiac troponin C
DNA	Deoxyribonucleic acid
ECG	Electrocardiography
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GIP	Glucose-insulin-potassium
H_2O_2	Hydrogen peroxide
OH^-	Hydroxyl radical
HTK	Histidine-triptophan-ketoglurate
ICU	Intensive care unit
IL-8	Interleukin-8
I-R	Ischemia-reperfusion
LAD	Left anterior descending artery
LCX	Left circumflex artery
MPP	Mitochondrial permeability pore
MRI	Magnetic resonance imaging
OPCAB	Off-pump coronary artery bypass grafting
PCI	Percutaneous coronary intervention
POST	Postoperative
PRE	Preoperative
PROM	Predicted risk of mortality
RCA	Right coronary artery
RNA	Ribonucleic acid
0_2^-	Superoxide anion
TEE	Transesophageal echocardiography
TNF-α	Tumor necrosis factor alpha
TnI	Troponin I
TnT	Tropinin T
TUNEL	Terminal transferase mediated ddUTP nick end-labeling

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications

I Malmberg M, Vähäsilta T, Saraste A, Kytö V, Kiss J, Kentala E, Kallajoki M, Savunen T. Cardiomyocyte apoptosis and duration of aortic clamping in pig model of open heart surgery. Eur J Cardiothorac Surg 2006;30:480-4.

II Malmberg M, Pärkkä J, Vähäsilta T, Saraste A, Laitio T, Kiss J, Latva-Hirvelä J, Saukko P, Savunen T. Cardiomyocyte apoptosis after cardioplegic ischemia: comparison to unprotected, regional ischemia-reperfusion. Eur Surg Res 2011;46:19-25.

III Malmberg M, Vähäsilta T, Saraste A, Koskenvuo JW, Pärkkä JP, Leino K, Laitio T, Stark C, Heikkilä A, Saukko P, Savunen T. Intracoronary levosimendan during ischemia prevents myocardial apoptosis. Manuscript. Submitted.

IV Vähäsilta T, Malmberg M, Saraste A, Koskenvuo J, Pärkkä J, Valtonen M, Leino K, Nuutila K, Saukko P, Kuttila K, Savunen T. Cardiomyocyte apoptosis following antegrade and retrograde cardioplegia during aortic valve surgery. Ann Thorac Surg 2011;92:1351-7

1. INTRODUCTION

Open heart surgery with cardioplegic arrest and cardiopulmonary bypass (CPB) has an important role in modern clinical practice (Chu et al. 2009, Braathen et al. 2011, Kappetein et al. 2011). During surgery, ischemia and reperfusion (I-R) cause injury to the myocardium, which can be seen as several clinical manifestations, such as reversible postoperative myocardial dysfunction (stunning), irreversible myocardial damage, and arrhythmias (Kloner et al. 1974, Bolli 1990, Creswell et al. 1993, Gottlieb et al. 1994). Apoptotic death of myocytes is induced by I-R injury during heart surgery, but the clinical significance of apoptosis remains still unclear (Schmitt et al. 2002, Gaudino et al. 2007, Oka et al. 2008).

The protection of myocardium and preservation of its function during open heart surgery have key roles in achieving the best available clinical outcome (Braathen et al. 2010). There are several ways to protect the myocardium such as hypothermia, cardioplegia and pharmacological agents, and many of them can also reduce cardiomyocyte apoptosis (Feng et al. 2005, Meybohm et al. 2009, Caimmi 2011). However, the clinical significance of prevention of cardiac apoptosis during I-R injury on, e.g. postoperative myocardial dysfunction is not clear (De Hert et al. 2004, Yao et al. 2010, Tempe et al. 2011).

Novel pharmacological agents such as intracellular calcium sensitizer levosimendan have improved the recovery of patients after surgery and have also provided us with new knowledge about protecting the heart during surgery (Kopustinskiene et al. 2004, Tripatete et al. 2009). Levosimendan is used in heart failure patients to improve myocardial contractility, and in heart surgery especially in patients with impaired left ventricle function (Hasenfuss et al. 1998, Eriksson et al. 2009).

During cardiac surgery, the cardiolegia solution can be administered antegradely to the coronary ostia or retrogradely to the coronary sinus. Retrograde cardioplegia is associated with impaired perfusion of the myocardium, particularly on the right side of the heart (Winkelmann et al. 1995). Depending on the patient and the type of the procedure, a combination of these two techniques might be the most effective way to protect the myocardium. However, these techniques can also be used separately (Ruengsakulrach et al. 2001, Onorati et al. 2003).

In these studies we wanted to investigate the effects of different myocardial protection strategies on cardiomyocyte apoptosis in I-R injury associated with open heart surgery, in experimental models and in patients undergoing aortic valve replacement (AVR). In addition, we wanted to study the role of cardiac apoptosis in postoperative ventricular dysfunction.

2. REVIEW OF THE LITERATURE

2.1 Anatomy of the human heart

The human heart is a four-chamber muscle pump situated in the lower part of the mediastinum and it is shaped like a three-sided pyramid. The base of the heart lies in an oblique plane behind the sternum, and the apex of the heart extends outward into the left hemithorax. The heart is usually positioned with one third to the right and two thirds to the left from the midline.

The right atrium receives deoxygenated blood from the inferior and superior vena cava. From the right side of the heart, the blood is pumped into lungs and oxygenated. The oxygenated blood flows from the lungs into the left atrium through pulmonary veins. The left ventricle is filled through the mitral valve from the left atrium, and from the left ventricle it is pumped further back into the systemic circulation through the aorta. The left ventricle works against higher pressure than the right one, and therefore it has a thicker ventricular wall.

The inside of the heart is covered by an internal layer called the endocardium and on the outside by the pericardium. The pericardium is formed by two parts, the fibrous and the serous, and it also covers the great vessels near the heart. Between these two layers, the endocardium and the pericardium, lies the myocardium.

The myocardial blood flow is supplied by the coronary arteries, originating in the ascending aorta, Figure 1. The left main coronary artery is divided into two main braches: the anterior interventricular (the left anterior descending artery, LAD) and the circumflex artery (LCX). The right ventricle blood flow is mostly supplied by the right coronary artery (RCA), as well as most of the right atrium and sinoartrial- and artrioventricular nodes. The left coronary artery with its branches supplies the blood flow to most of the left ventricle and the left atrium. In approximately 90 % of the population, the RCA branches to the posterior descending artery, which supplies blood to the posterior part of the left ventricle. This is called the right coronary artery dominance. Domination of the left coronary artery (the inferior interventricular artery originating from the LCX) is found in only 10 % of the population.

The coronary veins drain blood from the myocardium. The larger veins accompany the major coronary arteries through the coronary sinus into the right atrium. Arterioluminal vessels are direct communications from the coronary arterioles to the chambers of the heart. Venoluminal vessels are analogous communications on the venous side. Thebesian veins are connections between the capillaries and the right atrium and ventricle. All of these “shortcuts” of myocardial blood flow are more often seen on the right side of the heart. (Ansari 2001, Mao et al. 2005, Sellke et al. 2010).

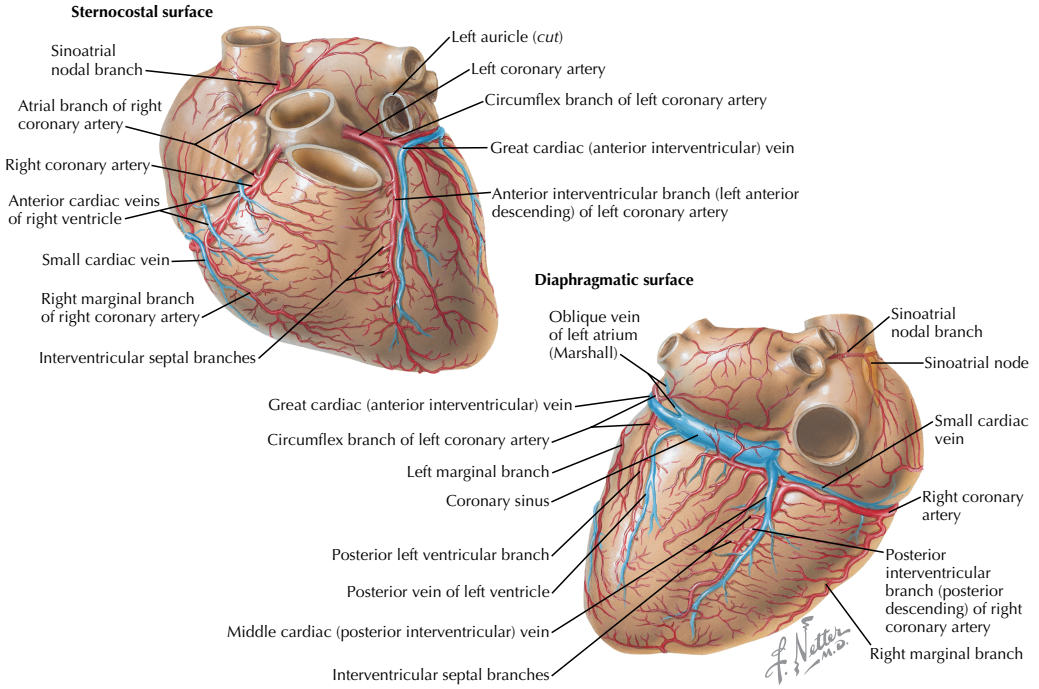

Figure 1. The anatomy of coronary arteries and cardiac veins as illustrated by Frank H. Netter. Netter illustration used with permission of Elsevier, Inc. All rights reserved. www.netterimages.com

2.1.1 Comparison with the pig heart

The anatomy of the pig heart is almost identical to the human heart with a few important exceptions. The left ventricle wall is much thicker than in the human heart and the apex of the pig heart is entirely formed by the left ventricle. In the porcine, the heart is rotated more to the right on its longitudinal axis so that the anterior interventricular groove is faced more anteriorly. The pig heart is also in a more supine position. The ascending aorta lies behind the pulmonary trunk, which is often quite a prominent structure. In the pig, there are only two pulmonary veins draining into the left atrium. The left azygous vein drains into the coronary sinus in the porcine heart but otherwise the coronary circulation is principally comparable with human anatomy. (Weaver et al. 1986, Crick et al. 1998).

2.2 Myocardial ischemia-reperfusion injury in open heart surgery

In order to keep the target still during on-pump open heart surgery, the heart is connected to the CPB and stopped by disabling the blood flow to the myocardium using an aortic cross-clamp. Myocytes are dependent on the constant oxygen flow and, therefore, the prevention of the coronary perfusion leads to an injury called ischemia (Antman et al. 1996). Prolonged myocardial ischemia results eventually in cell necrosis, impaired ventricular function, and ventricular arrhythmias (Fallavollita et al. 2005). The severity

of the injury depends upon the degree and the duration of ischemia (Buja 1998). The myocardium is commonly protected from the ischemia injury during the operation by hypothermia and cardioplegia solution (Braathen et al. 2010). At the end of the operation, blood flow is restored to the myocardium by opening the aortic cross-clamp. However, reperfusion has been described as the "double-edged sword", because reperfusion itself causes additional injury to the myocardium (Braunwald et al. 1985). This damage, caused to the myocardium first by disabling and then restoration of the myocardial blood flow, is called I-R injury (Jennings et al. 1960, Weman et al. 2000). It is clinically manifested as irreversible myocyte death, arrhythmias, myocardial stunning, microvascular dysfunction, and the no-reflow phenomenon (Kloner et al. 1974, Bolli 1990, Creswell et al. 1993, Gottlieb et al. 1994). I-R injury is also seen during acute myocardial infarct (AMI) and its rescue treatments, but during heart surgery the I-R injury is caused in a controled environment (Bolognese et al. 2004). Severe I-R injury is associated with impaired outcome of the surgery (Mathew et al. 2004, Doenst et al. 2008).

2.2.1 Mechanisms of ischemia-reperfusion injury

The underlying pathophysiological mechanisms of I-R injury are complex and at least partially still unclear, and therefore remain under intense research (Turer et al. 2010). Several factors have been suggested to transmit the mechanisms of I-R injury; here the most commonly observed mechanisms are summarized.

The myocardium requires large amounts of energy in the contraction process. This energy is generated in the myocardial cells by mitochondrial oxidative metabolism, mostly in the form of adenosine triphosphate (ATP) (Bell et al. 2006). During ischemia, the mitochondrial oxidative phosphorylation rapidly stops with loss of oxygen, causing ATP levels to fall, and the energy metabolism to switch to anaerobic glycolysis. Lactic acid increases, while intracellular pH is reduced as a result of anaerobic metabolism. An increased amount of positively charged protons in the cytoplasm leads to activation of sodium/proton (Na^+ - H^+) exchanger and accumulation of intracellular Na^+ concentration. This results in sodium/calcium (Na^+ - Ca^{2+}) exchanger inhibition and, thereby, the intracellular and mitochondrial calcium concentrations are increased. (Tani et al. 1989, Piper et al. 2003). At reperfusion, the blood flow and oxygen levels are restored and reactive oxygen species such as hydroxyl (OH^-), hydrogen peroxide (H_2O_2) and superoxide (0_2^-), are produced within the first few minutes of reperfusion (Kim et al. 1994, Xia et al. 1995). In addition to other factors, increasing pH, high contraction of calcium, and reactive oxygen species result in the mitochondrial permeability pore (MPP) opening (Di Lisa et al. 2006, Kim et al. 2006). The inner mitochondrial membrane is normally an impermeable barrier between the inner mitochondrion and cytoplasm, and the formation of these pores results in increasing release of reactive oxygen species, depletion of ATP products and, e.g. formation of cytochrome c and other pro-apoptotic proteins in the cytoplasm (Crompton et al. 2002). The formation of MPPs is believed to be one of the key features in I-R injury, Figure 2 (Di Lisa et al. 2006). In moderate injury, the opened

pores close, ATP production continues, and the myocytes result in apoptosis. In more severe injury, the pores stay open, ATP production stops, and the cardiomyocytes die by necrosis (Halestrap et al. 2004, Honda et al. 2005). Opening of these mitochondrial pores is believed to occur only during reperfusion (Griffiths et al. 1995).

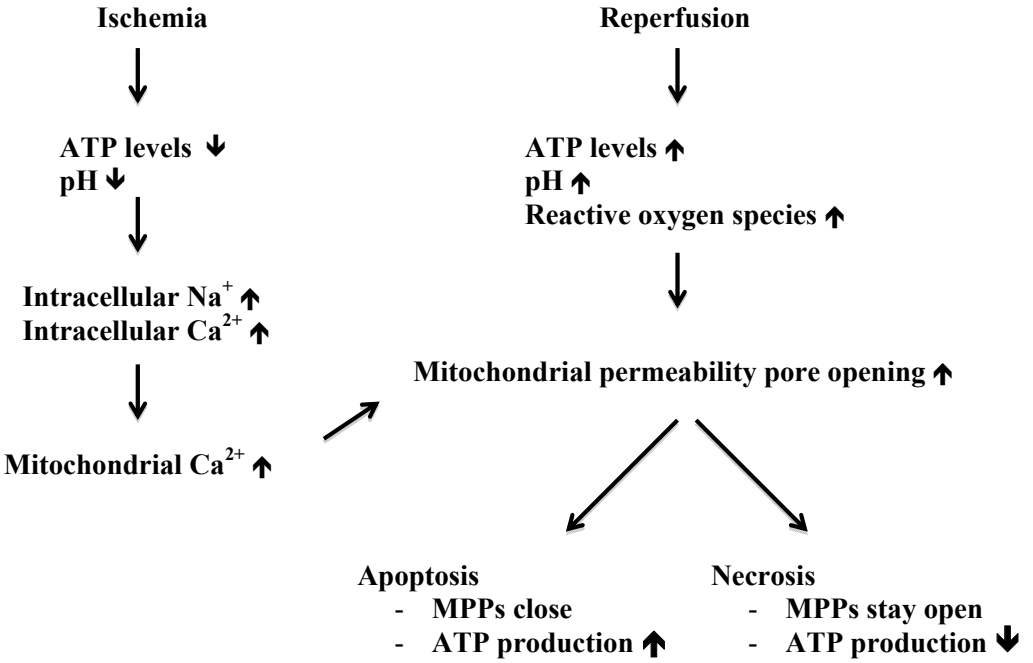

Figure 2. Mechanisms of ischemia-reperfusion injury and the relation of the mitochondrial permeability pore (MPP) opening to the myocardial apoptosis and necrosis. ATP= adenosine triphosphate, Na^+=sodium, Ca^{2+}=calcium.

In addition to significant impact on MPPs, highly reactive oxygen species and calcium itself can cause severe damage to the myocardial cells. Free oxygen radicals cause damage to the cells by reacting with lipids, proteins and nucleic acids (Zweier et al. 2006). Reactive oxygen species are shown to be present only during reperfusion in I-R injury (Lazzarino et al. 1994). Intracellular calcium overload has been associated with the post-operative development of myofibrillar hypercontraction also known as "the stone heart" (Loughrey et al. 2002).

I-R injury has been shown to induce inflammation as generated by endothelial cells and cardiomyocytes during open heart surgery (Valen et al. 2001). Shortly after reperfusion, neutrophil cell activation and accumulation occurs in the damaged myocardium (Kloner et al. 1991). These neutrophils and other inflammatory cells further induce the I-R injury by releasing free oxygen radicals, proteases, and pro-inflammatory mediators, such as IL-8. Embolization of microvessels leading to the no-reflow phenomen, secondary ischemia, and further increasing endothelial damage, are associated with increased

neutrophil concentration (Smedly et al. 1986). CPB itself induces inflammatory responses during I-R, which can be seen as increased levels of interleukins and other inflammatory mediators, and increased activation of inflammatory genes (Finn et al. 1993, Valen et al. 2001). As a part of the inflammation reaction, the complement system is activated during I-R (Ascione et al. 2000). The activation of the complement system leads to further induction of inflammatory cells and mediators and also to direct tissue damage (Park et al. 1999)

2.2.2 Arrhythmias

Postoperative cardiac arrhythmias are frequently seen after cardiac surgery, while atrial arrhythmias are most common. After coronary artery bypass grafting (CABG), the incidence of atrial arrhythmias is approximately 30 %, after CABG and AVR 48 %, and after CABG and mitral valve replacement 60 % (Creswell et al. 1993). Risk factors associated with postoperative atrial fibrillation are increasing patient age, chronic obstructive pulmonary disease, preoperative use of digoxin, history of rheumatic heart disease, increasing aortic cross-clamp time, valve surgery, post-operative withdrawal of a β-blocker or an angiotensin-converting enzyme inhibitor, and hypertension (Ormerod et al. 1984, Caretta et al. 1991, Creswell et al. 1993, Aranki et al. 1996, Mathew et al. 2004). The pathophysiology of I-R induced arrhythmias is multifactorial and still unclear, but free oxygen radicals and inflammation have been suggested to be the key mechanisms (Berner et al. 1989, Abdelhadi et al. 2004). Of patients who have postoperative atrial fibrillation, 70 % develop the arrhythmia during the first four postoperative days (Aranki et al. 1996).

Ventricular tachycardia after CABG is less common than atrial arrhythmias (3.1 %), but the in-hospital mortality rate is significantly higher (25 %). Bypass graft placed across a noncollateralized total occlusion in the infarcted myocardium has been associated with ventricular tachycardia, as well as reduced left ventricular function (ejection fraction ≤ 30 %) (Steinberg et al. 1999, Kaul et al. 1998).

2.2.3 Microvascular dysfunction and no-reflow phenomenon

Kloner et al. first described the no-reflow phenomenon in an animal model of regional ischemia in 1974 (Kloner et al. 1974). Since then it has been shown to occur also in humans, especially after percutaneous coronary interventions (PCI) due to AMI, but it has been suggested that it might also be a consequence of global I-R injury in heart surgery (Boyle et al. 1996, Bolognese et al. 2004). The no-reflow phenomenon is defined as the inability to perfuse previously ischemic myocardium, even when the blood flow has been restored to the large arteries supplying the tissue (Kloner et al. 1974). No-reflow is caused by four interacting components: 1) distal atherothrombotic embolization, 2) ischemic injury, 3) reperfusion injury, and 4) vulnerability of coronary microcirculation to injury. No-reflow seems to cause both reversible and irreversible injury to the myocardium (Niccoli et al.

2009). In patients, the microvascular dysfunction after PCI is associated with impaired long-term outcome (Bolognese et al. 2004).

2.2.4 Myocardial stunning

Postischemic myocardial dysfunction, stunning, was first described by Heyndrickx et al. in 1975 after brief, regional, myocardial ischemia (Heyndrickx et al. 1975). In postischemic myocardial stunning the mechanical dysfunction of the heart persists after restoring normal blood flow, although irreversible myocardial damage cannot be seen. Myocardial stunning is a completely reversible form of I-R injury (Bolli 1990). Stunning has also been shown to occur after I-R injury induced by heart surgery (Roberts et al. 1980, Schmitt et al. 2002). Several mechanisms have been studied and hypothesized to explain the cellular events of myocardial stunning. It seems that at least free oxygen radicals and increased intracellular calcium levels play an important role in the development of stunning (Myers et al. 1985, Bolli et al 1999).

2.2.5 Irreversible myocyte death

Myocardial cells can tolerate short periods of ischemia. If the blood flow is restored to the myocardium within 20 minutes, the damage to the myocardial cells can be reversible (e.g. depressed myocardial contractility). If the ischemia continues, myocardial cells start to die. Most of the myocardial damage during I-R injury is reversible, but there is also irreversible loss of myocardial cells due to necrosis and apoptosis (Park et al. 1999, McCully et al. 2004). Necrosis is an uncontrolled form of cell death, which is manifested as swelling of the cell, increased membrane permeability, swelling and vacuolization of organelles and inflammation (Majno et al. 1995). In heart surgery, this can be seen as elevated postoperative levels of troponin I (TnI), troponin T (TnT) and creatine kinase, and specially its MB isoenzyme (CK-MB) (Katus et al. 1991, Costa et al. 2001, Landoni et al. 2007). During I-R injury myocardial cells also die due to apoptosis (Gottlieb et al. 1994, Wu et al. 2003, McCully et al. 2004). This will be discussed in the next section.

2.3 Cardiomyocyte apoptosis

Apoptosis is a morphologically distinct type of cell death, which is genetically controlled and requires energy (Kerr et al. 1972, Saraste et al. 2000). Compared to necrosis, apoptosis is associated with shrinkage of the cell and phagocytosis by neighboring cells without inflammation (Saraste et al. 2000). It is considered as natures own way to destroy cells that are no longer needed or function abnormally, and was first described by Kerr et al. in 1972. Since then apoptosis has been shown to occur in several conditions, diseases and organs, including the human heart, e.g. in heart failure, AMI, hibernating myocardium, myocarditis, and hypertension (Chen et al. 1997, Olivetti et al. 1997, Saraste et al. 1997, Gonzalez et al. 2002, Kytö et al. 2004). Cardiac apoptosis has been shown to have a role

in left ventricle remodelling and also in progressive dilation of the heart after AMI in a diabetic experimental model (Palojoki et al. 2001, Bäcklund et al. 2004, Dorn 2008).

Several animal and human studies have shown that myocardial apoptosis is induced after local and global I-R injury (Gottlieb et al. 1994, Saraste et al. 1997, Vähäsilta et al. 2001, Schmitt et al. 2002). It seems that apoptotic cell death in the myocardium is initiated during ischemia, but the energy needed for the execution is provided during reperfusion (Gottlieb et al. 1994, Golenhofen et al. 1999, Zhao et al. 2000). The meaning of cardiomyocyte apoptosis is still unclear, but it might provide a potential target for cardiac protection since viable cells are lost during I-R injury in heart surgery (Wu et al. 2003, Oka et al. 2008). There is some evidence that the amount of myocardial apoptosis might correlate with postoperative stunning (Schmitt et al. 2002, Gaudino et al. 2007). In addition, there is evidence that inhibition of cardiomyocyte apoptosis reduces infarct size and improves post-ischemic contractile dysfunction during reperfusion in an animal model of regional I-R (Zhao et al. 2003).

The signal transduction pathways in the regulation of apoptosis in myocardial cells are complex and not fully clarified. However, at least two different pathways can be identified: the external (death receptor) pathway and the internal (mitochondrial) pathway, Figure 3 (Logue et al. 2005, Lopez-Neblina et al. 2005, Scarabelli et al. 2006).

In the external pathway, pro-inflammatory cytokines such as tumor necrosis factor alpha, bind to the receptor complex expressed on the surface of the myocardial cells (Torre-Amione et al. 1995, Krown et al. 1996). This activates upstream cysteine proteases such as cysteine-dependent aspartate-directed proteases (caspases) 2, 8, 9 and 10, which in turn activate downstream caspases 3,6 and 7 in the caspase cascade. It is the downstream caspases that eventually execute the apoptotic cell death. Especially the activated form of caspase-3 has a crucial role in the induction of proteins needed for cell cleavage in the process of apoptosis (Saraste et al. 2000, Gustafsson et al. 2003, Valen et al. 2003, Scarabelli et al. 2006).

In the mitochondrial pathway, various cellular stress signals such as hypoxia, oxygen free radicals, calcium ions, and DNA damage can interact with the mitochondrial membrane and trigger apoptotic cell death. These signals provoke the opening of the MPPs, and result in mitochondrial swelling and distribution (necrosis) or at least in the releasing of pro-apoptotic factors such as cytochrome c and apoptosis-inducing factor (apoptosis) (Crompton et al. 2002, Logue et al. 2005, Lopez-Neblina et al. 2005). The release of cytochrome c activates several proteins in the apoptotic cascade, e.g. caspase-9, which further activates downstream caspases such as caspase-3 (Valen et al. 2003). The opening of MPPs and the release of cytochrome c are at least in part regulated by the Bcl-2 (B-cell lymphoma-2 protein, family of apoptosis regulator genes) family members located at the mitochondrial outer membrane. Of these Bcl-2 proteins, Bcl-2 and Bcl-X_L (B-cell lymphoma-extra large molecule) have been shown to have anti-apoptotic features, while Bax (Bcl-2-associated X protein), Bad (Bcl-2-associated death promoter

protein), Bik (Bcl-2-interacting killer gene) and Bnip3 (Bcl-3/adenovirus E1B19 kd-interacting protein) are considered to be pro-apoptotic (Lee et al. 2009). Expression of these genes is induced during I-R injury (Feng et al. 2004). Exactly how the Bcl-2 family proteins regulate apoptosis in myocardial cells in not known, but it is believed that the controlling of the mitochondorial functions is a particularly important feature (Gustafsson et al. 2007, Lee et al. 2009).

These two pathways have also been shown to cross-talk during I-R injury, leading to apoptotic cell death (Lee et al. 2009). Activation of caspace-8 by the external pathway activates caspase-3 as previously described, but also induces the release of cytochrome c from the mitochondria (the internal / mitochondrial pathway) (Lee et al. 2009).

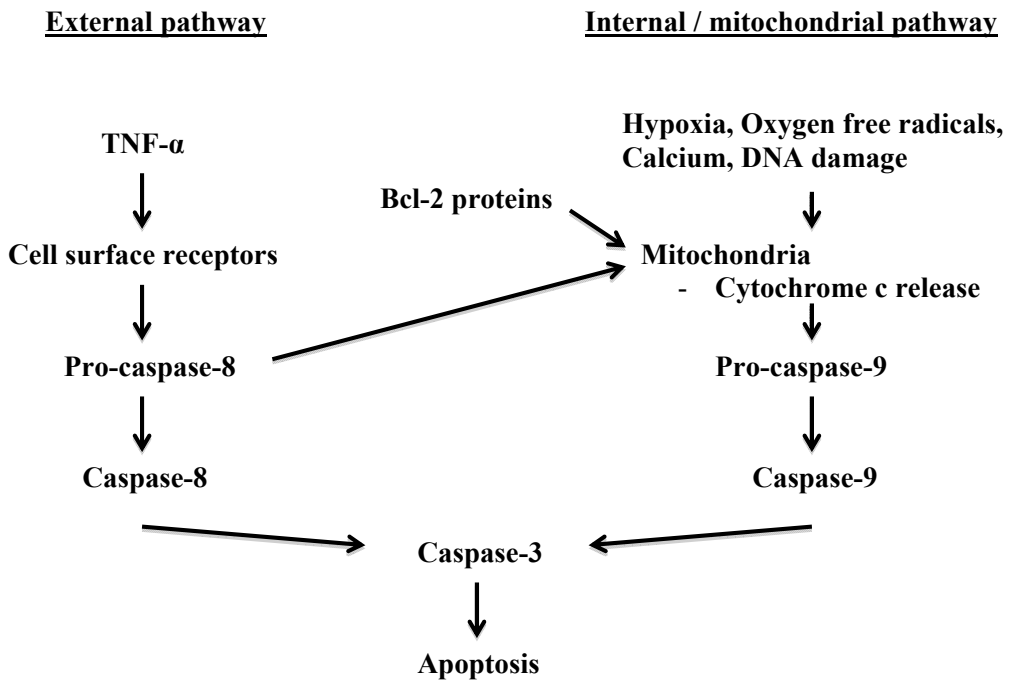

Figure 3. A simplified schematic presentation of the main intracellular pathways of apoptosis. TNF-α=tumor necrosis factor alpha, Bcl-2=b-cell lymphoma-2.

2.4 Detection of ischemia-reperfusion injury

The consequences of I-R caused by heart surgery can be detected clinically as described above. Myocardial changes caused by I-R injury can also be seen at microscopic level (apoptosis and necrosis). In detection of apoptotic cells in tissue samples several techniques can be used, including electron microscopy, immunohistochemistry, and biochemical assessments (Western blott, Annexin-V) (Saraste et al. 2000, Thimister et al. 2003, Jugdutt et al. 2005). The most preferred method is carefully standardized terminal

transferase mediated ddUTP nick end-labeling assay (TUNEL) (Saraste et al. 2000). TUNEL assay is based on immunohistochemistry and labeling of DNA fragmentation as DNA strand breaks become visible in light microscopy (Saraste et al. 2000, Jugdutt et al. 2005). Specific proteins (markers) of apoptosis such as activated caspase-3, Bcl-2, Bax, Bad, can be assessed using either immunohistochemistry or biochemical analysis by Western immunoblotting (Saraste et al. 2000, Jugdutt et al. 2005). In general, it is preferred to use at least two different methods in detection of apoptotic cells, e.g. activated caspace-3 proteins in myocardial cells have shown to co-localize with TUNEL-positive cardiomyocytes after I-R injury (Black et al. 1998. Jugdutt et al. 2005). The level of I-R injury can also be estimated from blood samples: increased levels of CK-MB, TnT and TnI indicate cardiac cell necrosis after CABG and predict impaired outcome (Steuer et al. 2004, Newall et al. 2006, Thygesen et al. 2007).

2.4.1 Imaging of myocardial function and viability

Myocardial viability has been assessed using single photon emission computed tomography with tracers such as 99mTc-sestamibi, position emission tomography with e.g. fluorine-18 fluorodeoxyglcose and dobutamine stress echocardiography (Miller et al. 1995, Pedone et at. 2008, Boehm et al. 2010). More commonly, echocardiography is used to evaluate cardiac anatomy and function (Bellenger et al. 2000). Also, in addition to myocardial anatomy and function, cardiac magnetic resonance imaging (MRI) is an accurate method to evaluate myocardial viability (Kim et al. 1999, Bellenger et al. 2000). With delayed-enhancement technique, using intravenous administration of contrast agent such as gadolinium-diethyenetriamine pentaacetic acid, it is possible to detect and quantify infarcted and hypoperfused areas after AMI or CABG or in chronic infarction (Knuesel et al. 2003, Ibrahim et al. 2005, Steur et al. 2004). Moreover, it is possible to quantify the no-reflow phenomenon after AMI using contrast-enhanced MRI (Gerber et al. 2000).

2.5 Myocardial protection in open heart surgery

Ever since Dr. Gibbon first established his heart-lung machine and CPB in the clinical practice of heart surgery in 1953, myocardial protection has taken major steps to improve the outcome of cardiac surgery (Gibbon 1954, Cordell 1995, Hausenloy et al. 2007). With aortic cross-clamp it is possible to achieve a bloodless and still operating field, but in normothermic conditions without sufficient myocardial protection this results in ischemic contraction (stone heart) (Cooley et al. 1972, Loughrey et al. 2002). Deep systemic hypothermia and topical hypothermia have been previously used successfully during open heart surgery, and the use of hypothermia still continues (Drew et al. 1959, Shumway et al. 1960, Bavaria et al. 2010). In addition, hypothermia has been shown to reduce cardiomyocyte apoptosis (Meybohm et al. 2009). Ventricular fibrillation induced by intermittent aortic cross-clamp is a rather rarely used method in CABG to create a still

operating field, but this technique seems to work in selective patients and in experienced hands (Anderson et al. 1994, Liu et al. 1998).

2.5.1 Cardioplegia

Although the term cardioplegia was first introduced in 1958 and the first cardioplegia solutions were invented already in the 1950´s, the use of cardioplegia as an inductor of cardiac arrest and protector of the myocardium during open heart surgery started to become more and more popular in the 1970´s (Brown et al. 1958, Melrose et al. 1955, Hearse et al. 1976). Today, cardioplegia can be considered a corner-stone of cardiac surgery (Bavaria et al. 2010). The basic idea of cardioplegia solution is to achieve protected cardiac arrest during heart surgery by: 1) energy conservation through rapid induction of diastolic arrest with agents such as potassium, 2) slowing metabolic and degenerative processes using hypothermia, and 3) preventing ischemia-induced changes with specific protective agents (Hearse 1983).

Sevelar clinical studies have compared different types of cardioplegia (cold crystalloid/ cold blood/warm blood), and the current data seem to indicate that cold blood cardioplegia might have some benefits over crystalloid and warm blood cardioplegia at least in adult cardiac surgery (Martin et al.1994, Braathen et al. 2010). However, the evidence is not fully convincing (Ovrum et al. 2004 and 2010). When comparing cold crystalloid with cold blood cardioplegia, at least few studies show smaller postoperative cardiac enzyme release and less low output syndrome with cold blood cadioplegia, but no differences in death or other clinical outcomes (Guru et al. 2006, Braathen et al. 2010). In addition, there is indication of increased risk for postoperative neurological events with warm blood cardioplegia (Martin et al. 1994).

The contests of crystalloid and blood cardioplegia solutions have varied during the years since the development to the crystalloid St. Thomas I cardioplegia solution (Hearse et al. 1976). The contests of standard modern crystalloid (modified St. Thomas II) and blood cardioplegia solutions are presented in Table 1 (Ovrum et al. 2010, Braathen et al. 2010, Chambers et al. 2010). With hyperkalemic cardioplegia solutions, the depolarized cardiac arrest is induced by increased extracellular potassium, while procaine hydrochloride is used as a membrane stabilizer and acetate as a buffer (Chambers et al. 1999 and 2010).

Cold crystalloid cardioplegia can also be administered as a single dose, with a comparable result (Scrascia et al. 2011). This Bretschneider-HTK (histidine-triptophan-ketoglurate, Custodiol®, Table 1) solution has been shown to protect the myocardium at least in aortic, mitral valve and pediatric surgery especially with longer ischemia times (Modi et al. 2003, Braathen et al. 2011, Scrascia et al. 2011). With Bretscheiner´s solution the cardioplegic arrest is induced by zero calcium concentration combined with low sodium concentration (Chambers et al. 1999). In HTK, histidine has good buffering capacity, triptohan protects the cells by stabilizing the cell membrane, and ketoglurate is induced as an energy substrate (Fridell et al. 2009). Mannitol is added to the solution to reduce

the amount of free oxygen radicals (Fridell et al. 2009). In Langendorff rat hearts HTK solution prevented myocardial apoptosis (Jin et al. 2009).

Table 1. The comparisons of the St. Thomas II cardioplegia, cold blood cardioplegia and Bretscheider-HTK (histidine-triptohan-ketoglurate, Custodiol®) solutions.

1 litre of cardioplegia	**St. Thomas II cardioplegia**	**Blood cardioplegia**	**HKT (Custodiol®)**
Potassium (K^+) mmol/l	19.6	21.5	9 (KCl)
Magnesium (Mg^{2+}) mmol/l	16.7	18.2	4 ($MgCl_2$)
Calcium (Ca^{2+}) mmol/l	2.0	2.2	0.015($CaCl_2$)
Sodium (Na^+) mmol/l	128	145.1	15 (Nacl)
Procaine hydrochloride mmol/l	1	1.1	-
Acetate	29.4	6.5	-
Chloride (Cl^-) mmol/l	154.9	154.9	-
pH	6.3	7.3 – 7.4	7.02 – 7.2
Temperature °C	4 – 8	4 – 8	8 – 12
Hydrogen carbonate mmol/l		28.9	-
Histidine / histidine hydrochloride mmol/l	-	-	18 / 180
Triptophan mmol/l	-	-	2
Potassium hydgoren 2-ketoglurate mmol/l	-	-	1
Mannitol mmol/l	-	-	30

In addition to other evidence of suggested better myocardial protection, experimental studies have shown that cold blood cardioplegia protects the myocardium from cardiomyocyte apoptosis induced by I-R injury, when compared to cold crystalloid or warm blood cardioplegia (Yeh et al. 2003, Feng et al. 2004, Feng et al. 2005).

2.5.1.1 Antegrade and retrograde cardioplegia

Retrograde cardioplegia administered into the venous system of the heart is associated with inadequate myocardial perfusion and protection when used alone, and compared to the antegrade cardioplegia administration in experimental and clinical studies (Winkelmann et al. 1995, Allen et al. 1995, Tian et al. 2003). Especially the right ventricular free wall and septum are inadequately perfused with retrograde cardioplegia (Winkelmann et al. 1995). The main reason for the differences between antegrade and retrograde cardioplegia perfusion is suggested to be the anatomical differences in the venous circulation when compared to the arterial circulation, for example the Thebesian veins (Ruengsakulrach et al. 2001). In experimental studies of I-R injury, retrograde cardioplegia has been shown to induce more cardiomyocyte apoptosis in the right ventricle than antegrade cardioplegia (Vähäsilta et al. 2005). On the other hand, there are also advantages in using retrograde cardioplegia, such as clear operating field, no

risk of coronary ostial injury, and effectiveness in the presence of aortic regurgitation (Ruengsakulrach et al. 2001). It seems that CABG patients with left main coronary artery stem disease might benefit most when antegrade and retrograde cardioplegia are used as a combination (Onorati et al. 2003).

2.5.2 Conditioning

Conditioning during heart surgery means preserving the myocardium from I-R injury, and it was first established as ischemic preconditioning (Murry et al. 1986, Yellon et al. 1993). It is characterized as therapeutical attempts to prepare the myocardial cells for better resistance towards ischemia and reperfusion (Rimpiläinen 2011). Since it was first used, conditioning has developed further in cardiac surgery and has several clinical and experimental implications such as remote ischemic conditioning, postconditioning and pharmacological conditioning.

2.5.2.1 Ischemic conditioning

Brief episodes of ischemia and reperfusion before aortic cross-clamp, e.g. 2 minutes of ischemia followed by 3 minutes of reperfusion, have been shown to reduce the postoperative cardiac enzyme release (TnI) after CABG with aortic cross-clamp fibrillation and cardioplegic arrest (Jenkins et al. 1997, Ji et. al 2007). Ischemic preconditioning protects the myocardium against postoperative stunning after CABG, and the same protective effect can be seen with regional ischemic preconditioning during OPCAB (Wu et al. 2001, Laurikka et al. 2002). In addition, ischemic preconditioning reduces the occurrence of postoperative atrial fibrillation and ventricular tachyarrhythmias after CABG with cold blood cardioplegia (Wu et al. 2002, Wu et al. 2003). Ischemic preconditioning has been shown to reduce cardiomyocyte apoptosis in patients during CABG and in an animal model of regional I-R without myocardial protection (Piot et al. 1997, Vohra et al. 2006).

In remote conditioning, the cardioprotective effect is obtained by using short periods of I-R in other than the ischemic area of the myocardium (local ischemia) or in another organ, e.g. lower limb (Przyklenk et al. 1993, Birnbaum et al. 1997). The exact mechanism of the cardioprotective effect is still unclear, but remote ischemic preconditioning has been used successfully in CABG and in pediatric cardiac surgery, although opposite results have also been published (Cheung et al. 2006, Hausenloy et al. 2007, Rahman et al. 2010). There is some evidence that remote limb ischemia during aortic cross-clamping (perconditioning) reduces TnI release in patients during AVR (Li et al. 2010).

In postconditioning the myocardial protection is achieved by starting the reperfusion with short cycles of I-R before final reperfusion (Zhao et al. 2004). Postconditioning has shown to be cardioprotective during adult AVR, and in an experimental regional I-R

model, postconditioning protects the myocardium from apoptosis (Luo et al. 2008, Sun et al. 2010).

2.5.2.2 Pharmacological conditioning

Several pharmacological agents have been studied in clinical and experimental settings in order to preserve optimal myocardial function during heart surgery. Here some of the most commonly studied are birefly summarized.

Preconditioning with volative desflurane in patients undergoing elective CABG showed lower TnI values and better left ventricle function after surgery (Meco et al. 2007). Also, there are clinical studies indicating the cardioprotective effect of sevoflurane and isoflurane, and in addition, in an experimental setting, sevoflurane prevented myocardial apoptosis (De Hert et al. 2004, Yao et al. 2010, Tempe et al. 2011).

Adenosine has been shown to be cardioprotective in patients undergoing CABG, but despite the promising results, it has not reached normal clinical practice (Lee et al. 1995, Shalaby et al. 2008). The anti-apoptotic effect of adenosine in a clinical setting is still unclear (Shalaby et al. 2008).

There is evidence indicating myocardial protection with glucose-insulin-potassium (GIP) infusion in patients undergoing CABG and AVR, although the evidence of reduction in cardiac enzymes postoperatively is not clear (Quinn et al. 2006, Howell et al. 2011). GIP prevents cardiomyocyte apoptosis at least in an experimental I-R model (Zhang et al. 2004).

Preconditioning with cyclosporine A, a specific inhibitor of MPP opening, and bradykinin have been shown to be anti-apoptotic in experimental cardioplegic I-R injury (Oka et al. 2008, Yeh et al. 2010).

Antioxidants, calcium channel blocker nicardipine and anti-inflammatory drugs such as nitroprusside and corticosteroids have all shown properties for myocardial protection in clinical studies (Dhalla et al. 2000, Freyhold et al. 2003, Halonen et al. 2007, Casthely et al. 2008)

In an experimental model, intracoronary administered insulin-like growth factors IGF-II could prevent cardiomyocyte apoptosis, and also the postoperative TnI levels were significantly reduced (Salminen et al. 2011). New agents such as tetracycline antibiotic minocycline and urocortin have indicated anti-apoptotic features in experimental settings (Lawrance et al. 2004, Scarabelli et al. 2004).

2.5.2.2.1 Levosimendan

Levosimendan is an intracellular calcium sensitizer, which improves myocardial contractility in heart failure patients (Hasenfuss et al. 1998, Kivikko et al. 2002). In

cardiac surgery, it has been shown that perioperative levosimendan infusion facilitates weaning from CBP in CABG patients with impaired left ventricle function (Eriksson et al. 2009). In addition, levosimendan pre-treatment before CABG results in a reduction in tracheal intubation time, a shorter length of ICU stay, less requirement for inotropic support, and lower postoperative TnI concentration (Tritapepe et al. 2009). In patients undergoing elective CABG, presichemic levosimendan infusion has been shown to have preconditioning effects (Tritapepe et al. 2006). Levosimendan causes arteriolar and venous dilatation and therefore decreases systemic vascular resistance and preload, and it also reduces pulmonary arterial pressure, pulmonary vascular resistance, and pulmonary capillary wedge pressure (Bergh et al. 2010).

The positive inotropic effects of levosimendan are explained by its ability to sensitize myocardial filaments to calcium, without increasing the amount of intracellular calcium, and by inhibiting phosphodiesterase III (Toller et al. 2006). The target protein for levosimedan is cardiac troponin C, which the drug also stabilizes (Haikala et al. 1995). The vasodilating mechanism of levosimedan is proposed to transmit by activation of ATP-sensitive potassium channels (De Witt et al. 2002). Several studies indicate that levosimendan has a protective effect on the myocardium during I-R injury, and it is transmitted through mitochondrial ATP-sensitive potassium channels (Kopustinskiene et al. 2004, Metzsch et al. 2007, du Toit et al. 2008). Levosimendan has been shown to prevent cardiomyocyte apoptosis, and this mechanism is also believed to transmit through the same mitochondrial ATP-sensitive potassium channels (Akoa et al. 2001, Louhelainen et al. 2007, Ozturk et al. 2010).

Intravenous administration of levosimendan might cause systemic hypotension and, therefore, it has been suggested that intracoronary administration might be a safer way to achieve higher concentration of levosimendan in the myocardium without severe systemic side effects. In previous studies, the intracoronary administration of levosimendan has been started after the ischemia during the reperfusion period, and it has been shown to be an adequate method to improve postoperative cardiac dysfunction (Grossini et al. 2005, Caimmi et al. 2006, Grossini et al. 2010, Caimmi et al. 2011). Moreover, intracoronary administration of levosimendan has been shown to prevent cardiomyocyte apoptosis (Caimmi et al. 2011).

Levosimendan has two metabolites, OR-1855 and OR-1896, of which OR-1896 is the active metabolite and has the same pharmacological effects as levosimendan (Antila et al. 2004 and 2004).

2.5.3 Off-pump coronary artery bypass graft surgery

CABG performed without CBP (off-pump coronary artery bypass grafting, OPCAB), has been shown to be less harmful to the myocardium than conventional CABG with cardioplegic arrest when comparing the post-operative values of cardiac enzymes and post-operative stunning (Penttilä et al. 2001, van Dijk et al. 2001, Selvanaygam

et al. 2004). Although there seem to be no significant differences between these two techniques in long-term health outcomes (graft patency, major adverse cardiac-related events or death, mean health-related quality of life), in higher risk patients OPCAB is associated with lower operative mortality, and this benefit increases with increasing risk of mortality (The Society of Thoracic Surgeons Predicted Risk of Mortality, PROM) (Angelini et al. 2009, Puskas et al. 2009, Chu et al. 2009). In addition, there is evidence indicating that women might benefit more from off-pump surgery than men (Puskas et al. 2007). In low-risk patients, at five-year follow up, there were no differences in the patients´ cognitive or cardiac outcomes when on-pump CABG was compared to OPCAB (van Dijk et al. 2007).

3. AIMS OF THE STUDY

The objective of this study was to investigate the effects of different cardioprotective strategies on cardiomyocyte apoptosis during I-R injury associated with open heart surgery, both in experimental models and in patients. In addition, the role of cardiomyocyte apoptosis in post-operative left ventricle dysfunction was an object of interest. The specific aims of the study were to test:

1. whether the duration of myocardial ischemia is associated with the amount of cardiomyocyte apoptosis in the left ventricle in a pig model of cardioplegic ischemia (I)

2. how global, cardioplegia-protected I-R injury differs from regional, unprotected I-R injury in terms of myocardial apoptosis and left ventricle dysfunction (II)

3. how intracoronary levosimendan administered during ischemia protects the myocardium from cardiac apoptosis and left ventricle dysfunction after I-R injury when compared with pre-ischemic, intravenous levosimendan infusion (III)

4. whether protection of the left ventricular myocardium by antegrade or retrograde cardioplegia has different effects on cardiomyocyte apoptosis and preservation of post-operative left ventricle function in patients undergoing AVR (IV)

4. MATERIALS AND METHODS

4.1 Experimental studies (I-III)

All animals used in these studies were Finnish land race pigs, aged 12 weeks, their weight being between 28-31 kilograms. Altogether 52 animals were included in the final analysis and an additional seven animals were used to assess the levosimendan dose (III). The study protocols were reviewed and approved by the Ethical Committee for Animal Experiments of the University of Turku, and all animals received humane care in compliance with the European Convention on Animal Care.

4.1.1 Study designs

In the animal studies the pigs were randomly divided into three groups.

I In this study, all animals were connected to the CPB. The first group underwent cardioplegic arrest for 60 minutes (n=4) and the second group for 90 minutes (n=4). After the cardioplegic arrest, the animals were weaned from the CPB, followed by a reperfusion period of 120 minutes. In the control group (n=5), the animals were connected to the CPB without ischemia for 120 minutes.

II In the first group of this study, the animals (n=5) underwent unprotected regional warm ischemia caused by occlusion of terminal branch either RCA or LCX for 20 minutes and distal LAD for another 20 minutes, followed by reperfusion. In the second group, the animals (n=6) were connected to the CPB and they underwent cold, cardioplegic arrest for 40 minutes followed by reperfusion. The third group served as control and the animals (n=4) were connected to the CPB for 65 minutes without ischemia. Cardiac MRI was performed on all animals after 120 minutes of reperfusion.

III In this study, all animals were connected to the CPB, and they underwent cardioplegic arrest for 40 minutes. The reperfusion period after ischemia was 240 minutes. Echocardiography was performed on all animals at the beginning and at the end of the experiment. In the L-IV group the animals (n=8) received intravenous levosimendan (65μg/kg) for 40 minutes before cardioplegic arrest. In this group, the levosimendan was administered using a 10-minute infusion (35 μg/kg) followed by a 30-minute infusion (1 μg/kg/min). In the L-IC group, the animals (n=8) received the same dose of levosimendan (65 μg/kg) during ischemia administered intracoronary, mixed with 1000 ml of cardioplegia solution, given in two doses. The control group (n=8) did not receive levosimendan.

4.1.2 Surgical management

On the morning of the experiment day, the animals were transported to the animal laboratory and they were fasted for 12 hours before the experiment. The weight was measured before the transport. The animals were premedicated with 34 mg/kg (1000 mg) of ketamine (Ketanest-S (I) or Ketalar (II, III), Pfizer AB, Täby, Sweden) given intramusculary. After a peripheral vein cannulation, the animals received 0.7 mg/kg (20 mg) diazepam (Stesolid Novum, A/S Dumex, Denmark) intravenously. The intubation was performed openly after the trachea was surgically exposed and the animals had received a 4 mg bolus of pancuronium (Pavulon, Organon, The Netherlands). Anesthesia was maintained with a continuous infusion of ketamine (0.27 mg/kg/min) and pancuronium (0.007 mg/kg/min). In addition, a continuous infusion of succinylated gelatin (Gelofusin, B. Braun Melsungen AG, Melsungen, Germany) was maintained. The animals were connected to a respirator and ventilated with room air (I, II) or 60 % oxygen (III). The right common carotid artery and both external jugular veins were cannulated for drug administration, blood sampling, and hemodynamic monitoring. The heart was exposed from median sternotomy and the pericardium was opened and lifted. A pediatric thermodilution catheter (Swan-Ganz, Edwards Life sciences LLC, Irvine, CA, USA) was guided to the pulmonary artery through the external jugular vein. In order to avoid severe ventricular arrhythmias, the animals received 100 mg of lidocain hydrochloride (Xylocard, Hässle Läkemedel AK, Mölndal, Sweden) before manipulating the heart. A 3.4 mg/kg bolus of heparin (Heparin, Lövens, Ballerup, Denmark) was given to all animals before cannulating the heart (I, II, III) or occluding the coronary arteries (II). After the operations, the effect of heparin was antagonized with 3.4 mg/kg protamine (Protaminsulfat Leo, LEO Pharma A/S, Ballerup, Denmark). At the end of the experiments, the animals were sacrificed with potassium chloride injection (3000 mg).

4.1.2.1 Global I-R injury (I-III)

Animals were surgically prepared for the CPB by placing purse string sutures on the ascending aorta and the right atrium. On the aortic line, an 18 F single cannula and on the venous side, a two-stage cannula was used. A pediatric membrane oxygenator (Midiflo Pediatric D705, Dideco, Mirandola, Italy) was primed with 1500 of fresh pig blood containing 3800 mg of sodium citrate and 50 mg of heparin. During CPB, the flow was adjusted to 2.5-3 l/min (85-100 ml/kg/min) according to blood gas analysis and venous blood oxygen saturation percentage (60-70 %, Oxysat, Meter SM-0200, Baxter, Bentley, Irvine, SA, USA). The left hemiazygos vein draining to the coronary sinus was ligated in study I. The heart was stopped by placing the aortic cross-clamp and infusing cold (+5°C), crystalloid (modified St. Thomas Hospital N:o II) cardioplegic solution 500 ml to the proximal ascending aorta through an 18 G cannula (Venlon 2, Viggo AB, Helsinborg, Sweden). An additional dose of cardioplegia (250 ml (I, II) or 500 ml (II)) was given every 30 (I) or 20 (II, III) minutes. After the ischemia period, the aorta was declamped

and cardioversion was performed in case of ventricular fibrillation. The animals were weaned from the CPB after declamping and followed through the reperfusion period (120 minutes (I, II) or 240 minutes (III)). Control animals undergoing only CPB without ischemia (I, II) were operated accordingly.

4.1.2.2 Regional I-R injury (II)

In this study the heart was lifted from the apex using a heart stabilizer designed for off-pump surgery (Guidant Xpose™ 4 Device, Guidant Corporation, Santa Clara, CA, USA or Starfish 2 Heart Positioner, Medtronic, Inc., Minneapolis, MN, USA). The left posterior descending coronary artery (n=2) or the right posterior descending coronary artery (n=3) was occluded distally with a suture and a pledget for 20 minutes to produce small myocardial ischemia in the infero-posterior area. In addition, the distal LAD was ligated for another 20 minutes after stabilizing the anterior wall of ventricles with a heart stabilizer (Octopus 4 Tissue Stabilizer, Medtronic, Inc., Minneapolis, MN, USA) to produce a small area of ischemia in the anterior wall of the apex.

4.2 Patient study (IV)

This study was performed with 20 volunteer patients who were subjected to elective AVR due to aortic stenosis. Exclusion criteria were significant coronary artery disease (stenosis >50 % in one or more major coronary arteries in angiography), need for additional surgical procedures, and impaired left ventricle function in echocardiography (ejection fraction <50 %). The study was approved by the Committee of Ethics of the Southwest Finland Health Care District and all patients gave their signed informed consent.

4.2.1 Study design and surgical management

The patients were openly randomized to receive only either antegrade (n=10) or retrograde cardioplegia (n=10) during AVR. Cardiac MRI was performed in all patients one day before surgery and repeated nine months after surgery. Transesophageal echocardiography (TEE) was performed before the operation and repeated immediately after surgery in the operating theatre and the following morning in the ICU. Postoperative clinical events including inotropic medication, myocardial infarction, need for intra-aortic back pulsator, need for resternotomy, stroke, and mediastinitis were recorded. Duration of stay in the ICU and duration of hospital stay were compared between the groups.

The surgical protocol was identical in all patients, except for the cardioplegia administration. Anaesthesia was induced using intravenous sufentanil and propofol, and maintained with sevoflurane and sufentanil. Muscle relaxation was achieved using

rocuronium. AVR was performed through median sternotomy. During CPB, urinary bladder temperature was kept at 33°C. In the antegrade group the patients received cold blood cardioplegia using only antegrade delivery, and in the retrograde group using only retrograde delivery. To achieve asystole, the cardioplegia solution (12°C) was mixed with blood in a ratio of 1:4 and delivered as an initial dose of 1000 ml. In the retrograde group, a continuous low flow of cardioplegia (ratio 1:8) into the coronary sinus was maintained. In the antegrade group, additional doses of cardioplegia (500 ml, ratio 1:8) were given every 20 minutes into the coronary ostia. Patients older than 70 years received a biological valve prosthesis, and younger patients a mechanical valve. A warm (36°C) cardioplegia dose ("hot shot", 400 ml) was administered in both groups before removing the cross-clamp. In case of ventricular or atrial fibrillation the heart was defibrillated after declamping the aorta. The postoperative follow up was carried out at first in the ICU and later in the cardio-thoracic surgery ward.

4.2.2 Hemodynamic monitoring and laboratory analyses

Intraoperative hemodynamic monitoring included radial artery and pulmonary catheters. Cardiac index, central venous pressure, arterial pressure, pulmonary artery and pulmonary artery wedge pressures were monitored throughout the operation. Also electrocardiogram (ECG) and TEE were followed. In order to compare postoperative cTnI and CK-MBm values between the groups, blood samples were collected 2, 12 and 24 hours after the operation.

4.3 Myocardial samples and assessment of cardiomyocyte apoptosis

In order to detect cardiomyocyte apoptosis, transmyocardial samples were taken with a needle (Tru-Cut, Cardinal Health, McGaw Park, Il 60085 USA) in the beginning of the experiment from the anterior wall of both ventricles (IV) or from the apex in the left ventricle (I, II, III). In the patient study (IV), the myocardial samples were taken with a needle before cross-clamping the aorta, and at the end of the experiment after declamping the aorta before weaning from the CPB. In the animal studies (I, II, III), the postoperative myocardial samples from the left ventricle were taken by removing the heart at the end of the reperfusion period after sacrificing the animals. Cardiomyocyte apoptosis was assessed from the myocardial samples using TUNEL (I-IV), caspase-3 (I-IV) and Bcl-2, Bax and Bad (IV). The samples were fixed in neutral buffered formalin overnight, embedded in paraffin and cut into 4 μm sections for TUNEL and caspase-3 analyses. The analyses of apoptosis were performed blinded.

4.3.1 TUNEL (I-IV)

Briefly, in the TUNEL assay, paraffin-embedded myocardial sections were heated in sodium citrate solution and digested with proteinase-K to expose DNA. The DNA

strand breaks were then labeled using terminal transferase with digoxigenin-conjugated ddUTP and visualized using alkaline phosphatase immunohistochemistry. The assay was standardized with the use of serial sections treated with DNase I to induce enzymatic DNA fragmentation as a positive control of apoptosis (Saraste et al. 2000, Vähäsilta et al. 2005).

4.3.2 Caspase-3 (I-IV)

In order to confirm cardiomyocyte apoptosis assessment, also the activation of apoptosis-specific caspase-3 with an antibody specific for large (17-20 kDa) fragments of cleaved caspace-3 was analyzed. Sections that were deparaffined and hydrated were treated in a microwave oven for 10 minutes in sodium citrate buffer (pH 6.0) to expose antigens, followed by inhibition of endogenous peroxidase activity by 1 % H_2O_2. Using the avidine-biotine immunoperoxidase technique with diaminobenzin, the primary antibody (1:100) was visualized with a Vecstain ABCElite Kit (Vector Laboratories, Burlingame, CA, USA). As positive controls for the assay we used sections of inflamed human tonsil showing staining in some lymphocytes. Sections incubated without primary antibody showed no staining and served as a negative control (Saraste et al. 2000, Vähäsilta et al. 2005). The quantitative analyses of activated caspase-3 were performed in studies I and II. In studies III and IV, the analyses of activated caspase-3 served as a positive control for the TUNEL assay.

4.3.3 Bcl-2, Bax, Bad (IV)

In the analysis of apoptosis-regulating proteins, the expressions of anti-apoptotic Bcl-2 and pro-apoptotic Bax and Bad genes were determined. From the biopsy samples, the ribonucleic acid (RNA) was extracted with RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE Tissues (Applied Biosystems). The RNA concentration and quality were determined using a NapoDrop spectrophotometer. The RT-PCR assay included incubation at 50°C for 2 minutes and denaturing at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute. RNA from fresh tonsil tissue served as control.

4.4 Assessment of myocardial function

4.4.1 Hemodynamic monitoring (I-III)

Standard hemodynamic monitoring in animal studies included arterial pressure, central venous pressure, pulmonary artery pressure, pulmonary capillary wedge pressure, cardiac output, and ECG.

4.4.2 Magnetic resonance imaging (II, IV)

Cardiac MRI was performed in one animal study (II) and in all patients (IV). In brief, the both ventricles were covered by 8-12 slices at short axis orientation. Volumetrics were analyzed according to previous validation (Koskenvuo et al. 2007). Myocardial perfusion was assessed using gadoterate meglumine injection into the central vein at rest (II, IV) and during adenosine-induced hyperemia (II). Using the late enhancement technique, the size of the infarction scar was measured quantitatively (Mewton et al. 2009). Perfusion, wall motion and delayed-enhancement were analyzed visually for 17 segments, and segments 13-15 were considered as the area of risk (Cerqueira et al. 2002). The technique of cardiac MRI is described in the original article (II) in more detail. The analyses of the measurements were done blinded and off-line.

4.4.3 Echocardiography (III, IV)

Echocardiography was performed epicardially in all animals in study III and transesophegeally in all patients (IV). In study IV, TEE measurements were performed pre-operatively in the operating theater (time-point 1), immediately after surgery (time-point 2) and the morning after the operation (time-point 3). The TEE analyses included amplitude of the systolic motion of the lateral mitral annulus, cardiac output, early (E) and late (A) diastolic mitral valve inflow velocities, and early diastolic velocity of the mitral annulus (E´). All TEE measurements were performed in anesthetized patients and averaged over three cardiac cycles (Flachskampf et al. 2001). In addition, in the animal study, the left ventricle ejection fraction and coronary flow from mid LAD were measured (Kiviniemi et al. 2007, Saraste et al. 2007).

4.5 Assessment of myocardial and blood levosimendan concentrations

In study III, the concentrations of levosimendan and its metabolites OR-1896 and OR-1855 were measured from the frozen plasma samples collected during the experiment and from the myocardial samples collected at the end of the experiment. Plasma samples were collected during the experiment 110, 170, 260 and 320 minutes after starting the levosimendan. The bioanalytic methods are described in the original article (III) in more detail. The measurements of the concentrations were performed by Orion Pharma, Espoo, Finland.

4.6 Statistical analyses

To determine whether the data were normally distributed, the Shapiro-Wilk test was applied. Normally distributed, continues variables were expressed as mean ± standard error of mean, and non-parametric variables were expressed as median and interquartile range. In the patient study (IV), characteristics of the patient groups were compared

using chi-square test of Fisher´s exact test. The differences between groups were tested with 2-tailed T-test or Mann-Whitney U-test. Differences were considered significant when the p-value was <0.05. All analyses were performed using a SPSS software package (Version 16.0 or 20.0; SPSS, Inc., Chicago, Illinois, USA). Power analyses were calculated for studies III and IV. A biostatistician was consulted for a biostatic review (I-IV).

5. RESULTS

5.1 Experimental global and regional I-R injury (I-II)

5.1.1 Cardiomyocyte apoptosis and the duration of aortic clamping (I)

The amounts of TUNEL-positive cardiomyocytes at the end of the experiments in study I are presented in Figure 4. Ninety minutes of cardioplegic ischemia induced more apoptosis than 60 minutes or CPB (control). Moreover, CPB alone induced myocardial apoptosis in the left ventricle.

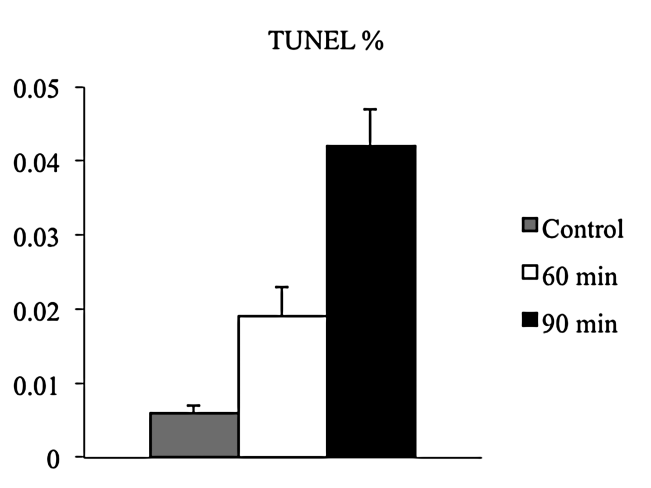

Figure 4. Cardiomyocyte apoptosis after I-R and CPB detected by TUNEL assay in study I. Control vs. 60 minutes p=0.031, control vs. 90 minutes p<0.001, 60 minutes vs. 90 minutes p=0.001.

The quantitative analysis of cardiomyocytes containing active caspase-3 in the nucleus and in the cytoplasm was performed from the left ventricle samples in study I, Figure 5. Ninety minutes of cardioplegic ischemia induced significantly more apoptosis than CPB (control).

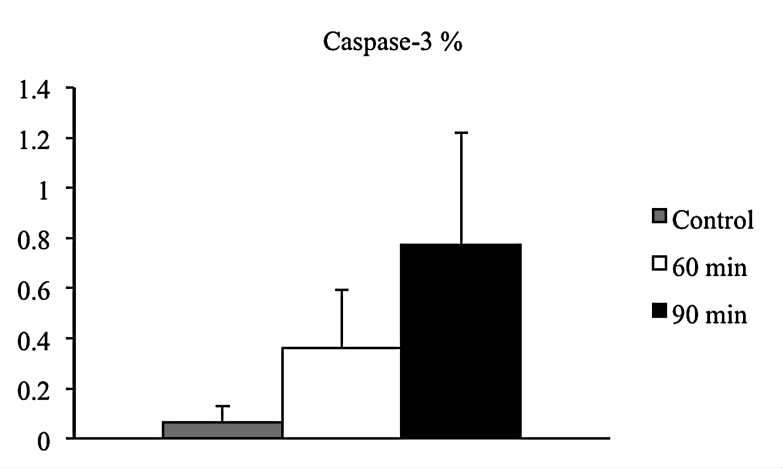

Figure 5. Cardiomyocyte apoptosis (caspase +/10 fields) in the left ventricle after I-R and CPB (control) detected by caspase-3 activity in study I. Control vs. 60 minutes p=0.362, control vs. 90 minutes p=0.019, 60 minutes vs. 90 minutes p=0.172.

5.1.2 Cardiomyocyte apoptosis during unprotected and cardioplegic ischemia (II)

In study II, cardioplegia protected the myocardium from apoptosis during global ischemia, when compared to unprotected, local ischemia, Figure 6. Moreover, global, cardioplegic ischemia induced more apoptosis in myocardial cells in left ventricular biopsies than CPB (control).

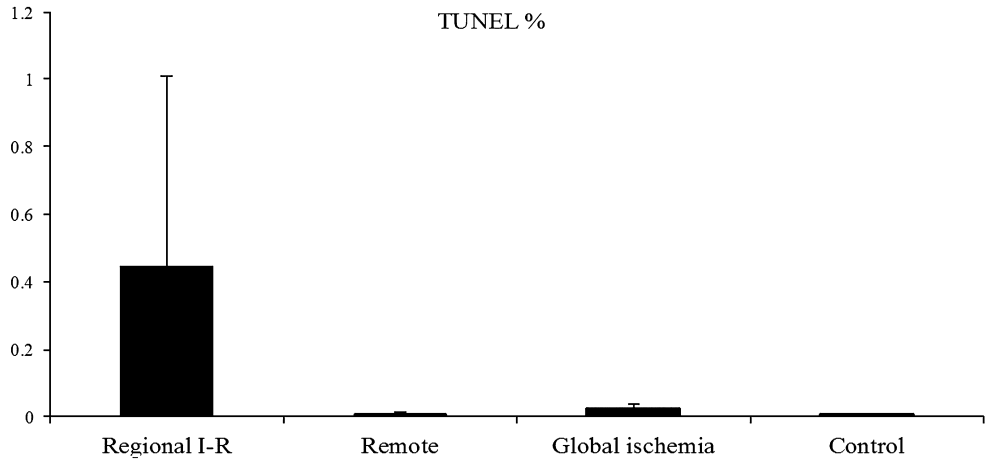

Figure 6. Cardiomyocyte apoptosis in left ventricle biopsies after I-R detected by TUNEL assay in study II. Remote samples are from the non-ischemic part of the left ventricle in the regional I-R group. Regional I-R vs. remote p< 0.001, regional I-R vs. global ischemia p=0.003, regional I-R vs. control p< 0.001, global ischemia vs. control p=0.03.

In study II, the degree of caspase-3 activation was semi-quantitatively analyzed as numbers of positively stained cardiomyocytes / 100 microscopic fields, Figure 7. Compared with CPB (control), there were significantly more caspase-3 positive cardiomyocytes in the ischemic areas of the unprotected, regional I-R.

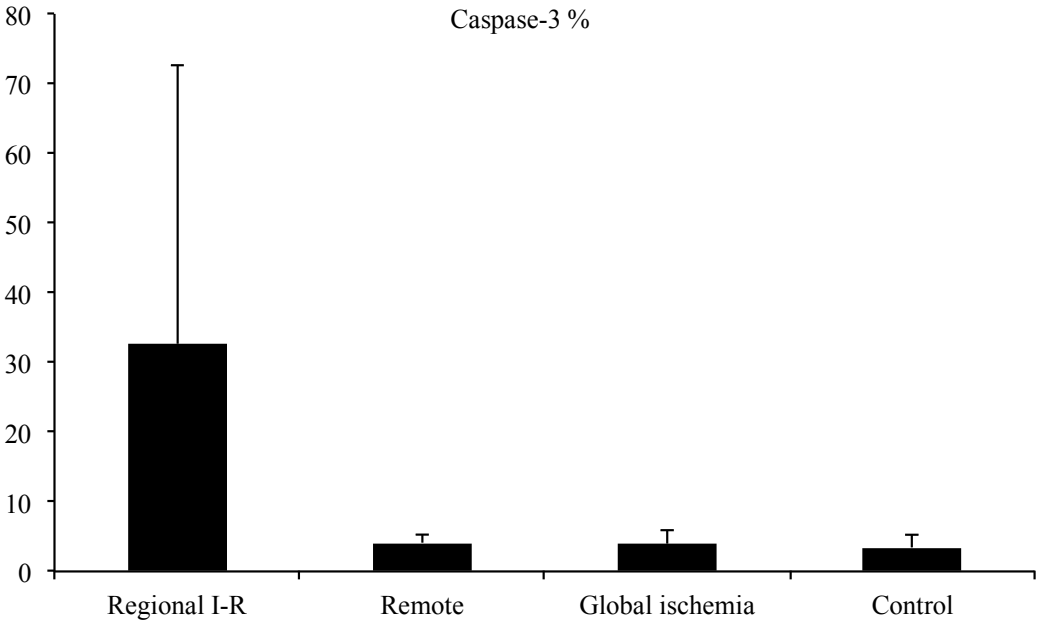

Figure 7. Cardiomyocyte apoptosis (caspase +/100 fields) after I-R detected by caspase-3 activity in study II from the left ventricle. Remote samples are from the non-ischemic part of the left ventricle in the regional I-R group. Regional I-R vs. remote p=0.88, regional I-R vs. global ischemia p=0.002, regional I-R vs. control p=0.004, global ischemia vs. control p=1.0

5.1.3 Cardiac function (II)

The left ventricle ejection fraction and wall motion in segments 13-15 detected by MRI after 120 minutes of reperfusion in study II are presented in Figure 8.

The ejection fraction was significantly higher in the control group than in the global ischemia group (p=0.02). The differences between regional I-R and global ischemia (p=0.59) or between regional I-R and control (p=0.11) were not significant.

The left ventricle wall motion score was significantly higher in the regional I-R group than in the global ischemia (p=0.03) or in the control group (p=0.004), indicating locally impaired myocardial function. The score was also higher after global ischemia when compered to the controls (p=0.05).

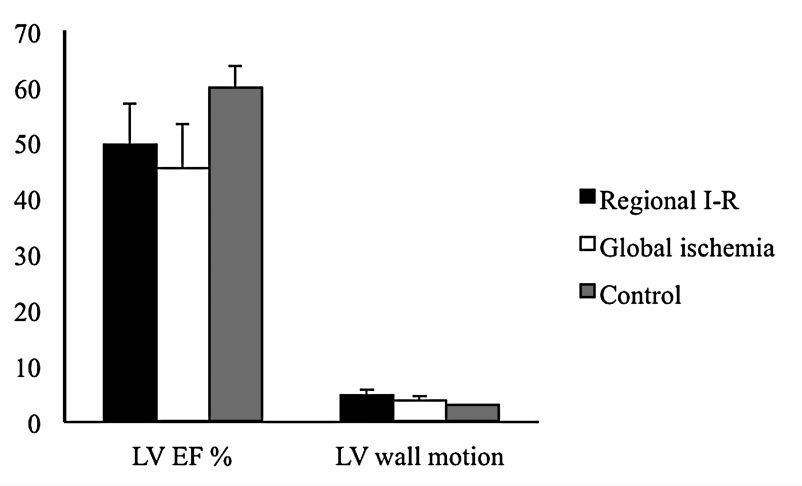

Figure 8. Left ventricle (LV) ejection fraction (EF) and wall motion in segments 13-15 detected by MRI 120 minutes after reperfusion in study II.

Focal delayed enhancement was detected in one pig in the global ischemia group, while delayed enhancement was found in altogether 5 of the 8 ischemic myocardial areas in the regional I-R group

5.1.4 Hemodynamic measurements (I, II)

Detailed results of hemodynamic measurements are presented in the original articles.

In study I, the mean arterial pressure and heart rate were significantly lower after I-R in the 90-minute ischemia group than in the 60 minutes group (50±11.5 mmHg vs. 75±12.4 mmHg $p=0.04$, 111±5.9 /min vs. 152±2 /min, $p<0.001$).

In study II, hemodynamic measurements were recorded before the operation and at 30, 90 and 210 minutes after the operation. At 90 minutes after the ischemia period, the mean arterial pressure was lower in the regional I-R group (57.4±8.1 mmHg) than in the global ischemia group (72.8±7.2 mmHg) or controls (80.8±6.1 mmHg, $p=0.01$). In the control group, 210 minutes after the operation, the heart rate (91.5±16.5 /min) was lower than in the global ischemia group (132.5±24.6 /min) or in the regional ischemia group (117.8±13.5 /min, $p=0.04$).

5.2 Effects of levosimendan in experimental I-R injury (III)

5.2.1 Pharmacological measurements

In study III, effective doses of levosimendan or active metabolite OR-1896 were detected in the plasma and myocardium samples of both levosimendan treatment groups, Figures

9 and 10. In the myocardial samples there were no traces of OR-1896 or OR-1855, and in the plasma samples we did not find traces of levosimendan or metabolite OR-1855.

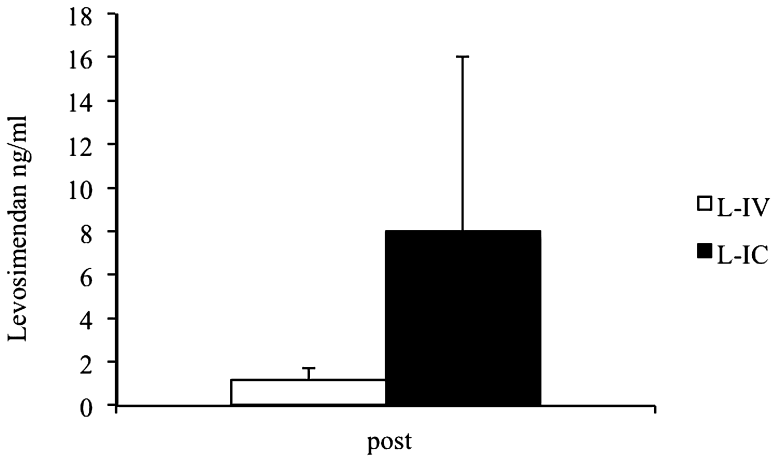

Figure 9. Levosimendan concentrations at the end of the experiment in the left ventricular myocardial samples. L-IV=levosimendan infusion–group, L-IC=intracoronary levosimendan-group, p=0.001.

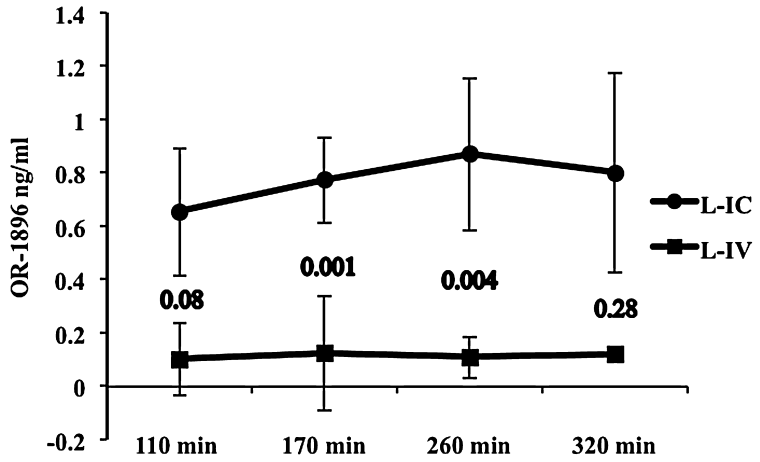

Figure 10. Concentration of active metabolite OR-1896 in the plasma samples 110, 170, 260 and 320 minutes after the onset of levosimendan administration. L-IV=levosimendan infusion-group, L-IC=intracoronary.

5.2.2 Cardiomyocyte apoptosis

In study III, during ischemia the administered intracoronary levosimendan protected the left ventricle myocardium equally well from apoptosis when compared with pre-ischemia intravenous infusion, Figure 11. In the control group, there were three times more apoptotic myocardial cells than in the levosimendan treatment groups in TUNEL assay. Cardiac apoptosis was significantly induced in all three groups during the experiment (pre L-IV vs. post L-IV p=0.02, pre L-IC vs. post L-IC p<0.001, pre control vs. post control p=0.01).

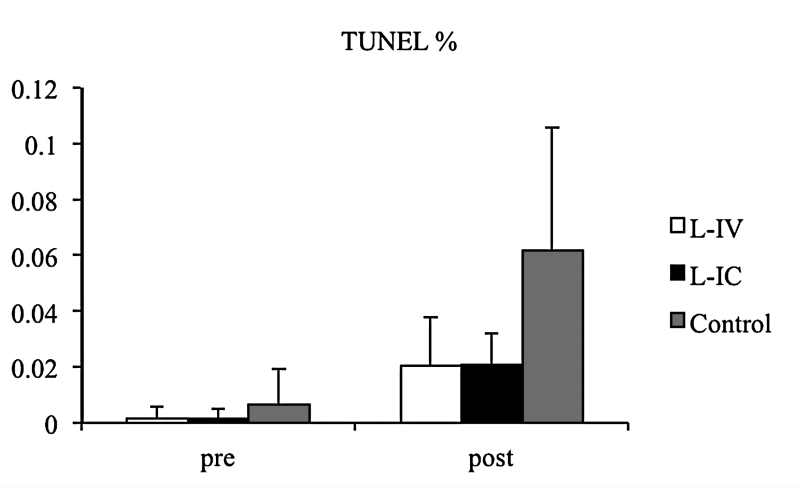

Figure 11. Cardiomyocyte apoptosis before (pre) and after (post) I-R detected by TUNEL assay in study III. Myocardial samples are from the left ventricle. Post L-IV vs. post control p=0.03, post L-IC vs. post control p=0.03, post L-IV vs. post L-IC p=1.00.

5.2.3 Cardiac function

The summarized echocardiography results at the end of the experiment of study III are presented in Table 2**.** The mitral inflow E-wave velocity was significantly reduced after the I-R injury in all three groups (pre L-IV vs. post L-IV p<0.001, pre L-IC vs. post L-IC p<0.001, pre control vs. post control p=0.015).

In addition, when E-wave velocity was compared between the groups at the end of the experiment, there was a significant difference between L-IV and L-IC groups (p=0.01), but not between L-IV and control (p=0.85), or between L-IC and control (p=0.06) groups.

Between the groups, there were no significant changes in EF, but, however, the EF was reduced during the experiment in the L-IC group (pre vs. post p=0.04) and in the control group (pre vs. post p=0.02) but not in the L-IV group (pre vs. post p=0.07).

The longitudinal systolic motion of the lateral mitral annulus was significantly reduced at the end of the experiment in the L-IC and the control groups when compared to the L-IV group (post L-IV vs. post L-IC p=0.003, post L-IV vs. post control p=0.01, post L-IC vs. post control p=0.97).

In other echocardiography variables no significant differences were detected.

Table 2. Summarized echocardiography data at the beginning (pre) and at the end of the experiment (post) in study III. Data presented as mean±standard error of mean or median and [interquartile range]. L-IV=levosimendan infusion -group, L-IC=intracoronary levosimendan–group. E=early, A=late mitral inflow velocity, EF=ejection fraction, Longit lat=longitudinal systolic motion of the lateral mitral annulus. *p=0.01, ^p=0.003, °p=0.01.

TEE variable	**L-IV**	**L-IC**	**Control**
E Pre (m/s)	0.67±0.09	0.63±0.12	0.71±0.1
E Post (m/s)	0.46±0.07*	0.35±0.06*	0.44±0.04
pre vs. post	**p<0.001**	**p<0.001**	**p<0.001**
A Pre (m/s)	0.67±0.14	0.57±0.12	0.71±0.19
A Post (m/s)	0.61±0.15	0.48±0.11	0.49±0.1
pre vs. post	p=0.5	p=0.1	p=0.05
EF Pre (%)	71.07±2.89	69.12±6.37	68.13±6.64
EF Post (%)	62.62±10.9	59.42±4.52	60.07±4.47
pre vs. post	p=0.07	**p=0.01**	**p=0.02**
Longit lat Pre (mm)	0.76±0.20	0.78±0.13	0.82±0.14
Longit lat Post (mm)	0.75±0.12^°	0.53±0.11^	0.54±0.11°
pre vs. post	p=0.9	**p=0.001**	**p<0.001**

5.3 Comparison of antegrade and retrograde cardioplegia (IV)

5.3.1 Clinical outcome and postoperative enzyme release

In study IV, there were fifteen patients in the final study group (antegrade n=7, retrograde=8): one patient refused the post-operative MRI (antegrade), one patient received both antegrade and retrograde cardioplegia (antegrade), one patient had to be connected twice to the CBP due to surgical bleeding (antegrade), one patient was treated for a malignant tumor (retrograde), and one patient was given a permanent pacemaker (retrograde).

There were no significant differences between the groups in the immediate post-operative clinical course, none of the patients died or had perioperative myocardial infarction, and the release of cardiac enzymes was comparable.

5.3.2 Cardiomyocyte apoptosis

In study IV, the percentages of cardiomyocyte apoptosis were increased in the left ventricle after retrograde (pre LV retro vs. post LV retro p=0.01) but not after antegrade cardioplegia (pre LV ante vs. post LV ante p=0.14) in the TUNEL assay, Figure 12. There was no significant induction of myocardial apoptosis in the samples from the right ventricle after antegrade (pre RV ante vs. post RV ante p=0.26) or retrograde cardioplegia (pre RV retro vs. post RV retro p=0.45). Compared with antegrade cardioplegia, there were no significant differences after retrograde cardioplegia in the left (p=0.13) or in the right ventricle (p=1.0). Also, there were no significant differences in apoptosis between the ventricles after antegrade (p=0.28) or retrograde (p=1.0) cardioplegia.

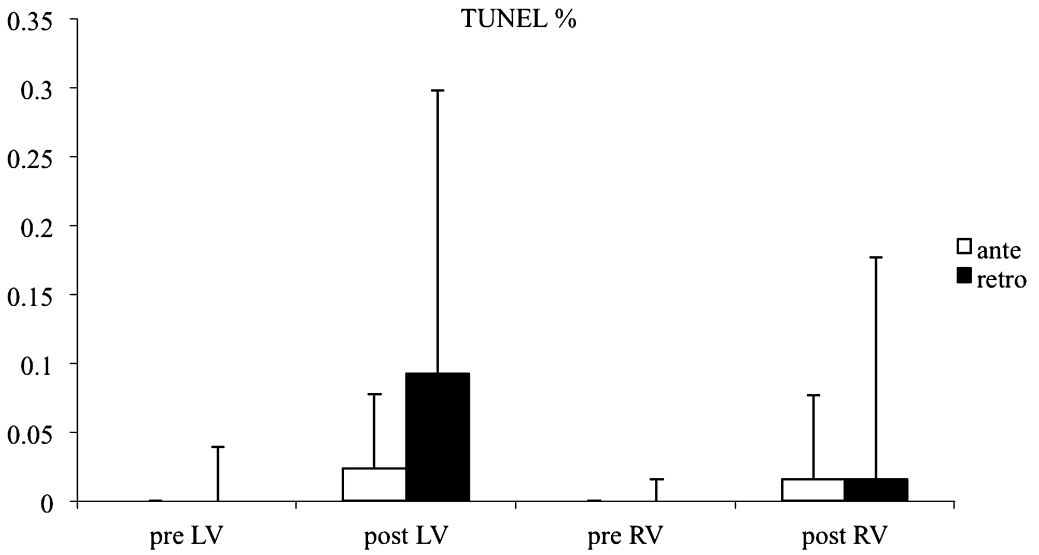

Figure 12. Cardiomyocyte apoptosis before (pre) and after (post) antegrade (ante) or retrograde (retro) cardioplegia detected by TUNEL assay in study IV. Samples are from the right (RV) and left ventricle (LV). Significant induction of apoptosis was found in the LV in the retrograde cardioplegia group (pre LV retro vs. post LV retro p=0.01).

The levels of Bcl-2, Bax and Bad gene expression normalized to glyceraldehyde 3-phosphate dehydrogenase (gene/GAPADH) were analyzed in study IV, Figure 13. There were no significant differences between ante- and retrograde cardioplegia groups in the LV or RV before or after the operation.

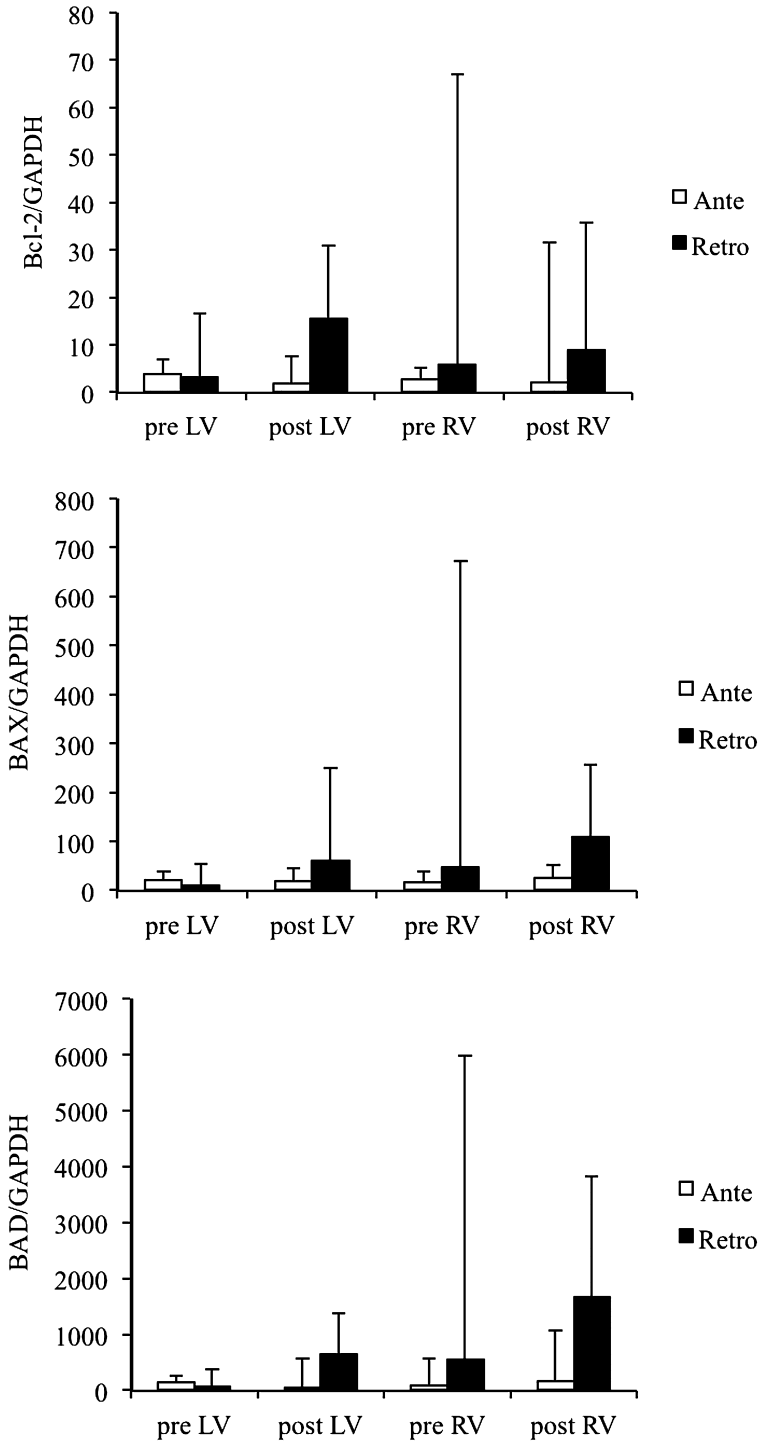

Figure 13. The levels of Bcl-2, Bax and Bad normalized to GAPDH before (pre) and after (post) antegrade (ante) or retrograde (retro) cardioplegia in study IV. Samples are from the right (RV) and the left ventricle (LV). There were no significant differences.

5.3.3 Cardiac function

The summarized results of the cardiac MRI measurements in study IV are presented in Table 3. MRI was performed in all patients one day before the operation and repeated 265±117 days after the operation (ante 233±74 vs. retro 293±144 p=0.32).

The left ventricle mass adjusted to the body surface area was significantly reduced after the operation in the antegrade but not in the retrograde group (pre ante vs. post ante p=0.03, pre retro vs. post retro p=0.12).

The cardiac output was reduced after the operation in the antegrade but not in the retrograde group (pre ante vs. post ante p=0.048, pre retro vs. post retro p=0.75).

There were no significant differences in the left ventricle end systolic or diastolic volume or in the ejection fraction, and neither were there significant differences between antegrade and retrograde groups at any time-point.

Table 3. Results of MRI 1 day before the operation and 9 months after the operation before (pre) and after (post) antegrade (ante) or retrograde (retro) cardioplegia in study IV. EF=ejection fraction, CO=cardiac output, LVMASS=left ventricle mass. LVMASSI=left ventricle mass adjusted to body surface area.

MRI parameter	Ante (n=7)	Retro (n=8)	p-value
Pre LVEF (%)	63.01±8.76	62.22±8.58	0.86
Post LVEF (%)	64.52±9.33	65.07±7.56	0.90
pre vs. post	p=0.76	p=0.49	
Pre LVCO (l/min)	6.72±0.82	6.88±1.12	0.79
Post LVCO (l/min)	5.85±0.63	6.67±1.21	0.12
pre vs. post	**p=0.048**	p=0.75	
Pre LVMASS (g)	161.8±30.17	177.41±41.29	0.41
Post LVMASS (g)	129.07±27.85	145.8±31.99	0.3
pre vs. post	p=0.06	p=0.11	
Pre LVMASSI (g/m^2)	86.08±14.06	87.48±16.05	0.86
Post LVMASSI (g/m^2)	68.98±10.88	74.14±16.04	0.48
pre vs. post	**p=0.03**	p=0.75	

The TEE results of study IV are presented in Table 4. The ratio early mitral inflow velocity to early diastolic velocity on the mitral annulus (E/E´) indicating increased left ventricle filling pressure, was higher immediately after the operation in the retrograde group (time-point 1 vs. time-point 2 p=0.05) but not in the antegrade group (time-point 1 vs. time-point 2 p=0.95).

The amplitude of longitudinal systolic motion of the lateral mitral annulus was lower immediately after the operation than before in the retrograde (time-point 1 vs. time-point 2 p=0.03) but not in the antegrade group (time-point 1 vs. time-point 2 p=0.78).

In cardiac output, in early or late mitral inflow velocities or in their relations, there were no significant differences between the measurements or between the groups.

Table 4. The summarized results of the TEE performed before sternotomy (time-point 1), immediately after the operation (time-point 2) and on the first post-operative day (time-point 3) using antegrade (ante) or retrograde (retro) cardioplegia in study IV. E=early diastolic mitral inflow velocity, E´=early diastolic velocity on the mitral annulus, Long. Lat=longitudinal systolic motion of the lateral mitral annulus, CO=cardiac output.

TEE parameter	**Ante**	**Retro**	**p-value**
E/E´ mean			
Time-point 1	17.64±7.35	16.24±3.54	0.69
Time-point 2	17.12±10.04	24.41±7.86	0.23
1 vs. 2	p=0.78	**p=0.03**	
Time-point 3	21.10±7.94	16.45±4.26	0.25
Long. Lat. (mm)			
Time-point 1	0.91±0.36	0.94±0.18	0.85
Time-point 2	0.84±0.42	0.73±0.10	0.56
1 vs. 2	**p=0.05**	p=0.95	
Time-point 3	0.63±0.12	0.71±0.24	0.56
CO (l/min)			
Time-point 1	4.39±2.62	6.38±4.01	0.43
Time-point 2	4.73±8.02	4.19±7.58	0.31
Time-point 3	5.80±1.61	5.65±2.73	0.91

6. DISCUSSION

In these studies we wanted to investigate the role of cardiomyocyte apoptosis in different strategies of myocardial protection during I-R injury associated with open heart surgery. In addition, the role of cardiomyocyte apoptosis in post-operative left ventricle dysfunction was an object of interest.

These studies provided the following new findings on the role of cardiomyocyte apoptosis and cardioprotection during open heart surgery.

1) In the animal model, longer ischemia time induced more myocardial apoptosis. 2) Local, unprotected ischemia induced more cardiomyocyte apoptosis in the animal model than global, protected ischemia, but was not associated with impaired ejection fraction. 3) Levosimendan infusion resulted in reduced cardiomyocyte apoptosis and improved postischemic cardiac function in the experimental model of global, cardioplegic ischemia. Intracoronary levosimendan administered during ischemia did not equally protect the myocardium when compared with intravenous infusion before ischemia. 4) During elective AVR, retrograde cardioplegia was associated with inferior myocardial protection when compared with antegrade cardioplegia, as shown by increased cardiomyocyte apoptosis and impaired postoperative cardiac function and left ventricular remodeling.

6.1 Methodological considerations

In this thesis, three studies were carried out with animal models and one study was conducted clinically.

In the experimental studies, we used a previously established animal model and developed it further (Vähäsilta et al. 2001 and 2005). Our animal model has been shown to be reproducible and closely related to clinical practice. In addition, the pig anatomy and physiology closely resembles to human. The porcine heart is delicate to handle and the surgical procedures are demanding, and therefore 11 animals were lost during the process. A further seven animals had to be excluded from the studies due to missing or inadequate data (MRI, study II). To ensure the success of our studies, it was necessary to limit the time of ischemia and reperfusion.

It is safe to take myocardial samples from both ventricles during standard AVR operation, as has been shown earlier (Wu et al. 2003). Although the number of patients was small, it was possible to find significant differences in myocardial apoptosis and function.

Quantitative analyses of TUNEL assay, as well as semi-quantitative analyses of activation caspase-3 enzyme, are well established and reliable methods to assess myocardial apoptosis from ventricular biopsies, while TUNEL assay still remains the golden standard in analyzing cardiac apoptosis (Saraste et al. 1997, Saraste 2000, Vähäsilta et

al. 2005). Expression of anti-apoptotic (Bcl-2) and pro-apoptotic (Bax, Bad) genes RNA analysis (IV) is also a reliable method to measure apoptosis; however, like other methods it can not be used on its own (Yeh et al. 2010).

Cardiac MRI and TEE are both valid methods to measure myocardial function and viability (Kim et al. 1999, Bellenger et al. 2000). In our models, epicardially performed echocardiography has shown to be a reproducible and accurate method to assess myocardial function and coronary flow in experienced hands.

6.2 Myocardial protection and cardiomyocyte apoptosis

Apoptosis is a part of normal human physiological functions, when it removes cells that function incorrectly or are destined to terminate (Kerr et al. 1972, Thompson 1995). Although it is clear that I-R injury caused by heart surgery induces cardiomyocyte apoptosis, and there is some evidence that cardiac apoptosis might correlate with postoperative stunning, the clear meaning of myocardial apoptosis in I-R injury is not yet fully clarified (Gottlieb et al. 1994, Schmitt et al. 2002, Ramlawi et al. 2006, Gaudino et al. 2007). The increased amount of apoptosis is more likely to be a part of general I-R injury, which raises the idea of apoptosis as an indicator of I-R injuries severity. Cardiac apoptosis might provide a potential target for myocardial protection, because viable myocardial cells are lost as a consequence of I-R injury (Gottlieb et al. 1994, Valen 2003). There is no clear evidence that only cardiomyocyte apoptosis could be prevented during I-R injury. However, if myocardial protection could be targeted more towards apoptosis, it might provide a beneficial effect on the outcome, although it seems that the amounts of apoptotic myocytes induced by I-R injury are often small (Wu et al. 2003, Vähäsilta et al. 2005).

6.2.1 Ischemia-reperfusion injury

In our experimental study (I) we have shown from left ventricular myocardial biopsies, that longer global, cardioplegic ischemia causes more cardiomyocyte apoptosis than shorter ischemia, when the reperfusion times are comparable. Moreover, our results show that CPB it self induces cardiomyocytes apoptosis, as previously indicated (Valen 2003). There have been studies indicating the same kinds of results, and our results further encourage the idea of apoptosis as a marker of I-R injury (Schmitt et al. 2002). Generally, aortic cross-clamp time has been shown to be an independent predictor of mortality (Doenst et al. 2008).

In study II, we showed that local, unprotected myocardial ischemia induced more apoptosis than global, cardioplegic ischemia. In addition, our study shows that hypothermic, cardioplegic arrest protects the myocardium from apoptosis when compared to unprotected ischemia. These findings also support the idea of apoptosis as a part of I-R injury, since cardioplegia has been shown to protect the myocardium and

to reduce the amount myocardial apoptosis (Cordell 1995, Schmitt et al. 2002, Yeh et al. 2003, Feng et al. 2004, Feng et al. 2005, Hausenloy et al. 2007). In the light of current evidence, it is not clear whetter the reduction of apoptosis improves the clinical outcome in the long term. However, it has been hypothesized that preconditioning, ischemic or pharmacological, would be able to save up to 50 to 90 % of tissue that would otherwise be lost through I-R injury (Fisher et al. 2004, Scarabelli et al. 2006).

6.2.1.1 Ante- and retrograde cardioplegia

As has been previously indicated, retrograde cardioplegia used on its own might provide inferior myocardial protection compared with antegrade cardioplegia, particularly in the right ventricle (Winkelmann et al. 1995, Allen et al. 1995, Tian et al. 2003). In addition, a previous experimental study showed that retrograde cardioplegia was associated with a higher amount of cardiomyocyte apoptosis in the right ventricle than antegrade cardioplegia (Vähäsilta et al. 2005). In our study with elective AVR patients (IV), we found more cardiomyocyte apoptosis in the left ventricle with retrograde compared to antegrade cardioplegia. Our results might be partially explained by a hypertrophied myocardium due to aortic valve stenosis, since higher amounts of apoptotic cardiomyocytes have been found in similar situations (Gaudino et al. 2007). However, inadequate perfusion of the myocardium with retrograde cardioplegia is more likely to be the main reason for the increased amount of apoptosis after AVR (Winkelmann et al. 1995). This finding is supported by the postoperative cardiac MRI result, where only in the antegrade cardioplegia group was the left ventricle mass index reduced nine months after surgery. Reduction of the left ventricle mass after AVR has been documented previously as a part of ventricular remodelling and in addition, a few studies have indicated that also cardiomyocyte apoptosis has a role in remodeling (Pela et al. 1997, Palojoki et al. 2001, Dorn 2008).

6.2.1.2 Levosimendan

While the use of levosimendan in open heart surgery is increasing, its benefits can be limited due to systemic hypotension associated with intravenous infusion (Kivikko et al. 2002, Tasouli et al. 2007, Lahtinen et al. 2011). Therefore, it has been suggested that intracoronary administration might be beneficial; this has indeed been successfully used after global or regional ischemia to prevent postoperative ventricular dysfunction and cardiomyocyte apoptosis (Grossini et al. 2010, Caimmi et al. 2011). In our experimental animal study in vivo (III), we found, that levosimendan reduced the amounts of cardiomyocyte apoptosis after I-R to one third when compared to cardioplegic ischemia. In addition, there was higher intracellular concentration of levosimendan in the myocardial cells with intracoronary administration.

During 24-hours infusion of levosimendan in humans, the steady state concentration is achieved commonly 4-8 hours, and the highest concentration approximately 24 hours,

after starting the infusion, while the maximum concentration of metabolites OR-1896 and OR-1855 can be seen approximately within 24 hours after terminating the infusion (Antila et al. 2004). In our study, the intracoronary administration significantly increased the intracellular levosimendan concentration after only four hours, while the plasma concentrations were comparable to those of humans, suggesting that intracoronary administration can be an effective way to increase the amount of levosimendan in the myocardium (Lehtonen et al. 2007).

The opening of mitochondrial ATP-sensitive potassium channels seems to be in a key feature in protecting the myocardium. Although the intracellular mechanisms of levosimendan are various and still not fully clarified, it seems that the cardioprotective mechanism of levosimendan is transmitted through these channels, as are its anti-apoptotic effects (Kopustinskiene et al. 2004, Maytin et al. 2005, Pollosello et al. 2007). Levosimendan also has other cardioprotective effects than preventing apoptosis and some of its benefits are transmitted through favorable hemodynamic effects (Eriksson et al. 2009, Tritapepe et al. 2009). However, the intracellular mechanisms of levosimendan raise interesting questions about the role of cardiomyocyte apoptosis in myocardial protection.

6.2.2 Myocardial function

The preservation of myocardial function is the main object of cardioprotection in heart surgery. At least a few studies indicate that higher amounts of cardiomyocyte apoptosis associate with impaired postoperative left ventricular function (Schmitt et al. 2002, Gaudino et al. 2007). However, it is not entirely clear whether there is a connection between apoptosis and myocardial function. Encouraging results have been found in experimental studies, when inhibition of caspase activation has been shown to result in better ventricular function after cardioplegic arrest (Yarbrough et al. 2004, Mukherjee et al. 2004).

In our studies, we found more cardiomyocyte apoptosis and lower ejection fraction (MRI) with cardioplegic ischemia compared to the control group without ischemia (II). Also with local, unprotected I-R we could see locally impaired left ventricle function and more cardiomyocyte apoptosis when compared to global, protected I-R (II). Moreover, when comparing the postoperative myocardial functions, intravenous levosimendan protected the myocardium better than intracoronary administration or cardioplegia alone. This might be explained by the fact that pre-ischemic intravenous levosimendan has more time to affect than intracoronary levosimendan, but it does not answer the question of why the amounts of apoptosis were equal in the myocardial cells in the levosimendan treatment groups. In addition, with retrograde cardioplegia, there were more apoptotic myocytes, while the systolic mitral annulus movement, as well as the diastolic left ventricular function were, decreased immediately after the operation, although in the long term the results were comparable. However, although our result might indicate a

close relation between cardiomyocyte apoptosis and postoperative stunning, we did not find a correlation between them.

6.3 Research interests for the future

In the near future, with novel pharmaceutical agents, myocardial protection will very likely advance in preserving myocardial function during heart surgery. Anti-apoptotic treatment might be in a position to offer great potential. Levosimendan is one of the new drugs that could provide more possibilities in clinical practice. To study the short- and long-term cardioprotective effects of levosimendan administered intracoronarly during ischemia in patients undergoing open heart surgery could be one objective for the future. There are several interesting research possibilities when imaging the myocardial function with MRI and echocardiography and combining these results with cellular changes in the myocardium during I-R, e.g. is there truly a correlation between cardiomyocyte apoptosis and myocardial stunning.

7. CONCLUSIONS

On the basis of our experimental and clinical studies on myocardial apoptosis and cardioprotection during open heart surgery, we can draw the following conclusions:

1. Longer ischemia time induced more cardiomyocyte apoptosis in left ventricle biopsies in an experimental model of cardioplegia-protected global I-R.

2. Local, unprotected ischemia induced much more cardiomyocyte apoptosis than global, cardioplegia-protected ischemia in an experimental model of I-R injury. Local ischemia also induced regional left ventricular dysfunction.

3. Pre- and perioperative levosimendan treatment was associated with reduced cardiomyocyte apoptosis. Intracoronarly administered levosimendan and preischemic intravenously administered levosimendan were equally effective in preventing apoptosis, but intracoronary administration was associated with more impairment of systolic and diastolic left ventricular function.

4. Retrograde cardioplegia was associated with induction of more apoptosis in the left ventricular cardiomyocytes than antegrade cardioplegia in biopsies of patients undergoing AVR. Left ventricular systolic and diastolic functions were impaired immediately after surgery, and in the long-term, remodeling of the left ventricle was impaired with retrograde cardioplegia. These results provide evidence of inferior myocardial protection with retrograde cardioplegia alone.

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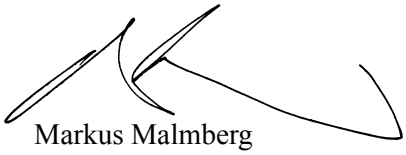

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