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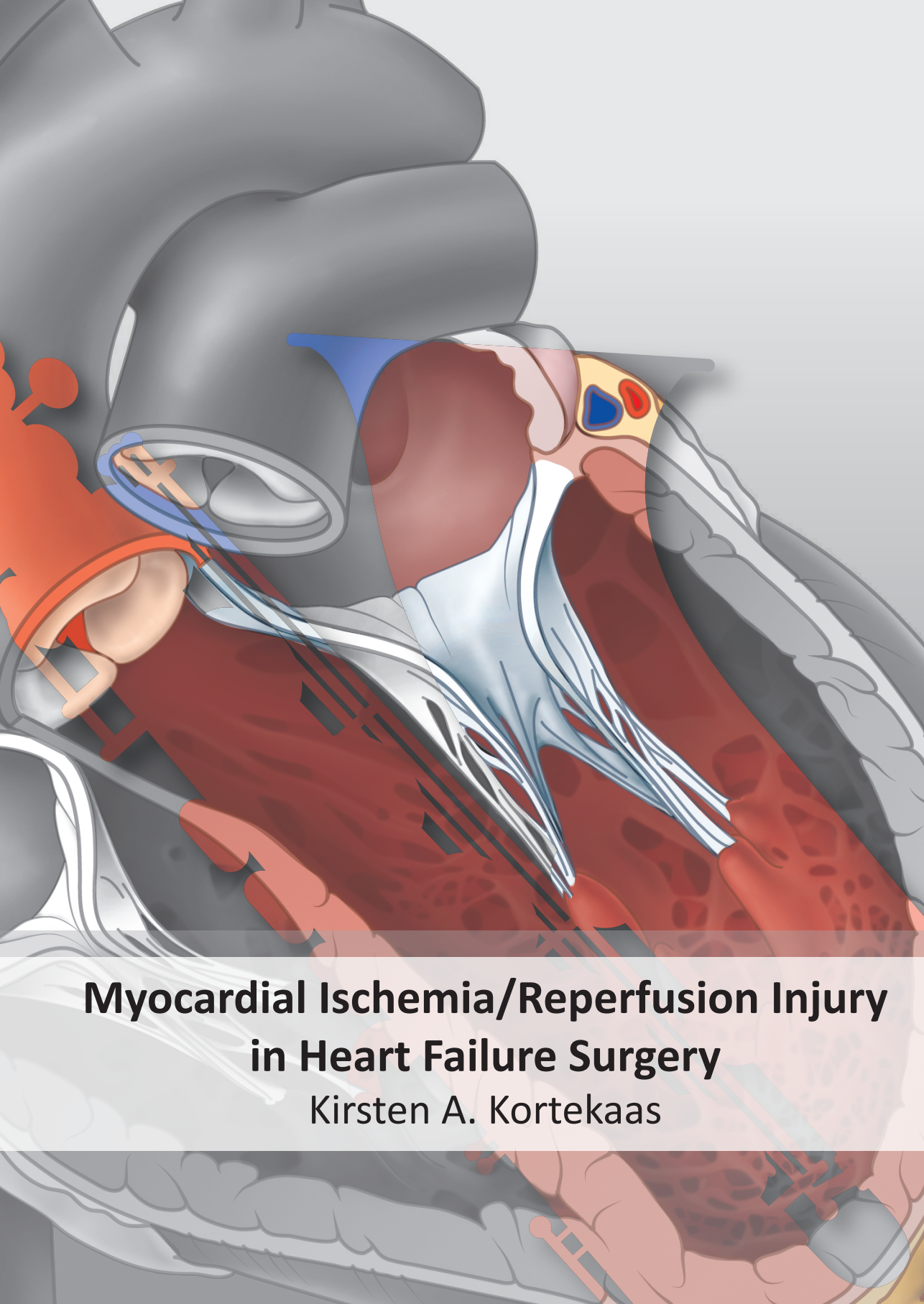


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Myocardial Ischemia/Reperfusion Injury in Heart Failure Surgery

Kirsten A. Kortekaas

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COLOFON

Myocardial Ischemia/Reperfusion Injury in Heart Failure Surgery
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Success is not the key to happiness.
Happiness is the key to success.
If you love what you are doing, you will be successful.

Albert Schweitzer (1875-1965)

Voor mijn ouders en Kim

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CHAPTER ONE

General Introduction

HEART FAILURE

Heart failure is described as the inability of the heart to pump sufficient oxygenated blood to meet the body's needs. Heart failure is one of the most common, costly, disabling, and potential lethal pathologies worldwide.[1, 2] Clinical manifestations of heart failure can be characterized by hemodynamic, autonomic, neurohumoral, and immunological abnormalities (Figure 1).[3, 4] Not all of these mechanisms activated in heart failure are deleterious. Various beneficial counter-regulatory mechanisms are activated to oppose sodium- and water retention, like the natriuretic peptide system. However, when the syndrome of heart failure progresses, the potent negative effects of the renin-angiotensin-aldosterone system and sympathetic nervous system outweigh these counter-regulatory mechanisms. As a result, patients experience symptoms of dyspnea – often orthopnea –, exercise intolerance with concomitant fatigue, and edema.

Aging of the population and improved survival of patients with acute myocardial infarction has led to an increased incidence and prevalence of heart failure. Of note, there is concern heart failure will become an important burden on the health care system the next decades. The magnitude of the problem can not be assessed precisely since consistent population-based estimates of its epidemiology and prognosis are lacking.[5, 6] According to data of the Dutch Heart Foundation, 180,000 patients out of a population of 16,5 million are diagnosed with heart failure with an incidence rate of 35,000 patients per year.

Drug therapies can be regarded as the cornerstone of treatment in patients with heart failure. In some patients this can be combined with resynchronisation and/or implantable cardioverter defibrillator (ICD) therapy. However, this approach is eventually insufficient for many patients, resulting in an important need for alternatives. Nowadays the annual mortality rate for patients with heart failure is approximately 10%, depending on the stage of the disease.[2] Due to the limited availability of donor organs, heart transplantation is an option for very few patients. Therefore, one of the aims of alternative surgical therapies is to improve the systolic pump function. Examples are: 1) revascularisation for the relief of objectively proven ischemia; 2) restrictive mitral valve annuloplasty for the treatment of functional mitral regurgitation; 3) surgical ventricular reconstruction after a large myocardial infarction; 4) external constraint devices for dilated cardiomyopathy; and 5) left ventricular assist devices as mechanical cardiac support.

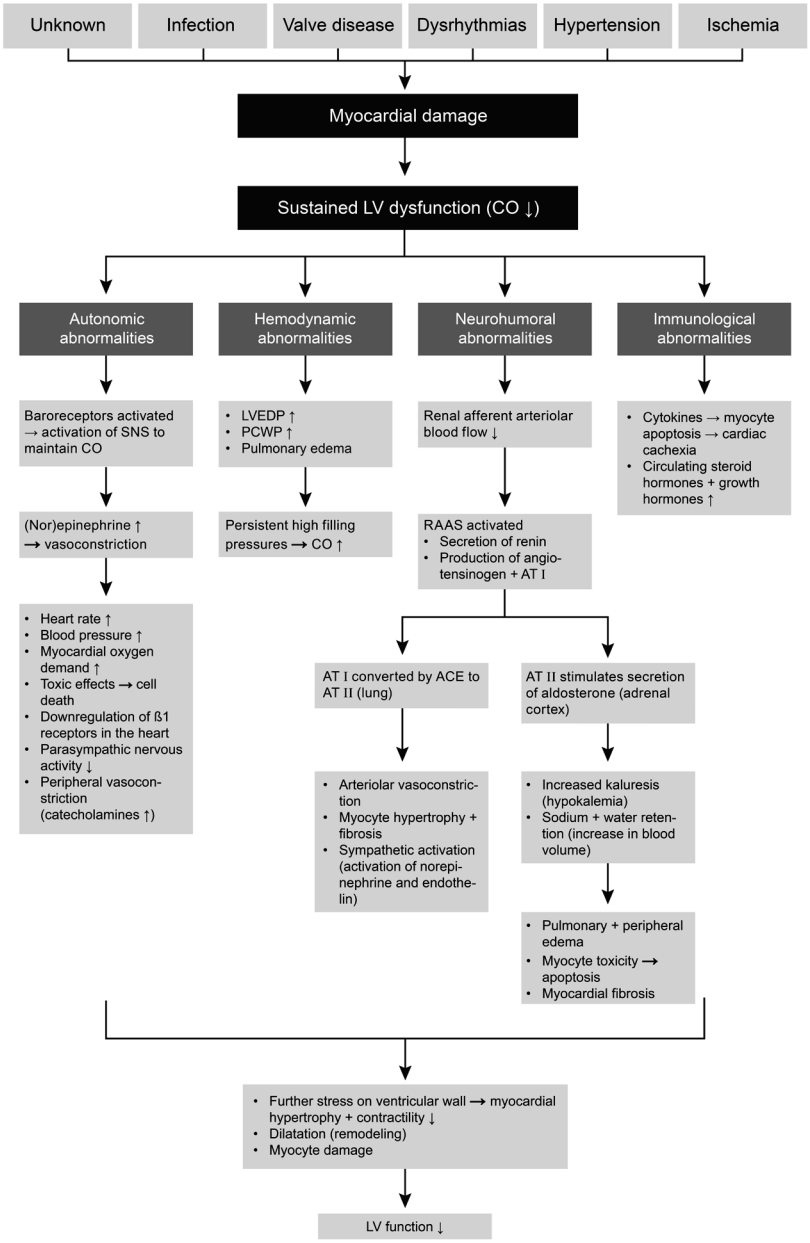


Figure 1. Overview of the pathophysiology of heart failure.
The syndrome of heart failure is characterized by hemodynamic, autonomic, neurohumoral, and immunological abnormalities.
Abbreviations: ACE, Angiotensin Converting Enzyme; AT I/II, Angiotensin I/II; CO, Cardiac Output; LV, Left Ventricular; LVEDP, Left Ventricular End-Diastolic Pressure; PCWP, Pulmonary Capillary Wedge Pressure; RAAS, Renin-Angiotensin-Aldosterone System; SNS, Sympathetic Nervous System.

Although the benefits of several surgical interventions have been established for patients with heart failure[7-9], perioperative morbidity and mortality remain significant and limit the widespread use of surgical interventions. Frequent observed complications in patients with heart failure undergoing cardiac surgery are ventilator-assisted pneumonias, kidney damage, therapy-resistant vasoplegia, stroke, bleeding, and infections. These complications in patients with preexisting heart failure seem to occur at a much higher frequency than after complex cardiac (valve) surgery in patients without heart failure. Furthermore, these complications appear to be largely independent from the high preoperative risk or the impaired cardiac function. Hence, these observations suggest that patients with preexisting heart failure have a different response to the surgical injury in general, and may reflect a different response of the failing heart to cardioplegic arrest and reperfusion. The purpose of this thesis was to study this response in more detail.

ISCHEMIA/REPERFUSION INJURY

Well-timed re-establishment of blood flow is essential to rescue ischemic tissues, preventing cell necrosis, and infarction.[10-12] However, reperfusion of ischemic myocardium is inevitably associated with ischemia/reperfusion (I/R) injury. This is the paradoxical increase of tissue damage upon re-oxygenation of previously hypoxic tissue. I/R is considered the major cause of tissue damage encountered during a variety of standard medical and surgical procedures, like revascularization therapy, organ transplantation, and cardiac surgery with use of cardiopulmonary bypass.

The pathophysiology of I/R injury is complex and not fully elucidated yet. Free radicals, thrombocytes, neutrophils, inflammatory mediators, complement components, and endothelial cell activation markers have frequently been suggested to play a role in the initiation of the complex cascade following I/R (Figure 2).[10-12] I/R injury can lead in its severest form to multiorgan dysfunction or even death. However, understanding of the pathophysiological mechanisms is mainly based on experimentally derived concepts obtained from pre-clinical, mainly murine, I/R experiments (Figure 3). Clinical studies concerning these mechanisms are sparingly performed, and interventions aimed at attenuating human I/R-induced damage fail to show a substantial benefit yet. Identification of the underlying mechanisms is difficult as a result of the large variation between different organ systems in their sensitivity and response to clinical I/R.

Myocardial I/R injury is an inevitable consequence of cardiac surgery with use of cardiopulmonary bypass. A period of ischemia (i.e. aortic cross-clamping with cardioplegic arrest) is followed by restoration of the blood flow during reperfusion (i.e. unclamping of the aorta). The clinical manifestations of myocardial I/R injury are diverse and may

include myocyte death and necrosis, myocardial hibernation or stunning, endothelial- and microvascular dysfunction including the no-reflow phenomenon, reperfusion arrhythmias, low cardiac output state, breakdown of the gastrointestinal barrier, and impaired cerebral function.[13, 14]

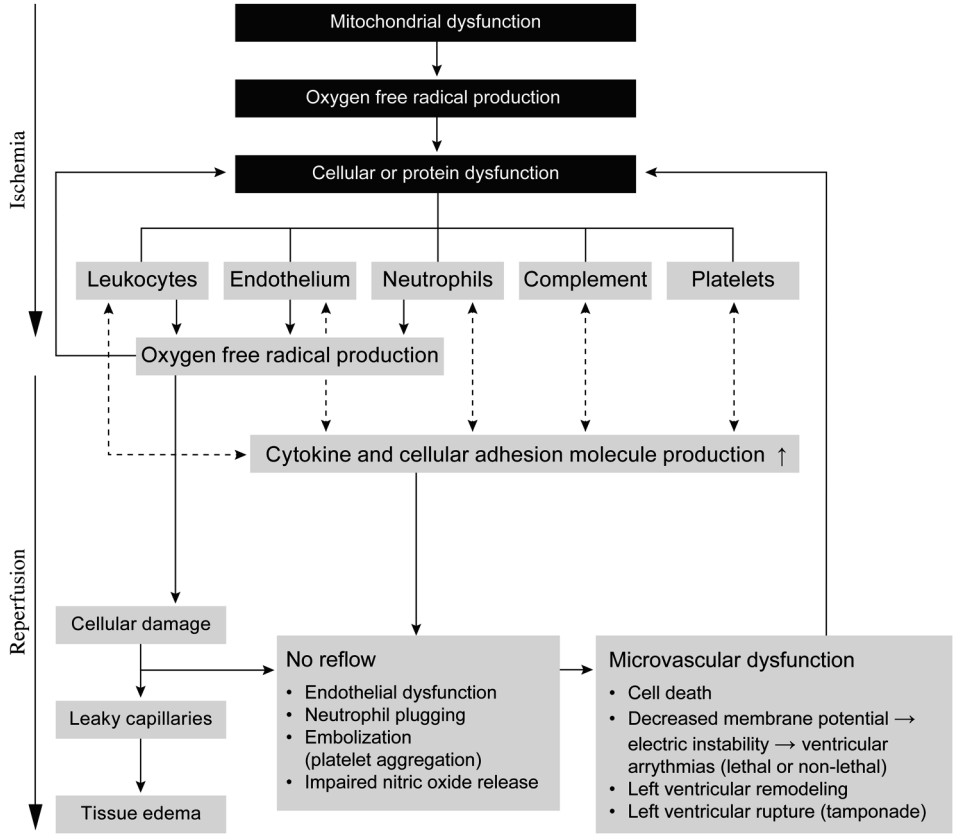


Figure 2. Hypothesis of the complex cascade following human myocardial ischemia and reperfusion. Free radicals, thrombocytes, neutrophils, inflammatory mediators, complement components, and endothelial cell activation have frequently been suggested to play a role in the initiation of the complex cascade following myocardial ischemia/reperfusion.

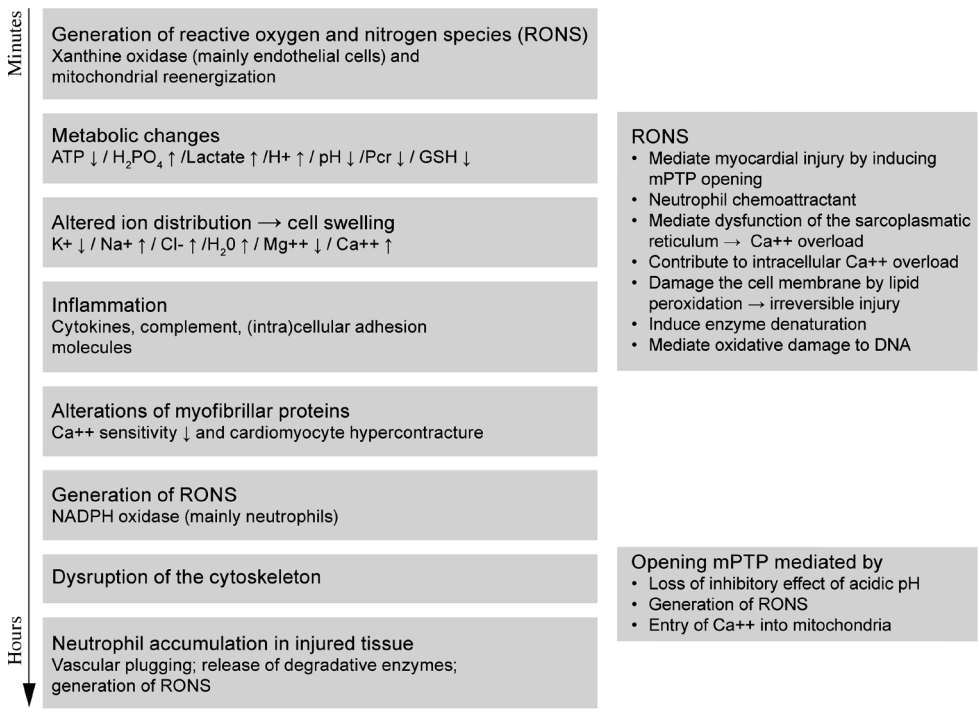


Figure 3. Sequence of biochemical and metabolic events upon myocardial reperfusion.
Abbreviations: ATP, adenosine triphosphate; DNA, deoxyribonucleic acid; GSH, Glutathione; mPTP, mitochondrial Permeability Transition Pore; NADPH, Nicotinamide Adenine Dinucleotide Phosphate; Pcr, Phosphocreatine; RONS, Reactive Oxygen and Nitrogen Species.

EFFECTS OF I/R INJURY ON REMOTE, NON-ISCHEMIC ORGANS

Taken together, the consequences of I/R injury may thus extend beyond the local effects. Reperfusion of ischemic tissue can lead to both a local and a systemic response that may be followed by a widespread altered tissue barrier function. Ultimately, it may even result in a systemic inflammatory response syndrome (SIRS) and a multiple organ dysfunction syndrome (MODS).[10, 15] These two systemic remote effects of reperfusion can be devastating and fatal.

SIRS is a generalized, nonspecific inflammatory response to injury in the absence of infection.[16] Two or more of the following conditions must be present to diagnose SIRS: a temperature > 38°C, a heart rate > 90 beats per minute, a respiratory rate > 20 breaths per minute or a carbon dioxide tension < 32 mmHg, and a white blood cell count > 12.000/mm^3 or < 4.000/mm^3 or immature forms accounting for > 10% of neutrophils. Surgical procedures

with the use of cardiopulmonary bypass already induce these hemodynamic, respiratory, and hematologic changes necessary for inclusion in the SIRS definition. Therefore, the definition of SIRS is complex in relation to cardiac surgery. The severity of this syndrome is variable in patients with heart failure undergoing cardiac surgery. It does manifest with severe hemodynamic disturbance in only a minority of the patients. Moreover, SIRS is not necessarily deleterious, it can be a controlled self-limiting inflammatory response. The postulated beneficial effects of SIRS are immune system priming, prevention of infection, and improved wound healing.[16]

MODS is a frequent complication of SIRS and is characterized by the development of progressive and potentially reversible physiologic dysfunction in two or more organs or organ systems.[17] Acute respiratory distress syndrome (ARDS), due to increased permeability in the lung vasculature, is often the first clinical sign of MODS.[10, 18] The start of this respiratory dysfunction is frequently characterized by an abnormal gas exchange, increased pulmonary vascular resistance, and increased alveolar-arterial oxygen pressure gradient.[19] This dysfunction, often called acute lung injury, commonly occurs within 24-72 hours after the initiating ischemic event.[10, 15] In daily clinical practice, this dysfunction leads to increased oxygen demands, greater dependence on artificial ventilation and prolonged stay in the intensive care unit. Pulmonary infiltrates are often seen at the chest X-ray with a delay of 24 hours.

In addition, MODS after myocardial I/R can involve hepatic, renal, gastrointestinal, and central nervous system dysfunction.[10] The mortality is directly correlated with the number of failed organ systems: 30-40% if one organ system fails, 50-60% if two organ systems fail and 80-100% when three or more organ systems fail.[13] Respiratory insufficiency and renal failure are the most common complications after cardiac surgery. The underlying mechanism is damage to vascular beds, causing fluid extravasation and tissue edema. Moreover, MODS is also characterized by dysfunction of the immune and coagulation systems.[10]

Neutrophils, complement components, adhesion molecules, reactive oxygen species, and cytokines have been implicated as key mediators of remote organ damage.[10, 18, 20] In specific, elevated levels of inflammatory markers, like tumor necrosis factor (TNF)-alfa, interleukin (IL)-1 beta, IL-6, and IL-8, are associated with increased mortality and increased risk for SIRS and MODS.[21, 22] Given the local and systemic effects of clinical I/R injury, understanding of the pathophysiological mechanisms is essential to develop effective methods of prevention and treatment.

PATHOPHYSIOLOGY OF MYOCARDIAL I/R INJURY

Reactive oxygen and nitrogen species

Reactive oxygen and nitrogen species (RONS) are considered key initiators of I/R injury. Previous studies have shown that the substrates of xanthine oxidase, xanthine and hypoxanthine, accumulate during ischemia and are responsible for the formation of free radicals in reperfused isolated rat hearts within minutes after reperfusion.[23, 24] In other words, ischemia-related metabolic adaptations and dysregulated mitochondrial homeostasis are thought to result in release of RONS upon reintroduction of oxygen. Zorov et al. showed that RONS can activate the opening of the mitochondrial permeability transition pore, resulting in a vicious circle of additional free radical release from the mitochondria of cardiac myocytes.[25] This RONS overload can overwhelm the endogenous antioxidant system, resulting in oxidative damage. This may trigger processes such as inflammatory responses.[26-29] As a result, it was hypothesized that antioxidant therapy attenuates reperfusion injury. The validity of this concept has been proven in various pre-clinical studies, which all demonstrate that antioxidant therapy ameliorates I/R injury.[12, 30, 31] Despite these findings, studies in humans consistently fail to show any clinical relevant effect.[12, 32-35] The basis for this discrepancy between human and animal studies is still unclear. An explanation could be that the contribution of RONS to I/R injury in humans is less than commonly thought.

Complement components

Complement activation is considered one of the key mediators of myocardial I/R induced tissue damage.[36-39] During cardiac surgery, the complement cascade is activated as a result of the surgical trauma, use of cardiopulmonary bypass, and I/R injury. There are three pathways of activation of the complement system (classical, alternative, and mannose binding lectin), whereby C5a and C5b-9 are believed to be primarily responsible for I/R injury.[40] C5a can induce chemotaxis of leukocytes and the amplification of the inflammatory response by initiating the production of TNF-α, IL-1, IL-6, and monocyte chemoattractant protein-1 (MCP-1).[13, 41] C5b-9 can activate endothelial nuclear factor-kappa B to increase leukocyte adhesion molecule transcription and expression, and can induce chemotactic mediators like IL-8 and MCP-1.[13, 41] In addition, complement components can induce cell injury by increasing the permeability of the cell. Moreover, complement components can release histamine and platelet activating factor, and can also stimulate neutrophil adherence, and superoxide production. Complement inhibitors are highly effective in reducing tissue damage in animal studies of myocardial I/R injury.[37, 42, 43] However, anti-

complement therapy in human myocardial I/R injury has led to differential results without clinical relevant benefits so far.[44-46] Whether the pathophysiology of human myocardial I/R injury is mainly based on complement activation can therefore be questioned. The limited human studies concerning complement activation in myocardial I/R injury have shown that there is an increased systemic activation of complement components the first hours after reperfusion.[38, 47] Those systemic levels show a biphasic pattern with inhibitory mechanisms from eight hours postoperatively onwards.[39] If the complement activation after cardiac surgery is well balanced and controlled, it contributes to defense against infection and wound healing. Nevertheless, extreme activation of the complement system can contribute to organ dysfunction resulting in increased morbidity and mortality postoperatively.[48]

Thrombocyte activation

Pre-clinical evidence points towards the possibility that platelets are involved in the pathogenesis of I/R injury.[49-52] Platelets are specialized cells of the innate immune system and modulators of the inflammatory response in addition to their evident role in hemostasis. Upon their activation, platelets secrete various mediators into the circulation by exocytosis, like leukotrienes, P-selectin, beta-thromboglobulin, platelet-derived growth factor, and Regulated upon Activation, Normal T-cell Expressed and Secreted (RANTES), thereby increasing inflammation and damage to cardiac myocytes.[53, 54] In addition, thromboxane A2 and serotonine[55], released by activated thrombocytes, may exacerbate microcirculatory spasms, leading to a decreased coronary flow. Moreover, the externalization of P-selectin mediates adhesion of neutrophils, which release free radicals and proteins that also injure the cardiac myocytes. In contrast to existing pre-clinical data, the involvement of platelets as key regulators of clinical I/R in general, and more specifically in myocardial I/R injury, is unclear.[56, 57]

Endothelial cell activation

Endothelial cells are activated during exposure to hypoxia and inflammatory mediators, leading to the release of vasoactive substances upon I/R, like endothelin and nitric oxide.[58] This causes either vasodilatation or vasoconstriction of underlying smooth muscle cells.[59] In high concentrations, nitric oxide promotes the formation of RONS. However, endothelial cells are not only a source of reactive species, but also a target for reactive species as shown by Arroyo et al. with spin trapping methods.[60] Experimental data show that increased permeability of the endothelium occurs due to reoxygenation upon reperfusion.[61] Endothelial cell activation and injury increase vascular permeability and activate the coagulation and immune systems through expression of chemokines, cytokines, and

adhesion molecules. Those adhesion molecules promote tissue invasion by inflammatory cells, mainly neutrophils, T-lymphocytes, and macrophages. Carden et al. and Panes et al. have shown that those inflammatory cells are harmful to the myocardium because they generate RONS, secrete proteases, and occlude the microvasculature.[10, 62] Yet, the role of the various endothelial cell activation markers and adhesion molecules in the clinical setting of I/R injury is unknown. An increased understanding of this role may allow therapies to be designed to further attenuate the post-reperfusion response.

Chemokines and cytokines

In response to tissue injury, an immune response is initiated that arranges homeostatic responses and is a prerequisite for subsequent healing of the lesion. However, an exaggerated inflammatory response contributes to maladaptive tissue damage. For myocardial I/R in specific, animal studies have shown that a complex cascade of events ultimately results in a myocardial inflammatory response upon reperfusion.[63-65] Whether this can be extrapolated to the more complex situation in humans remains in question. Overall, there is a correlation between elevated levels of pro-inflammatory cytokines and adverse events after cardiac surgery as outlined below.[66] The pro-inflammatory mediators TNF-α and IL-1β are elevated early after cardiac surgery, with IL-6 and IL-8 peaking thereafter.[66, 67] TNF-α is produced mainly by activated monocytes and plays a very important, and initiating role in inflammation. TNF-α can cause hypotension, coagulopathy, and renal dysfunction. Moreover, it plays a major role in increased microvascular permeability. Elevated levels of TNF-α are also associated with SIRS and MODS.[68] IL-1β is mainly produced by macrophages and monocytes, and is primarily responsible for the release of pro-inflammatory cytokines and the synthesis of endothelial adhesion molecules. The most significant effects of IL-6, produced by macrophages, are the coordination of the acute phase response and activation of lymphocytes. High IL-6 levels after elective cardiac surgery correlate with hepatic and renal dysfunction.[69] IL-8 is mainly produced by macrophages and plays a major role in polymorphonuclear trafficking (chemotaxis). High IL-8 levels after elective cardiac surgery correlate with respiratory dysfunction and duration of inotropic support.[69] IL-10, one of the anti-inflammatory cytokines, is produced mainly by macrophages and is a strong inhibitor of TNF-α, IL-1β, IL-6, and IL-8. IL-1 receptor antagonist is a specific antagonist of IL-1β, thereby limiting the inflammatory response. Overall, the clinical prognosis after cardiac surgery is depending on the balance between pro- and anti-inflammatory mediators.[70]

Inflammatory cells

Zuidema et al. and Linfert et al. outline in their reviews that inflammatory cells are involved in I/R injury.[71, 72] Upon tissue damage, the activation and recruitment of neutrophils are promoted by several cytokines, like IL-1, IL-6, IL-8, and TNF-α. In animals, accumulation and activation of neutrophils in the post-ischemic area occurred within the first hours after the initiation of myocardial reperfusion.[73, 74] Neutrophils can release free radicals, proteases, and pro-inflammatory markers. This directly amplifies the influx of other neutrophils in myocardial tissue, thereby contributing to damage.[73, 75] However, it is uncertain whether neutrophil accumulation is a cause or a consequence of I/R injury.[76, 77] Secondly, monocytes migrate from the circulation into tissue where they mature to macrophages and take up residence. Macrophages secrete a variety of pro-inflammatory cytokines, such as IL-1β, TNF-α, IL-6, IL-8, and IL-12. These inflammatory mediators also recruit neutrophils and other leukocytes, amplifying the inflammatory response. Moreover, recent data suggest that lymphocytes have a significant role in the response to I/R injury.[72] Animal models showed that T-lymphocytes are harmful during myocardial I/R injury.[78, 79] In general, the role of the various inflammatory mediators in clinical I/R injury is complex and not fully unraveled yet. They frequently play a detrimental role in I/R injury, although some also appear to have cardioprotective effects.

TREATMENT STRATEGIES FOR I/R INJURY

Although the precise mechanisms of I/R injury are still incompletely understood, numerous treatment strategies have been investigated targeting some of the postulated mechanisms of I/R injury. Most of them were promising in animal trials, but did not show a substantial clinical benefit in humans or still have to reach human clinical trials as outlined in Table 1.[12, 14, 80] Potential mechanisms contributing to the discrepancy between promising data of pre-clinical studies and disappointing results in clinical trials are outlined in Table 2.[12, 81, 82] Three often mentioned treatment options for human myocardial I/R injury will be outlined briefly: antioxidant therapy, anti-complement therapy, and preconditioning.

Experimental data clearly showed that antioxidant therapy effectively ameliorates I/R injury.[31, 83] Clinical trials on the other hand did not show any substantial, clinically relevant effect of antioxidant administration.[12, 33, 34, 83-85] At present, there is no clinical indication established recommending the routine use of antioxidants in I/R.[86] However, a large amount of data support the role of oxidative stress in myocardial I/R injury and stress the value of antioxidant defense mechanisms in tissue protection.

Table 1. Treatment strategies for human myocardial I/R injury.

Treatment	Positive clinical trials
Ischemic preconditioning[97-100]	Yes / No: reduced myocardial injury; beneficial effects on mortality await larger trials
Aprotinin[101-103]	Yes / No: modest reduction in risk of massive bleeding; higher mortality and adverse events like renal impairment, myocardial infarction, stroke and anaphylactic reactions[104, 105]
mPTP inhibitors (e.g. Cyclosporine)[106]	Yes / No: smaller infarct size after acute myocardial infarction; larger trials necessary and more specific inhibitors need to be developed
Activation of protein kinase C (e.g. KAI-9803)[107]	Yes / No: trends for smaller infarct size after acute myocardial infarction; larger trials necessary
Activation of the RISK pathway ▪ **Erythropoetin**[108-110] ▪ **Statins**[111, 112]	Yes / No: Improved myocardial function and reduced infarct size versus no reduced myocardial injury; larger trials necessary
Volatile anesthetics[113-116]	Yes / No: contradictory results concerning biochemical markers for myocardial dysfunction; larger trials necessary
Na-H-exchange-inhibition (e.g. Cariporide)[12, 117-120]	Yes / No: contradictory results concerning mortality and no beneficial effects concerning myocardial infarction in patients with unstable angina; increase in cerebrovascular events, only beneficial if administrated prior to the onset of ischemia
Adenosine (e.g. Acadesine)[121-128]	Yes / No: no difference in death or occurrence of congestive heart failure with anterior myocardial infarction; smaller infarct size; beneficial effects seen if administrated prior to the onset of ischemia
Anti-complement (e.g. soluble complement receptor type 1/C1-inhibitors)[12, 44-46, 89, 129, 130]	Yes / No: contradictory results concerning reduced myocardial I/R injury and improved cardiac function
Antioxidants (e.g. Super oxide dismutase, vitamin E)[12, 83, 86]	No
Anti-apoptosis[131]	No
Neutrophil depletion[77, 132]	No
Poly ADP-ribose polymerase (PARP)-inhibition[133]	No
Metabolic additives in cardioplegia solution (e.g. Pyruvate[134]**, amino acids, glutamate and aspartate)**[135, 136]	No
Steroids[137-139]	No
Calcium channel blockers (e.g. Verapamil)[140-143]	No

Abbreviations: mPTP, mitochondrial permeability transition pore; RISK, reperfusion injury signaling kinase.

Table 2. Major differences between animal models and clinical studies of myocardial ischemia/ reperfusion injury.

Type	Animal models	Clinical studies
Subjects	Homogenous, healthy, young, small animals, often males, no coexisting diseases	Heterogeneous, middle-aged and elderly, often collaterals formed, coexisting diseases like diabetes mellitus
Concomitant medication	No	Often multiple drugs
Period of ischemia	Same brief duration (30-60 minutes) and same severity	Variable longer duration (3-12 hours) and variable severity
Reperfusion time	Short (2 hours – 3 days)	Longer: infarct healing and left ventricular remodeling
Preconditioning	Heart not exposed to ischemia prior to cardiac surgery	Often repeated ischemic insults (angina, infarction) prior to cardiac surgery
Nutritional state	Non-fasting	Fasting
Timing of intervention	Similar in all animals	Variable between patients
Therapeutics	Low dose	High dose, timing essential
Expression of size of myocardial damage	Large volume of whole myocardium at risk	Small volume of left ventricular volume
Endpoints for cardioprotection	Often left ventricular function or infarct size	Short- and long-term effects on morbidity and mortality

Furthermore, anti-complement therapy showed inconsistent results in human trials. Although the recombinant soluble form of complement receptor 1 showed reduced local and remote organ injury in animals after I/R, clinical data were disappointing.[87, 88] Administration of pexelizumab, an anti-C5 complement antibody, fails to inhibit the assembly of C5b-9 in patients with ST-elevation myocardial infarction undergoing primary percutaneous coronary intervention.[89] In contrast, the same antibody showed to attenuate the activation of the complement system, activation of leukocytes, myocardial injury, and early postoperative mortality in humans undergoing coronary artery bypass grafting.[90, 91] Moreover, the administration of a C1-esterase inhibitor in patients undergoing coronary bypass surgery showed reduced myocardial I/R injury and improved cardiac function.[44, 45]

The most widely studied form of possible protection against I/R-induced myocardial injury is preconditioning. A special form of this preconditioning is ischemic preconditioning. Brief periods of ischemia followed by reperfusion induce cardioprotection, although the precise mechanisms remain elusive.[92] Ischemic preconditioned tissues have reduced energy requirements, altered energy metabolism, decreased intracellular calcium overload, improved endothelial function, better electrolyte homeostasis, less RONS and neutrophils, less apoptotic cardiomyocyte death, and diminished systemic dysfunction.[93] In addition, this form of preconditioning also protects against lethal reperfusion injury by activating the reperfusion injury signaling kinase pathway and by inhibiting the opening of the mitochondrial

permeability transition pore.[94, 95] Therefore, ischemic preconditioned tissue is better able to tolerate I/R. Nonetheless, clinical data about ischemic preconditioning are limited. Whether preconditioning has beneficial or harmful clinical effects still has to be elucidated.[96] Other studied forms of 'conditioning' are therapeutic pre- and postconditioning. Several targets have been postulated for these kinds of conditioning. Of these targets, non have made it from the laboratory to routine use in daily clinical practice.

In conclusion, no established targeted therapy is currently available to safely and significantly reduce the effects of clinical I/R injury despite amelioration of I/R injury in various pre-clinical studies. Timely reperfusion of ischemic tissue remains the cornerstone in daily clinical practice. The basis for the discrepancy between human and animal studies is still unclear, yet it may suggest that pathophysiological mechanisms are different or more complex in humans. Therefore, a clinical model of planned myocardial I/R injury in the setting of cardiac surgery was used in this thesis to study the pathophysiology in more detail. Myocardial release of various mediators associated with I/R injury were assessed during planned I/R by cannulation of the efferent vein, i.e. the coronary sinus. With this approach, sensitive and organ specific measurements of net changes in various mediators over the reperfused heart were conducted.

OUTLINE OF THIS THESIS

Chapter 2 of this thesis provides background information regarding systemic complications after cardiac surgery, such as vasoplegia, sepsis, prolonged ventilation, and organ dysfunction. The impact of preexisting heart failure on complications after correction for pump failure was evaluated by differentiating between complications categorized as not, possibly or definitely related to pump failure.

In **Part I** six studies are described reflecting the pathophysiology of I/R injury. **Chapter 3** challenges the prevailing paradigm on RONS-mediated I/R injury in both patients undergoing kidney transplantation and heart valve surgery. In **Chapter 4** the role of complement activation in the early myocardial reperfusion phase following cardiac surgery was evaluated. C5b-9 and C5a were assessed in patients with non-ischemic and ischemic heart failure, and patients without heart failure. Myocardial and endomyocardial depositions of C3d and C5b-9 were also studied and compared with pre-transplantation tissue from myocardial allografts. The role of platelet activation (**Chapter 5**) and endothelial cell activation (**Chapter 6**) upon myocardial reperfusion are described in two observational studies. **Chapter 7** reports about the myocardial inflammatory response after cardiac surgery in patients with non-ischemic and ischemic heart failure, compared with patients without heart failure. Specific attention is paid to the presence of inflammatory cells in myocardial tissue of all three patient groups. In

Chapter 8 the role of galectin-3 is described in relation to left ventricular reverse remodeling after mitral valve repair in patients with non-ischemic and ischemic heart failure.

To investigate the translation of pathophysiological mechanisms from bench to bedside, two studies were performed in the second part of this thesis (**Part II**). Effects of influencing the response to I/R injury were analyzed in more detail. **Chapter 9** describes the effects of intramyocardial delivery of sevoflurane on myocardial damage and inflammatory responses. In **Chapter 10** inhibition of interleukin-9 in a murine renal I/R injury model is reported.

Finally, the main findings of the different studies presented in this thesis are discussed in a general discussion section, including aspects for future studies towards unraveling the pathophysiology of clinical I/R injury and treatment of the (remote) effects.

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CHAPTER TWO

Neth Heart J 2012;20:202-207

Preexisting heart failure is an underestimated risk factor in cardiac surgery

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Theo **STIJNEN**, Robert AE **DION**, Robert JM **KLAUTZ**

ABSTRACT

Background: Heart failure is characterized as a strong risk factor for systemic failure after cardiac surgery. However, the impact has never been substantiated.

Methods: Patients with heart failure (n = 48) – scheduled for elective ventricular reconstruction or external constraint device – were compared with a one-to-one matched control group of patients without heart failure undergoing cardiac surgery between 2006 and 2009.

Results: As expected, patients with heart failure more frequently experienced complications definitely related to pump failure ($P = 0.01$). However, complications not related to their pump failure were also more often observed, such as prolonged mechanical ventilation, sepsis and vasoplegia ($P = 0.01$). Overall, organ dysfunction – circulatory, renal, and pulmonary failure – was often observed in heart failure patients, contributing to a prolonged stay in the intensive care unit ($P < 0.001$) as well as in hospital ($P = 0.01$).

Conclusions: The adverse postoperative course in patients with heart failure is not only directly related to circulatory failure, but merely reflects a systemic dysregulation. Our findings suggest that heart failure impacts outcome and should therefore be included in prevailing risk classification systems. Offensive perioperative treatment strategies, focused on the main complications in patients with heart failure, will lead to improved results after cardiac surgery.

INTRODUCTION

Heart failure is one of the most common, costly, disabling, and potentially deadly pathologies worldwide.[1, 2] Aging of the population and improved treatments of the causes of heart failure will lead to an increased incidence and prevalence of heart failure. Due to the limited availability of donor organs, heart transplantation is only an option for very few patients. Alternative surgical therapies are important and aimed at improving the myocardial function.

Although the efficacy of several surgical interventions has been established for patients with heart failure[3-5], perioperative morbidity and mortality remain significant and hamper widespread use. The observed complications can not only be explained by the preoperative high risk state of the patient or the low cardiac output state alone. They are rather explained by systemic responses such as vasoplegia and a systemic inflammatory response syndrome.

Our hypothesis is that postoperative complications in patients with preexisting heart failure are often not related to pump failure. In other words, the preoperative risk stratification, which only includes ejection fraction as a measure of pump failure, is inadequate to predict an adverse postoperative course. To test this hypothesis, we compared patients with and without heart failure undergoing cardiac surgery in order to have comparable surgical injury. Our aim was to systematically examine the impact of preexisting heart failure on complications after correction for pump failure by differentiating between complications not, possibly or definitely related to pump failure.

METHODS

Patient characteristics

Between January 2006 and December 2009, all patients with preexisting heart failure scheduled for an elective external cardiac support device or ventricular reconstruction were included in this retrospective study (n = 48). Heart failure was defined as an inadequate pump function of the heart with an echocardiographically estimated ejection fraction biplane <30% and New York Heart Association (NYHA) class III or IV. Exclusion criteria were minimal access procedures, previous sternotomy, emergency procedures or heart failure hospitalization prior to admission to our department. Hospitalization is often an indication for an aggravated acute state of heart failure, affecting outcomes. All patients were scheduled for a restrictive mitral annuloplasty in addition to their external constraint device or ventricular reconstruction. To examine the impact of heart failure on adverse outcomes, all 48 heart failure patients were matched on a one-to-one basis with patients without heart failure undergoing cardiac surgery (controls). All 48 controls had a normal pump function

(ejection fraction biplane ≥ 50%) and presented in NYHA class I or II. Each patient with heart failure was first matched for gender and year of surgery, and then for the most closely resembling age. Controls were scheduled for at least reconstructive mitral valve surgery to limit heterogeneity and to have a comparable duration of surgery between the groups. Moreover, mitral valve surgery is a common procedure in both patients with and without heart failure.[6-8] Our study protocol was approved by the local ethics committee, who waived the need for individual informed consent due to the retrospective and anonymous nature of the study.

Performed surgical procedures

Surgical procedures were performed according to the local standard. An external cardiac support device (CorCap cardiac support device, Acorn Cardiovascular, St. Paul, MN, USA) was placed if a left ventricular end-diastolic diameter above 65 mm was measured on the preoperative echocardiographic examination. Patients were considered eligible for ventricular reconstruction as described by Dor et al.[9] if they had a large area of anterior akinesia or dyskinesia.[10] Tricuspid valve annuloplasty, coronary artery bypass grafting and ablation procedures were performed if indicated.

Endpoints and data collection

The complicated course of patients with heart failure is of high interest in current pathophysiological investigations. Complications, with often unravelled etiology, were categorized prior to the start of our study and were based on the opinion of experts in the field of heart failure surgery, i.e. cardiac surgeons and intensivists. Primary endpoints were a composite of complications not, possibly and definitely related to pump failure in an attempt to differentiate between pump failure and systemic failure. Secondary endpoints included the individual components of these primary endpoints in addition to other complications (Table 1). Patients were followed until discharge from hospital or death. Mortality was compared with a preoperative risk-adjusted model (logistic EuroSCORE).[11-13] Data collection was performed by examination of the patients' electronic or hard copy medical records of standard postoperative procedures, or by interviewing their general practitioner by telephone. Serum creatinine levels (Roche Diagnostics, Mannheim, Germany) were assessed within three months prior to surgery and blood pressure was measured the day before surgery. Excessive bleeding was defined as blood loss of more than two liters within 24 hours postoperatively.[14]

Table 1. Definitions of the primary and secondary endpoints.

Composite endpoints	
Not related to pump failure	Mechanical ventilation ≥ 48 hours, sepsis and vasoplegia (norepinephrine ≥ 0.2 µg/kg/min, 12-24 hours in the ICU); 2 or 3 present
Possibly related to pump failure	Early mortality and acute kidney injury; both present
Definitely related to pump failure	Inotropic support (dobutamine ≥ 5 µg/kg/min, 12-24 hours in the ICU) and use of mechanical assist devices (intra-aortic balloon pump or ventricular assist device); both present
Mortality	
Early mortality	All-cause mortality occurring within 30 days or in hospital[12]
Late mortality	All-cause mortality occurring from 30 days to 1 year[12]
Organ dysfunction	
Circulatory failure	Use of an intra-aortic balloon pump or use of a ventricular assist device
Neurological events	Transient ischemic attack (fully reversible symptoms of short duration), stroke (neurological deficit persisting ≥ 72 hours) confirmed by clinical findings and computed tomography scan, and documented critical illness neuropathy
Renal failure	Acute kidney injury (50% increase in creatinine values ≤ 48 hours postoperatively[17] and/or a postoperative creatinine value ≥ 200 µmol/L) and use of continuous venovenous hemofiltration
Pulmonary complications	Mechanical ventilation ≥ 48 hours, re-intubation, tracheostoma, ventilator-associated pneumonia or pneumothorax
Sepsis	Clinical suspicion confirmed by a positive blood culture (gold standard)

Abbreviation: ICU, Intensive Care Unit.

Statistical analysis

The occurrences of the primary and secondary endpoints were compared between the two patient groups. All statistical analyses were performed with the Statistical Package for the Social Sciences 17.0 (SPSS Inc., Chicago, IL, US). Continuous variables were expressed as mean ± standard deviation (SD) and categorical data as number of patients. Continuous variables were analyzed with the unpaired *t*-test or the Mann-Whitney *U*-test where appropriate, and categorical variables with a chi-square test.

RESULTS

Patient characteristics

Baseline patient variables and an overview of surgical procedures are shown in Table 2 and the Supplementary data, Table 1, respectively. Equal age and gender distribution reflect the matching process. As expected, patients with heart failure had a higher logistic EuroSCORE and NYHA functional class. Heart failure patients had lower blood pressure values and higher creatinine values at baseline.

Table 2. Baseline variables of patients with heart failure and controls.

	HF (n = 48)	Controls (n = 48)	*P* value
Clinical characteristics			
Age (years)	62.6 ± 9.3	62.5 ± 8.2	0.96
Gender (male / female)	39 / 9	39 / 9	1.00
Diabetes mellitus (no. of patients)			
Insulin dependent	7 (14.6%)	2 (4.2%)	0.16
Non-insulin dependent	6 (12.5%)	4 (8.3%)	0.74
NYHA functional class	3.1 ± 0.3	1.7 ± 0.5	< 0.001
MI < 3 months prior to surgery (no. of patients)	1	0	1.00
Significant CAD (no. of patients)	28	19	0.10
Logistic EuroSCORE (%)	10.4 ± 6.3	3.3 ± 2.1	< 0.001
Systolic blood pressure (mmHg)	113.0 ± 17.7	133.6 ± 18.8	< 0.001
Serum creatinine (µmol/L)	114.4 ± 38.7	90.9 ± 18.4	< 0.001
Surgical characteristics			
Duration of surgery (min.)	357.5 ± 107.9	329.6 ± 82.5	0.27
Cardiopulmonary bypass time (min.)	213.1 ± 79.7	192.4 ± 55.5	0.14
Aortic cross-clamp time (min.)	134.3 ± 58.2	146.8 ± 51.0	0.16

Details noted as mean ± standard deviation (SD).
Abbreviations: CAD, Coronary Artery Disease; HF, Heart Failure; MI, Myocardial Infarction.

Primary endpoints

The three primary endpoints are listed in Table 3. The composite endpoint of complications not related to pump failure was observed more frequently in patients with heart failure (*P* = 0.01). In detail, this first endpoint was observed in 10/48 heart failure patients (20.8%) and 0/48 controls (0.0%). Secondly, the composite endpoint of complications possibly related to pump failure was observed in 6 of the 48 patients with heart failure (12.5%) and in 1 of the 48 controls (2.1%). This second endpoint was, although six times more often, not significantly different between the groups (*P* = 0.12). This result indicates that early mortality and acute kidney injury may be related to pump failure but may also be related to other factors influencing these outcomes. For example, acute kidney injury may also be drug-induced during the perioperative period. Thirdly, the composite endpoint of complications

definitely related to pump failure was, as expected, significantly different between the groups (P = 0.01). This last endpoint occurred in 8/48 heart failure patients (16.7%) and in 0/48 controls (0%).

Secondary endpoints

Secondary endpoints are shown in Table 3. Early mortality was more common in heart failure patients as compared with controls (8/48 patients (16.7%) versus 1/48 patients (2.1%), respectively). One heart failure patient died in the operating room due to circulatory failure after ventricular reconstruction. In both groups the primary causes of early mortality were septic and cardiogenic shock. Surprisingly, the early mortality in patients with heart failure exceeded the predicted mortality based on the logistic EuroSCORE (10.4%), while in the controls the early mortality was lower than the predicted mortality (3.3%). Late mortality was similar between the groups: 3/48 patients with heart failure (6.3%) and 1/48 controls (2.1%). Those three heart failure patients died as a result of terminal or acute heart failure. The cause of late mortality of the control patient is unknown.

Organ dysfunction was divided into circulatory failure, neurological events, renal failure and pulmonary complications. Circulatory failure was more often observed in patients with heart failure, as expected due to their pump failure. These patients more often needed an intra-aortic balloon pump as a temporary cardiac assist device. The occurrence of neurological events was similar between the groups. The incidence of acute kidney injury was higher, although not significant, in patients with heart failure as compared with controls. Use of renal replacement therapy was strongly associated with mortality: 5/8 heart failure patients who needed continuous venovenous hemofiltration died in hospital. Also the control patient who died early needed dialysis. Prolonged ventilatory support (≥ 48 hours) and ventilator-associated pneumonias were more often observed in patients with heart failure. The more complex postoperative course for patients with heart failure was also reflected by the use of high concentrations of norepinephrine to treat vasoplegia and dobutamine as inotropic support. Overall, organ dysfunction was frequently observed in heart failure patients, contributing to a prolonged stay in the intensive care unit (P < 0.001) as well as a longer total stay in hospital (P = 0.01; Figure 1).

The number of patients with excessive bleeding postoperatively was similar between the groups (P = 0.98); three heart failure patients versus two controls. Early and late re-explorations for excessive bleeding or tamponade were similar in both groups (one early and one late re-exploration in patients with heart failure versus two early and two late re-explorations in the controls, both P = 1.00). All patients with a late pericardial tamponade could be treated by pericardial puncture. Patients with heart failure underwent a reoperation five times more often, although this was not significant (n = 5 versus n = 0, respectively, P = 0.07).

Table 3. Postoperative outcomes of patients with heart failure and controls.

Postoperative outcomes (no. of patients)	HF (n = 48)	Controls (n = 48)	P value
Composite endpoints			
Not related to pump failure	10	0	0.01
Possibly related to pump failure	6	1	0.12
Definitely related to pump failure	8	0	0.01
Mortality			
Early mortality	8	1	0.04
Late mortality	3	1	0.61
Organ dysfunction			
Circulatory failure: mechanical assist devices	19	0	< 0.001
Neurological events			
Transient ischemic attack	0	1	1.00
Stroke	1	1	1.00
Documented critical illness neuropathy	5	1	0.06
Renal failure			
Acute kidney injury	10	5	0.24
Continuous venovenous hemofiltration	8	1	0.03
Pulmonary complications			
Mechanical ventilation ≥ 48 hours	13	4	0.03
Reintubation	4	2	0.65
Tracheostoma	1	0	1.00
Ventilator-associated pneumonia	12	3	0.02
Pneumothorax	2	0	0.47
Sepsis	7	1	0.06
Pharmacological support 12-24 hours in the ICU			
Norepinephrine ≥ 0.2 µg/kg/min	11	0	0.001
Dobutamine ≥ 5 µg/kg/min	16	3	0.002

Abbreviations: HF, Heart Failure; ICU, Intensive Care Unit.

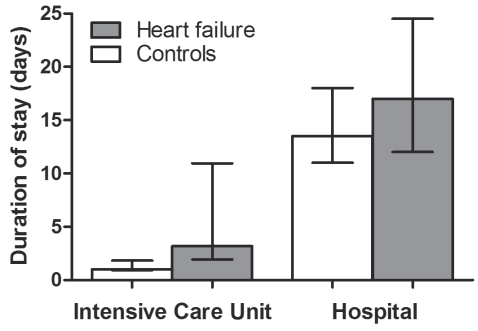


Figure 1. Intensive care unit stay and total stay in hospital of patients with heart failure and controls. Patients with preexisting heart failure experienced a longer stay in the intensive care unit ($P < 0.001$) than controls (median ± interquartile range of 3.2 (1.9-11.0) versus 1.0 (0.9-1.8) days). In addition, they experienced a longer total stay in hospital ($P = 0.01$) than controls (median ± interquartile range of 17.0 (12.0-24.0) versus 13.5 (11.0-18.0) days).

DISCUSSION

This study confirms the clinical observation that preexisting heart failure is a risk factor for systemic dysregulation after cardiac surgery. Not all adverse outcomes were related to the insufficient pumping capacity of the heart itself but merely to a cluster of organ dysfunction, suggesting systemic dysregulation. In an attempt to differentiate between pump failure and systemic failure, three composite endpoints were determined. As expected, the composite endpoint of complications definitely related to pump failure was significantly different between the groups. The composite endpoint of complications possibly related to pump failure – containing early mortality and acute kidney injury – was, however, not significantly different. This could first of all be the result of the relatively small patient numbers in this study, because this endpoint was observed six times more often in patients with heart failure. Moreover, during the postoperative period the serum creatinine values to assess acute kidney injury may not be accurate because of imbalances between creatinine production and elimination. Most interestingly to support our hypothesis was the composite endpoint of complications not related to pump failure. This endpoint was observed ten times more often in patients with heart failure, indicating systemic dysregulation.

The early mortality in patients with heart failure was higher than the preoperative expected mortality, based on the logistic EuroSCORE; while in the controls the early mortality was lower than the predicted mortality. In our opinion, the syndrome of heart failure is underestimated when using the logistic EuroSCORE to predict the mortality of patients with heart failure. This can be explained by the way the EuroSCORE was established.[11] There is an additive effect of heart failure on mortality, which is not well represented by only the ejection fraction in this scoring system. This suggests that heart failure surgery needs a modified risk stratification system. It is also the question if there were enough patients with heart failure included in the data collection for the development of the new version of the EuroSCORE system.

In this study it became clear that heart failure patients more often experience a systemic dysregulation postoperatively. The prolonged intensive care unit stay and total stay in hospital are mainly caused by sepsis, vasoplegia, prolonged mechanical ventilation with ventilator-associated pneumonias as a result, and organ dysfunction in general. The exact mechanisms of these outcomes are not yet unravelled, but were not directly related to the insufficient pumping capacity of the heart. Systemic disturbances are recognized to contribute to patient morbidity and mortality after cardiac surgery.[15] Those disturbances may comprise complement activation, immune cell activation, oxidative stress and others in addition to inflammation. One of the underlying mechanisms might be the different response to ischemia/reperfusion injury. In our opinion, this nearly inevitable phenomenon in cardiac surgery on top of susceptible vascular beds in patients with heart failure might

be the cause of the observed systemic dysregulation. However, further research is needed to assess the individual contribution of those underlying pathophysiological mechanisms to the observed systemic dysregulation.

Some issues merit mention. Patients with preexisting heart failure were frequently hospitalized prior to admission to our department. Although they are usually hospitalized at least once for adaptation of the treatment before they are considered for surgery, hospitalization in the days prior to surgery is often seen in patients with acute heart failure. In our opinion, including these patients would give an overestimation of the primary and secondary endpoints. However, excluding these hospitalized patients contributed to the relatively small patient numbers. Baseline differences for systolic blood pressures between the groups may reflect pharmacological treatment, because the therapy for heart failure is aimed at lowering the blood pressure with beta-blockers and ACE-inhibitors. The logistic EuroSCORE identified various preoperative risk factors related to mortality, but none of our statistically different baseline variables between the two groups are included in this scoring system. Compared with a study by Conlon et al.[16], our percentages of patients requiring renal replacement therapy are relatively high, but are influenced by local policy to treat acute kidney injury early and aggressively to prevent further deterioration of the kidney function.

Conclusion

This study shows that the majority of observed complications in patients with preexisting heart failure are not directly related to the insufficient pumping capacity of the heart, but merely reflect a systemic dysregulation. Further research is necessary to unravel underlying mechanisms for this adverse postoperative course. Moreover, the great impact of heart failure on adverse outcomes emphasizes the importance of adapting current risk stratification systems, such as the EuroSCORE. Offensive perioperative treatment strategies focused on the described complications will lead to improved results for patients with heart failure after cardiac surgery.

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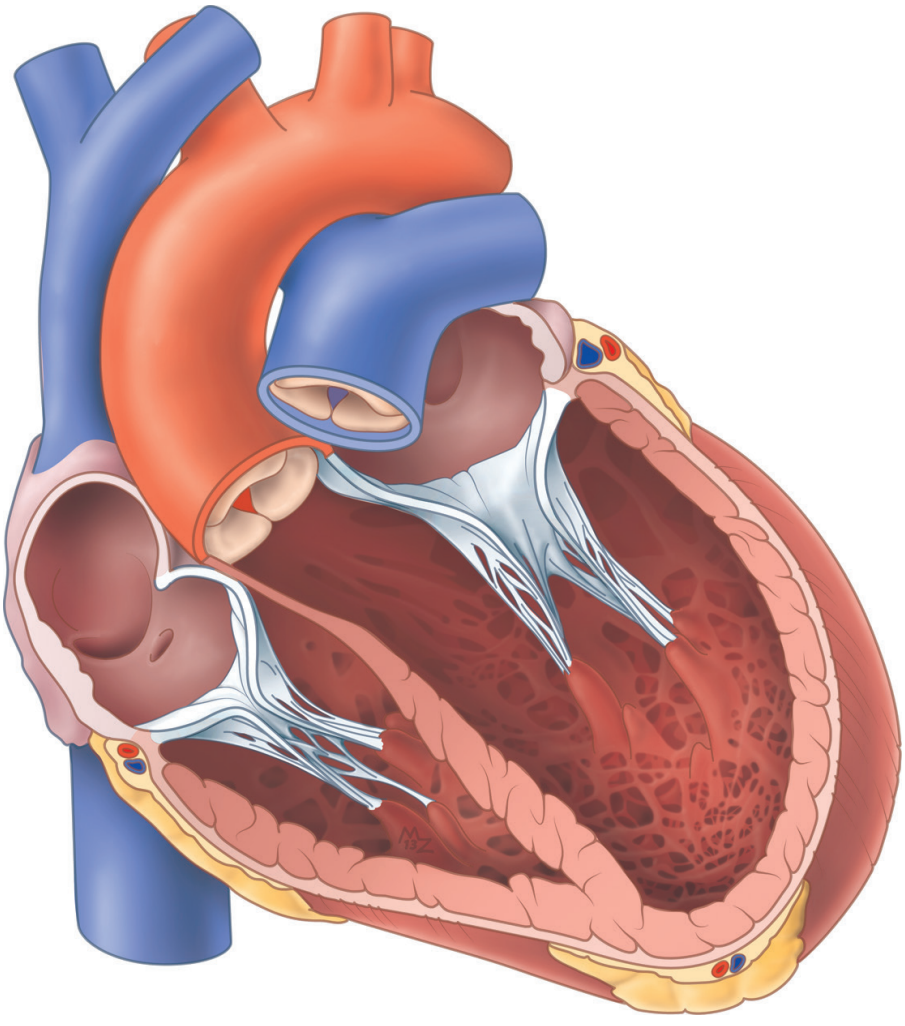
Supplementary data, Table 1. Performed surgical procedures in patients with heart failure and controls.

Surgical procedures (no. of procedures)	HF (n = 48)	Controls (n = 48)
External cardiac support device	23	0
Left ventricular reconstruction	25	0
Mitral valve repair	48	48
Tricuspid valve annuloplasty	32	20
Aortic valve repair / reconstruction	4	4
Coronary artery bypass grafting (average of bypasses per patient)	20 (2.3)	17 (2.8)
Ablation procedures		
Ventricular tachycardia	5	0
Atrial fibrillation	6	16

Abbreviation: HF, Heart Failure.

PART ONE

Pathophysiology of ischemia/reperfusion injury in cardiac surgery



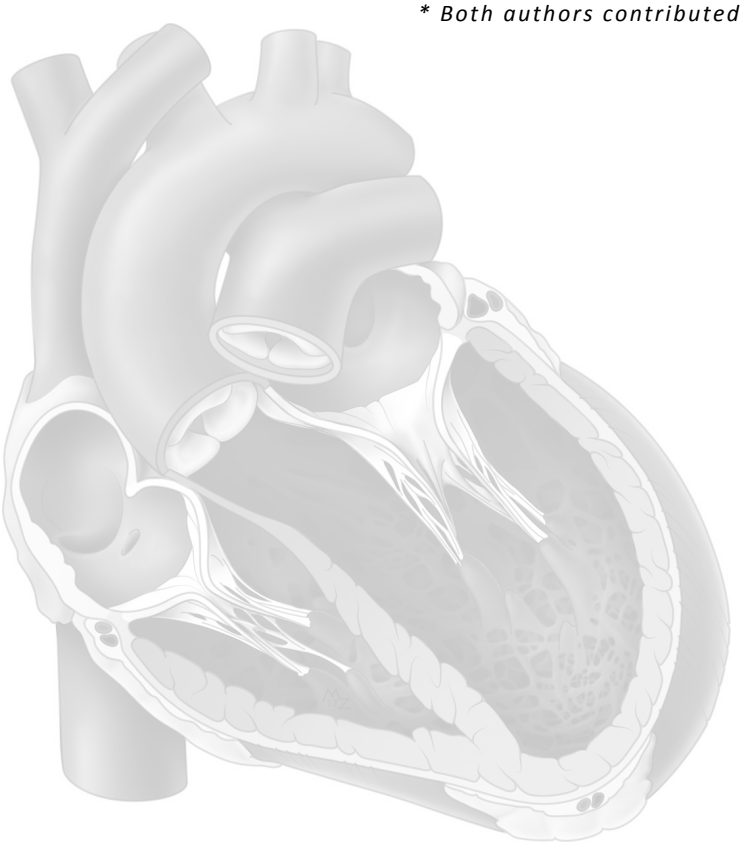
CHAPTER THREE

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Oxidative damage in clinical ischemia/ reperfusion injury: a reappraisal

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** Both authors contributed equally to this work*



ABSTRACT

Background: Ischemia/reperfusion (I/R) injury is a common clinical problem. Although the pathophysiological mechanisms underlying I/R injury are unclear, oxidative damage is considered a key factor in the initiation of I/R injury. Findings from preclinical studies consistently show that quenching reactive oxygen and nitrogen species (RONS), thus limiting oxidative damage, alleviates I/R injury. Results from clinical intervention studies on the other hand are largely inconclusive. In this study, we systematically evaluated the release of established biomarkers of oxidative and nitrosative damage during planned I/R of kidney and heart in a wide range of clinical conditions.

Results: Sequential arteriovenous concentration differences allowed specific measurements over the reperfused organ in time. None of the biomarkers of oxidative and nitrosative damage (that is, malondialdehyde, 15(S)-8-iso-prostaglandin F2α, nitrite, nitrate and nitrotyrosine) were released upon reperfusion. Cumulative urinary measurements confirmed plasma findings. As of these negative findings, we tested for oxidative stress during I/R and found activation of the nuclear factor erythroid 2-related factor 2, the master regulator of oxidative stress signaling.

Innovation: This comprehensive, clinical study evaluates the role of RONS in I/R injury in two different human organs (kidney and heart). Results show oxidative stress, but do not provide evidence for oxidative damage during early reperfusion, thereby challenging the prevailing paradigm on RONS-mediated I/R injury.

Conclusions: Findings from this study suggest that the contribution of oxidative damage to human I/R may be less than commonly thought and propose a re-evaluation of the mechanism of I/R.

INTRODUCTION

Ischemia/reperfusion injury (I/R) is the paradoxical increase of tissue damage upon reperfusion of ischemic tissue. I/R is considered a major contributor to tissue damage in multiple clinical situations such as myocardial infarction, stroke and organ transplantation. The pathophysiology of I/R injury is complex and incompletely understood, and effective treatment is currently lacking.

Reactive oxygen and nitrogen species (RONS) are considered key initiators of I/R injury. Ischemia-related metabolic adaptations and dysregulated mitochondrial homeostasis are thought to result in substantial RONS release upon reintroduction of oxygen. This RONS overload can overwhelm the endogenous antioxidant system, resulting in oxidative damage. This may trigger secondary processes such as a pro-inflammatory response.[1-4]

It has long been supposed that antioxidant therapy mitigates I/R injury. The validity of this concept has been proven in numerous animal studies, which all clearly demonstrate that antioxidant therapy ameliorates I/R injury.[5-7] Despite these findings, studies in humans consistently fail to show any clinically relevant effect.[5, 8-11] The basis for this discrepancy between human and animal studies is still unclear, yet it may suggest that the contribution of RONS to I/R injury in humans may be less than commonly thought.

In this study, a double approach was used to evaluate the role of RONS in clinical I/R injury. First, release of established biomarkers of oxidative damage from the human kidney and heart was assessed during planned I/R. By cannulation of the efferent vein, that is, the renal vein during kidney transplantation and the coronary sinus during cardiac valve surgery, sensitive and organ-specific measurements of net changes in oxidative damage biomarkers over the organ were conducted. Next, as none of the biomarkers were released during the reperfusion phase, we tested for presence of oxidative stress during early reperfusion, which did show activation of nuclear factor erythroid 2-related factor 2 (Nrf2), a critical transcription factor in oxidative stress signaling. All together, results from these studies, comprising different organs, clinical settings and durations of ischemia, are highly consistent and do indicate oxidative stress, but no RONS-related damage during the acute reperfusion phase in humans, suggesting that the endogenous antioxidant system is able to cope with I/R related oxidative stress.

METHODS

Patients and surgical procedures

This prospective observational study involved patients undergoing renal I/R during kidney transplantation with living donor (LD) and deceased donor (DD) grafts, and patients undergoing myocardial I/R (that is, during cardiac valve surgery) with and without preexisting heart failure. The study protocols were approved by the local ethics committee, and written informed consent was obtained from each patient.

Kidney transplantation

Twenty-four patients undergoing renal allograft transplantation were included: eight patients receiving a kidney from a LD and 16 patients receiving a kidney from a DD (nine brain-dead donors and seven cardiac-dead donors). LD and DD kidney transplantations were separately analyzed and compared because of large differences in ischemia times, pre-transplantation graft handling, and clinical outcome between these groups. Within groups variability of patient and graft characteristics was small for both donor types, rendering the comparison more reliable. LD kidney transplantations allowed for measurements in urine produced by the graft directly after reperfusion, providing a more cumulative measure than the arteriovenous measurements.

Kidney transplantations were performed according to the local standardized protocol. For technical reasons (renal vein sampling), only patients receiving a left kidney were included. In LDs a minimally invasive open nephrectomy was performed. The immunosuppressive regimen was based on induction therapy with basiliximab followed by maintenance therapy with tacrolimus or cyclosporine A in addition to mycophenolate mofetil and steroids.

Cardiac valve surgery

Myocardial I/R was studied in patients undergoing cardiac valve surgery with aortic cross-clamping (that is, cessation of blood flow in the coronary arteries). Twenty-four patients scheduled for elective mitral valve annuloplasty with use of cardiopulmonary bypass were included: 12 patients with preexisting heart failure and 12 patients without heart failure. Heart failure was defined as an inadequate pump function with an echocardiographically estimated ejection fraction biplane below 35%[12] and the presence of one or more clinical symptoms of heart failure (New York Heart Association classification).[13] Exclusion criteria were perioperative corticosteroid therapy, minimal invasive surgical procedures, emergency cardiac operations and previous cardiac surgery.

Cardiac surgery was performed according to the local standardized protocol. All surgical procedures were performed via a midline sternotomy under normothermic cardiopulmonary

bypass (Jostra Maquet, Maquet, Hirrlingen, Germany) with intermittent potassium-enriched antegrade warm-blood cardioplegia. This solution was administered every 15 to 20 minutes throughout the entire ischemic period. The cardiopulmonary bypass system was coated with a heparin softline coating. No antioxidants or other preservational or therapeutical substances were added.

During cardioplegia-induced cardiac arrest (myocardial ischemia), valve surgery was executed. Eventually, the aortic cross-clamp was removed to restore the blood flow through the heart (start of reperfusion).

Arteriovenous measurements

Arterial and venous blood samples were collected directly over the reperfused organ, that is, from the afferent and efferent blood vessel. Comparison of multiple biomarkers in these samples allowed for accurate and specific assessment of locally ongoing processes in the reperfusion phase.[14] This method was used to determine the release of biomarkers of oxidative damage in both kidney and heart I/R.

Kidney transplantation

Arterial and renal venous blood samples were obtained as described before.[14] Via an umbilical vein catheter placed in the renal vein, blood aliquots were sampled at 30 seconds, 3, 10, and 30 minutes after reperfusion. Paired arterial blood samples were simultaneously obtained via the iliac artery at 0, 3, 10, and 30 minutes after reperfusion (Figure 1A). The endpoint of sampling was reached 30 minutes after reperfusion by closing the abdominal wall. The initial urine produced by the graft in the first 10 minutes after reperfusion was collected directly from the graft's ureter. Control urine was collected from the urethral catheter of LDs during the nephrectomy procedure before kidney ischemia was induced. All samples were collected in pre-cooled tubes containing ethylenediaminetetraacetic acid (EDTA) (BD Vacutainer, Plymouth, UK) and placed on melting ice immediately. Blood and urine samples were centrifuged (1.550 *g*, 20 min, 4°C) within one hour after collection and the derived plasma or supernatant was re-centrifuged (1.550 *g*, 20 min, 4°C) to deplete it from remaining leukocytes and platelets. Material was aliquoted and stored at -70°C until analysis.

Cardiac valve surgery

A 5 French indwelling jugular vein catheter (PICC, Arrow International Inc., REF PS-01651, PA, USA) was inserted in the right atrium and placed in the coronary sinus during cardiac surgery (Figure 1B). As all patients underwent mitral valve surgery using a vertical transseptal incision, the coronary sinus could easily be cannulated during the surgical procedure. An arterial

catheter was routinely placed in the radial artery. Arterial and paired myocardial venous blood (coronary sinus) samples were obtained at 0, 15, 30, and 60 minutes after reperfusion as described recently.[15] All samples were collected in pre-cooled tubes containing EDTA (BD Vacutainer, Plymouth, UK) and placed on melting ice immediately. Blood samples were centrifuged (1.550 *g*, 10 min, 4°C) within one hour after collection and the derived plasma was re-centrifuged (10.000 *g*, 4 min, 4°C) to obtain leukocyte and platelet poor plasma. Aliquots were stored at -70°C until analysis.

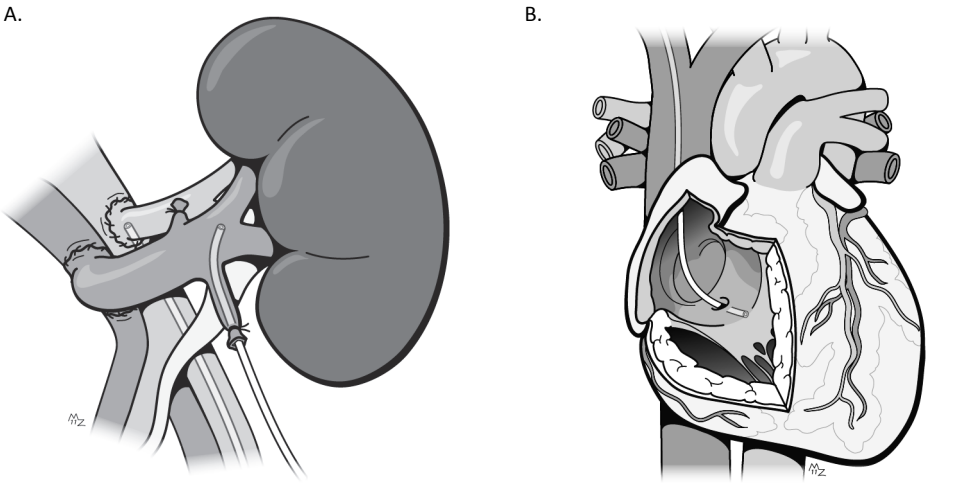


Figure 1. Schematic representation of the arteriovenous measurements of the kidney and heart. The figure shows the position of the arterial and renal venous catheter in the kidney (A), and the coronary sinus catheter in the heart (B).

Biopsy collection

A pre-transplantation renal cortical biopsy was obtained just before transplantation, when the graft was still on ice. A paired needle biopsy of the same kidney was collected 45 minutes after reperfusion. Myocardial biopsies were collected at start of cardioplegia-induced cardiac arrest and a paired, second biopsy was obtained at the end of the ischemic period, just before reperfusion of the heart. Because of technical reasons, a post-reperfusion biopsy could not be collected since most of the included heart failure patients were hemodynamically unstable in the early reperfusion phase. Lifting the heart, to take a post-reperfusion biopsy, was considered unsafe. All biopsies were snap-frozen in liquid nitrogen and stored at -70°C until analysis.

Laboratory plasma measurements

To validate the method of arteriovenous measurements and to assess whether there is oxygen consumption by the reperfused organ, the oxygen saturation level was assessed in arterial as well as venous blood samples of a LD by means of a routinely used validated blood gas analyzer. The extent of RONS-mediated damage was evaluated by measuring different well-established biomarkers of oxidative and nitrosative damage by state of the art stable isotope dilution gas chromatography-mass spectrometry (GC-MS) and gas chromatography-tandem mass spectrometry (GC-MS/MS) methods. Malondialdehyde (MDA) and 15(*S*)-8-*iso*-prostaglandin F2α (15(*S*)-8-*iso*-PGF2α) are widely recognized biomarkers of lipid peroxidation.[16] In humans, nitrite and nitrate are reliable indicators of nitric oxide synthesis and frequently measured breakdown products of RONS.[16-18]

MDA and total 15(*S*)-8-*iso*-PGF2α, that is, free and esterified 15(*S*)-8-*iso*-PGF2α, were assessed with an extensively validated GC-MS/MS method as described previously.[19, 20] In parallel, the thiobarbituric acid-reactive substances (TBARS) method was used to detect MDA in plasma as well as in urine samples.[21] Free 15(*S*)-8-*iso*-PGF2α in urine was measured by GC-MS/MS.[20] Nitrite and nitrate were measured simultaneously by GC-MS in plasma and urine aliquots as described elsewhere in detail.[19, 22] The prostaglandin E_2 (PGE_2) concentration in urine was measured by GC-MS/MS after immunoaffinity column chromatography extraction.[20] Urinary parameters were corrected for creatinine excretion.[23] Study samples were analyzed alongside quality control (QC) samples as described previously.[19, 22, 23] The MDA plasma concentration in the QC samples was determined to be 57.3 ± 7.8 nmol/L (mean ± standard deviation (SD), n = 25) corresponding to an imprecision (relative SD) of 13.7 %. The total 15(*S*)-8-*iso*-PGF2α plasma concentration in the QC samples was determined to be 157 ± 6.4 pmol/L (mean ± SD, n = 10) corresponding to an imprecision of 4.1%. In the QC samples for plasma nitrite and nitrate (n = 30), accuracy and imprecision were 90% to 112% and below 6%, respectively. 3-Nitrotyrosine in proteins, a biomarker of nitrosative damage, was measured by ELISA in accordance with the manufacturer's instructions (Biotech nitrotyrosine EIA, Oxis, Portland, Oregon, USA).[16]

Nrf2/ARE pathway

Nrf2 activation was assessed in whole-cell lysate. Snap-frozen renal biopsies were homogenized and whole-cell extracts were prepared according to the manufacturers' instructions (Nuclear extract kit, Active Motif, Carlsbad, CA, USA). Next, activated Nrf2 in cell lysates was quantified by DNA-binding ELISA according to the manufacturers' instructions (TransAM Nrf2, Active Motif, Carlsbad, CA, USA). Total RNA was extracted from renal or myocardial tissues as described earlier, using glyceraldehyde phosphate dehydrogenase

(GAPDH) as internal control.[24] For expression profiling, the integrity of each RNA sample was examined by Agilent Lab-on-a-chip technology using the RNA 6000 Nano LabChip kit and a bioanalyzer 2100 (Agilent Technologies, Amstelveen, The Netherlands). RNA preparations were considered suitable for array hybridization only if samples showed intact 18S and 28S rRNA bands, and displayed no chromosomal peaks or RNA degradation products (RNA Integrity Number > 8.0).[25] Subsequent microarray analysis was performed using Illumina whole-genome gene expression BeadChips (Illumina BeadArray®, San Diego, CA, USA) according to instructions of the manufacturer at Service XS (Leiden, The Netherlands).

Selected probes from the canonical Nrf2 pathway were used as an input for pathway analysis through the Ingenuity Pathway Analysis suite (http://www.ingenuity.com). Hierarchical clustering and heatmap visualization were carried out using Hierarchical Clustering Viewer and Heatmap Viewer modules within the GenePattern analysis suite.[26-28]

Histological analyses

In kidney biopsies collected before and after reperfusion, presence of various antioxidant enzymes and RONS generation by xanthine oxidase (XO) was assessed. Antioxidant enzymes superoxide dismutase (SOD) and peroxidases (catalase and glutathione peroxidase) were measured by histochemical methods as described previously in detail.[29] The XO activity was measured in renal tissue, based on the generation of superoxide anions from hypoxanthine, as described earlier.[30] Specificity of the assay was demonstrated by inhibition of XO by allopurinol. Rat liver tissue was included as positive control.

Statistical analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences 16.0 (SPSS Inc., Chicago, IL, USA). All data in the text and tables are expressed as mean ± SD. Patient characteristics were compared by paired *t*-test. The area under the curve (AUC) was calculated for the arterial and venous curve. AUC's and urinary measurements were compared by using the Wilcoxon signed rank test. Differential expression of probes was assessed using unpaired moderated *t*-test (LIMMA) through the Remote Analysis Computation for gene Expression data (RACE) suite at http://race.unil.ch.[31] A *P* value less than 0.05 was considered significant. Graph error bars indicate the standard error of the mean (SEM).

RESULTS

Patient characteristics and outcome

Patient characteristics for kidney transplantation and cardiac valve surgery are summarized in Tables 1 and 2, respectively. One-year patient and graft survival was 100% for the patients who underwent kidney transplantation. One-year survival was also 100% for all patients who underwent cardiac valve surgery.

Table 1. Transplantation and outcome characteristics in living donor and deceased donor kidney transplantations.

	LD	DD	*P* value
n	8	16	
Recipient age (years) Mean ± SD	41.1 ± 10.5	54.6 ± 12.2	0.02
Recipient gender (% male)	38 (n = 3)	56 (n = 9)	0.39
Cause renal failure			
ADPKD	2 (25%)	4 (25%)	
Renal fibrosis	2 (25%)	1 (6%)	
MPG	1 (13%)	1 (6%)	
IgA nephropathy	1 (13%)	1 (6%)	
BM nephropathy	0	2 (13%)	
Hypertension	0	2 (13%)	
FSG	0	1 (6%)	
Unknown	2 (25%)	4 (25%)	
Donor age (years) Mean ± SD	43.9 ± 10.6	53.5 ± 16.1	0.14
Donor gender (% male)	75 (n = 6)	44 (n = 7)	0.15
Cause of death donor	N/A		
BDD: trauma		3 (19%)	
BDD: SAH		2 (13%)	
BDD: hypoxic		3 (19%)	
CDD: traumatic brain injury		2 (13%)	
CDD: intracranial hemorrhage		4 (25%)	
CDD: SAH		2 (13%)	
Preservation fluids	HTK (n = 8)	UW (n = 11) HTK (n = 5)	0.001
CIT (min) Mean ± SD	179.1 ± 18.6	1117.7 ± 299.1	< 0.001
WIT (min) Mean ± SD	34.0 ± 6.3	33.5 ± 6.1	0.85

Abbreviations: ADPKD, Autosomal Dominant Polycystic Kidney Disease; BDD, Brain-Dead Donor; BM, Basement Membrane; CDD, Cardiac-Dead Donor; CIT, Cold Ischemia Time; DD, Deceased Donor; FSG, Focal Segmental Glomerulosclerosis; HTK, Histidine–Tryptophan–Ketoglutarate; LD, Living Donor; MPG, Membranous Glomerulonephritis; SAH, Subarachnoid Hemorrhage; UW, University of Wisconsin; WIT, Warm Ischemia Time.

Table 2. Characteristics of patients with and without heart failure undergoing cardiac surgery.

	Heart failure	No heart failure	*P* value
n	12	12	
Gender (% male)	75 (n = 9)	42 (n = 5)	0.11
Age (years) Mean ± SD	66.8 ± 8.9	62.7 ± 11.7	0.20
Ischemia time (min) Mean ± SD	128.3 ± 50.7	127.0 ± 32.6	0.89
Duration ICU stay (h)* Mean ± SD	62.5 ± 40.8	31.6 ± 23.9	0.02

Abbreviation: ICU, Intensive Care Unit.

Validation of arteriovenous measurements

The methodology of the arteriovenous sampling is schematically shown in Figure 1. The principle of the method is illustrated by arterial and venous oxygen saturation levels measured during early reperfusion, showing an effective and consistent consumption of oxygen (Figure 2).

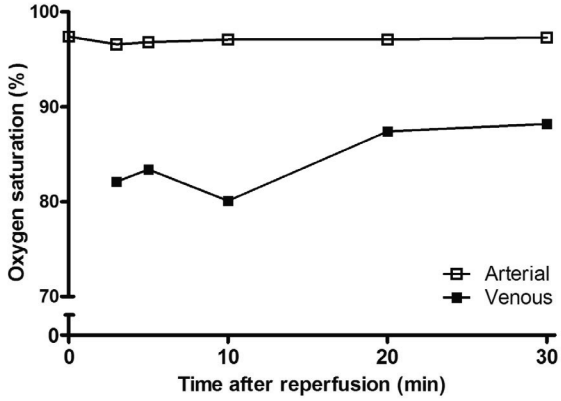


Figure 2. A typical example of arteriovenous oxygen saturation differences during reperfusion of the kidney.
Arterial and renal venous oxygen saturation was measured during the first 30 minutes of reperfusion. The consistently lower venous oxygen saturation level indicates the uptake of oxygen by the reperfused kidney, demonstrating its immediate metabolic activity.

Biomarkers of oxidative damage

Renal I/R is not associated with oxidative damage

MDA and 15(*S*)-8-*iso*-PGF2α were measured as established biomarkers of RONS-mediated damage. Arteriovenous measurements of MDA did not indicate its release from the kidney during the first 30 minutes of reperfusion of LD kidneys by either GC-MS ($P = 0.17$; Supplementary data, Figure 1) or TBARS method ($P = 0.06$). Urinary MDA levels (TBARS method) were also assessed in the first urine produced immediately after reperfusion (LDs only) as an aggregate measure of total MDA formation in the kidney. The MDA levels in this first urine collected after reperfusion were similar to the control urine, that is, urine produced by the kidney before its removal from the donor ($P = 0.40$).

Minimal release was observed for 15(*S*)-8-*iso*-PGF2α from reperfused LD kidney grafts ($P = 0.03$). However, clinically more vulnerable DD kidneys did not show any 15(*S*)-8-*iso*-PGF2α release upon reperfusion ($P = 0.16$; Figure 3). Arteriovenous measurements over the non-ischemic kidney in LDs, that is, before donor nephrectomy, revealed no release of 15(*S*)-8-*iso*-PGF2α ($P = 0.69$), demonstrating that its post-reperfusion release is specific for I/R. Urinary 15(*S*)-8-*iso*-PGF2α levels were not influenced by reperfusion ($P = 0.35$; Table 3).

Table 3. AUC venous minus AUC arterial (delta AUC) of the measured plasma biomarkers for oxidative and nitrosative damage during the early reperfusion phase.

	Kidney transplantation			Cardiac valve surgery	
Delta AUC*	**LD**	**DD**	**LD Urine†**	**Heart failure**	**No heart failure**
Oxidative damage					
Malondialdehyde (nmol/L×min)	138.2	Not		-4544	1758
(*P* value)	(0.17)	measured	(0.40)	(0.05)	(0.43)
15(*S*)-8-*iso*-PGF2α (pmol/L×min)	356	-1306		1056	934
(*P* value)	(0.03‡)	(0.16)	(0.35)	(0.02‡)	(0.18)
Nitrosative stress and damage					
Nitrite (nmol/L×min)	308	-910		-6949	-5878
(*P* value)	(0.50)	(0.87)	(0.06)	(0.002)	(0.03)
Nitrate (µmol/L×min)	-36	77		-14	75
(*P* value)	(0.50)	(0.98)	(0.69)	(0.53)	(0.81)
Nitrotyrosine (nmol/L×min)	-113	Not	Not	Not	Not
(*P* value)	(0.93)	measured	measured	measured	measured

* Delta AUC: AUC venous minus AUC arterial (*P* value) of the measured plasma markers during the early reperfusion phase. A positive number indicates a release from the reperfused organ, whereas a negative number indicates an uptake of the biomarker.

† Results in urine indicate *P* values of the difference of the pre- and post-reperfusion measure.

‡ Significant venous release ($P < 0.05$).

Abbreviation: AUC, area under the curve.

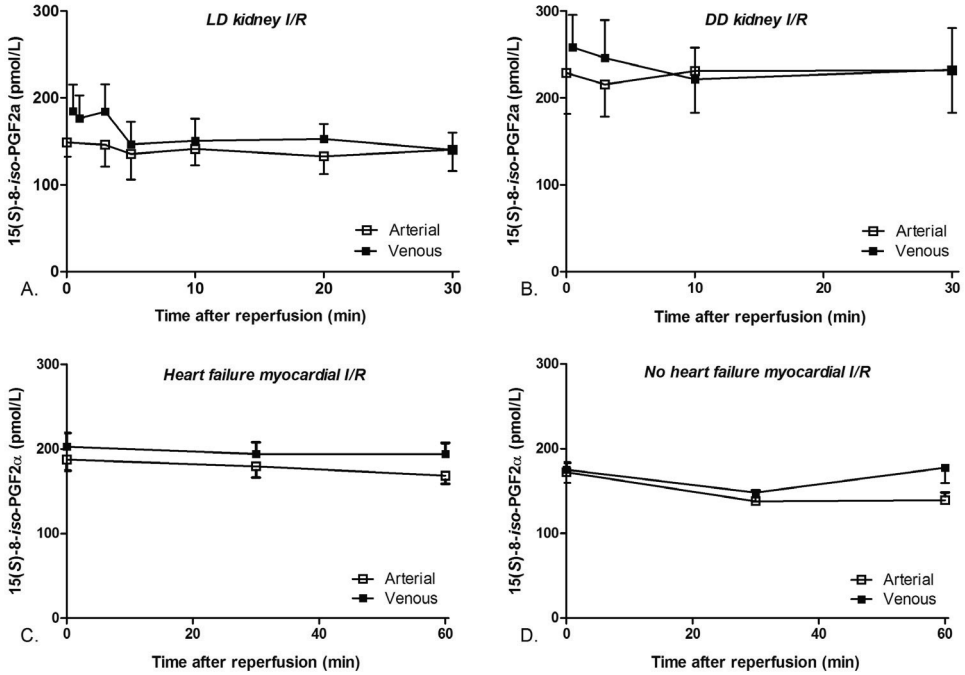


Figure 3. Arterial and venous plasma concentrations of 15(*S*)-8-*iso*-prostaglandin F2α.
(A) Arterial and renal venous plasma 15(*S*)-8-*iso*-PGF2α concentrations during the first 30 minutes of reperfusion in living donor (LD) kidney transplantation showed a small, but significant release from the kidney (P = 0.03); (B) In deceased donor (DD) kidney transplantation, 15(*S*)-8-*iso*-PGF2α was not released from the reperfused kidney during the first 30 minutes of reperfusion (P = 0.16); (C) Arterial and myocardial venous plasma 15(*S*)-8-*iso*-PGF2α concentrations during the first 60 minutes of reperfusion in patients with preexisting heart failure showed a small, but significant release from the myocardium (P = 0.02); (D) In patients without preexisting heart failure, 15(*S*)-8-*iso*-PGF2α was not released from the reperfused heart during the first 60 minutes of reperfusion (P = 0.18).

Urinary PGE_2 was measured as marker of cyclooxygenase-2 (COX-2) activity. PGE_2 levels in the first urine collected after reperfusion were higher than those of the control urine, that is, urine produced by the kidney prior to its removal from the donor (P = 0.02; Figure 4). This shows that in contrast to the absence of apparent oxidative *damage*, oxidative *stress* appears to be increased after renal reperfusion.

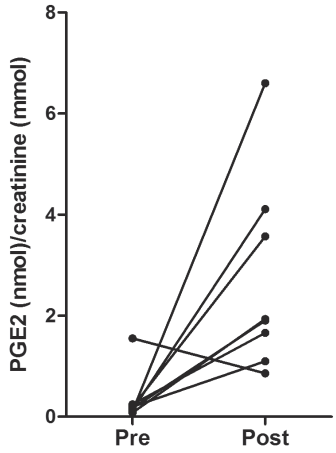


Figure 4. Prostaglandin E_2 excretion in urine.
Creatinine-corrected levels of prostaglandin E_2 (PGE_2) in the first urine collected after reperfusion (post) were higher than those of control urine (pre), that is, urine produced by the kidney before its removal from the donor (*P* = 0.02).

Myocardial I/R is not associated with oxidative damage

Results of myocardial I/R resembled those of the kidney. There was no myocardial MDA release during the first hour after reperfusion in both patients with and without heart failure (*P* = 0.05 and *P* = 0.43, respectively; Table 3, and Supplementary data, Figure 1). In fact, venous MDA levels tended to be lower than arterial levels in patients with heart failure.

15(*S*)-8-*iso*-PGF2α showed a small local release early after reperfusion in patients with preexisting heart failure (*P* = 0.02; Figure 3). In contrast, arteriovenous measurements of 15(*S*)-8-*iso*-PGF2α in patients without preexisting heart failure did not indicate a local release during the first hour after reperfusion (*P* = 0.18; Table 3).

Biomarkers of nitrosative damage

No evidence for nitrosative damage in human kidney transplantation

Nitrite concentrations were equal in renal arterial and venous blood samples (LDs *P* = 0.50, DDs *P* = 0.87; Supplementary data, Figure 2). Likewise, nitrate was not released from the reperfused graft either (LDs *P* = 0.50, DDs *P* = 0.98; Supplementary data, Figure 3). These findings were confirmed by the additional measurement of plasma nitrotyrosine in LD kidney grafts, indicating no such release (*P* = 0.93, Supplementary data, Figure 4). The integral urinary measurement of nitrite and nitrate remained similar after reperfusion in LD kidney transplantations (*P* = 0.06, *P* = 0.69, respectively; Table 3).

No evidence for nitrosative damage after reperfusion of the myocardium

Arterial nitrite concentrations were higher than venous concentrations in both patient groups during the early reperfusion phase (P = 0.002 preexisting heart failure, P = 0.03 no preexisting heart failure; Supplementary data, Figure 2), excluding myocardial nitrite release. Nitrate did not change over the reperfused myocardium in both patient groups (P = 0.53 preexisting heart failure, P = 0.81 no preexisting heart failure; Table 3, and Supplementary data, Figure 3).

Oxidative stress

Kidney

Since the above-mentioned results indicated no extracellular evidence for RONS-mediated damage, we tested for the occurrence of oxidative stress during I/R by assessing activation of Nrf2, the master regulator of the antioxidant stress response.

Activation of Nrf2 was quantified, as well as the upregulation of Nrf2 responsive genes, heme oxygenase-1 (HMOX-1), NAD(P)H quinone oxidoreductase (NQO1), and glutathione S-transferase A2 (GSTA2) in pre- and post-biopsies collected from both kidney and heart (Figure 5). Using a DNA binding ELISA for activated Nrf2, we found clear activation of this transcription factor upon reperfusion (P = 0.01; Figure 6), while Nrf2 gene expression increased as well in both LD and DD kidney grafts (P = 0.03, P = 0.04, respectively; Figure 7). Pathway-based analysis of Nrf2 downstream genes (Ingenuity Pathway Analysis suite, Supplementary data, Figure 5) showed highly significant ($P = 7.06*10^{-12}$) upregulation of the canonical antioxidant responsive element (ARE) pathway in kidneys from LDs.

Conversion of xanthine reductase to XO and accumulation of hypoxanthine during ischemia has been proposed as a major source of oxygen radicals during I/R injury. Histochemical analysis of kidney biopsies collected before and after reperfusion did not indicate the XO activity in renal tissue, whereas ample XO activity and abundant hypoxanthine were observed in the positive control (rat liver; Supplementary data, Figure 6).

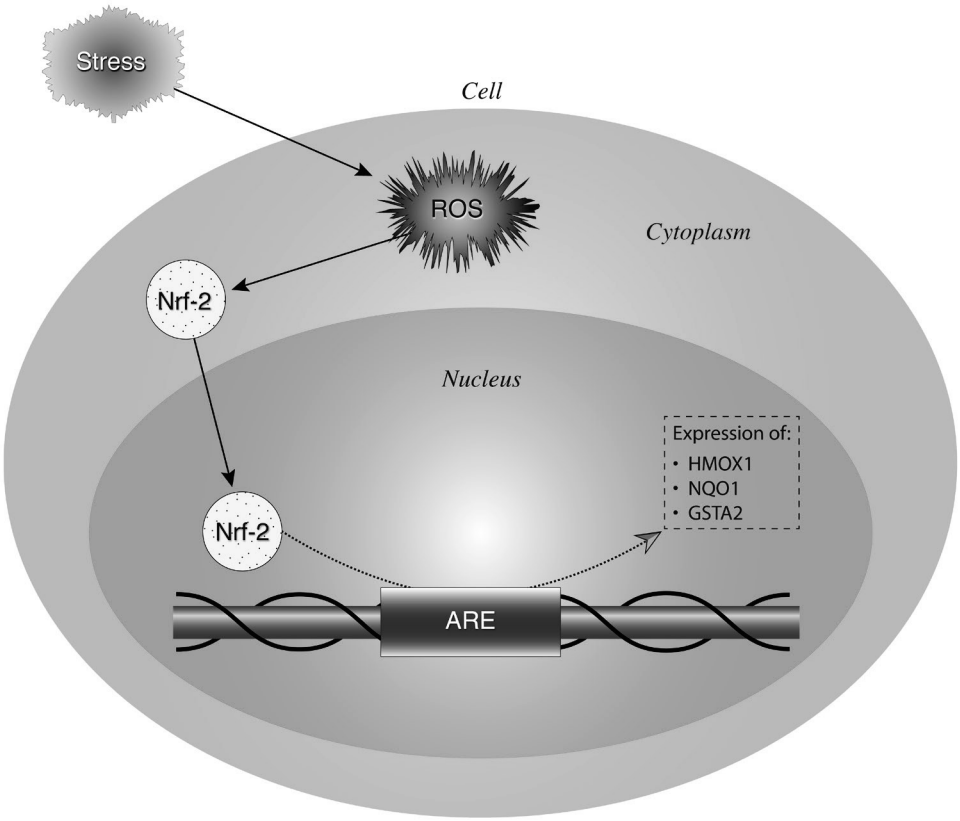


Figure 5. Schematic representation of the nuclear factor erythroid 2-related factor 2 / antioxidant responsive element pathway and antioxidant responsive element induced gene expression.
Environmental stress exerts oxidative stress on cells through production of reactive oxygen and nitrogen species (RONS). The oxidative stress response is largely coordinated by a transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2). Under homeostatic conditions, there is only an inactive minimal basal level of Nrf2-directed gene expression under tight control of protein Keap 1. RONS lead to the activation of mitogen-activated protein kinases (such as MAPK, ERK, p38), protein kinase C (PKC), phosphatidylinositol 3 kinase (PI3K), and others, which phosphorylate Keap1 and Nrf2. The Keap1-Nrf2 complex is then disrupted and the transcriptionally active Nrf2 is translocated to the nucleus where it binds to antioxidant responsive elements (AREs) in association with Maf proteins. This results in an increase of the transcriptional expression of Nrf2-inducible genes such as those encoding heme oxygenase-1 (HMOX1), NAD(P)H quinone oxidoreductase (NQO1), and glutathione *S*-transferase A2 (GSTA2). Together, these proteins scavenge RONS and limit oxidative damage.

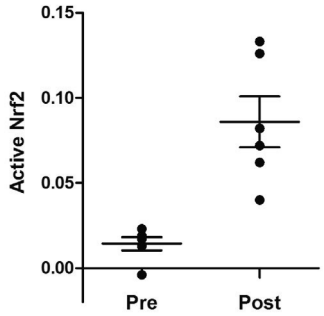


Figure 6. Nuclear factor erythroid 2-related factor 2 activation upon renal reperfusion.
Nuclear factor erythroid 2-related factor 2 (Nrf2) in renal tissue was significantly converted to its activated form after reperfusion ($P = 0.01$) during living donor (LD) kidney transplantation.

Antioxidant enzyme activities

SOD and peroxidase activities (catalase and glutathione peroxidase) were evaluated by histochemical analyses of kidney biopsies taken before and 45 minutes after reperfusion. Results of this analysis show abundant and similar antioxidant activities before and after reperfusion (Supplementary data, Figure 7).

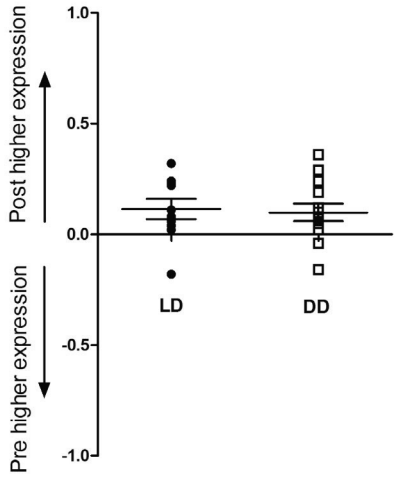


Figure 7. Changes in renal expression of nuclear factor erythroid 2-related factor 2 gene.
Renal expression of nuclear factor erythroid 2-related factor 2 (Nrf2) gene in biopsies collected before (pre) and 45 minutes after (post) reperfusion during living donor (LD) and deceased donor (DD) kidney transplantation. Y-axis shows the relative change in gene expression as calculated by $\log(1/2^{ddCt})$, where the dCt is the Ct of studied gene minus the Ct of GAPDH, and ddCt is dCt of post-biopsy minus dCt of pre-biopsy. A positive number indicates an increase in expression after reperfusion, whereas a negative number indicates a downregulation after reperfusion. Expression of the transcription factor Nrf2 gene NFE2L2 was significantly upregulated after reperfusion in both LD ($P = 0.03$) and DD ($P = 0.04$) kidney grafts.

DISCUSSION

Oxidative damage is long considered a key factor in the initiation of I/R injury. Although animal studies repeatedly showed the involvement of free radicals in I/R injury, studies in humans are scarce, and results of clinical intervention studies using antioxidants are inconclusive. Our study shows minimal release of markers of oxidative and nitrosative damage, and did not indicate upregulation of the classical oxidative response genes in both heart and kidney I/R. Results of our study are highly consistent and suggest that the role of oxidative and nitrosative damage in the initiation of *clinical* I/R injury may be less than commonly thought.

RONS have long been held responsible for the initiation and propagation of I/R injury in many different tissue types, including the kidney, liver, and heart.[32] Initial studies date back to 20 years ago when an increase in lipid peroxidation was shown after experimental reperfusion of rat kidneys.[33] Since then, further animal studies have supported a role for oxidative damage in I/R.[34-36] In contrast, studies in humans are scarce and findings are inconclusive. In a clinical study of Grech et al., it was confirmed that RONS are formed after I/R.[37] By using direct spin trapping methods, they demonstrated an immediate free radical production during the first four hours after myocardial reperfusion. However, whether RONS actually lead to tissue damage was not studied. Further studies on oxidative damage in I/R injury in humans show conflicting results. Zahmatkesh et al. showed that after kidney transplantation circulating lipid peroxidation biomarkers rise in peripheral blood.[38] Whether this can be attributed to oxidative damage in the kidney itself or in any other part of the body can not be distinguished by peripheral measurements. Some studies involving myocardial I/R used more specific methods, that is, arteriovenous measurements over the reperfused heart, after percutaneous transluminal coronary angioplasty (PTCA). F_2-isoprostanes were released from the reperfused myocardium after PTCA.[39, 40] However, results on MDA release were contradictory, showing no myocardial release after PTCA [41] and low extent MDA release during coronary artery bypass grafting.[42]

Although available antioxidant intervention studies are limited and results are inconclusive, antioxidant treatment appears a potential therapy to study in humans. Somehow, only few trials using antioxidants in human I/R injury are known, and it seems reasonable that a certain amount of publication bias has been introduced. El-Hamamsy et al. showed that administration of the antioxidant *N*-acetylcysteine during human coronary artery bypass surgery did not lead to improvement in clinical endpoints or decreased release of biochemical markers.[10] Administration of tirilazad mesylate, a non-glucocorticoid 21-aminosteroid inhibiting lipid peroxidation, to patients with acute ischemic stroke even increased the end-point of death instead of having beneficial effects.[8] In kidney I/R only two clinical trials have been published using antioxidants in renal transplantation. Pollak et al.

administered SOD intravenously before and one hour after reperfusion, but no beneficial effects were observed with respect to early graft function.[43] One year later, Land et al. administered a single, higher intravenous dose of SOD, immediately before reperfusion. The results demonstrated a significant reduction in first acute rejection.[44] However, up to now, none of both observations have been confirmed in other studies or have led to application in daily practice.

Thus, despite encouraging results of antioxidant I/R therapy in animals, published clinical studies lack an appreciable, clinically relevant effect thus far.[5, 8-11] Let alone minor differences in clinical set up, there appears to be a mechanistic disparity in the role of oxidative damage and endogenous antioxidant capacity in I/R injury between animals and humans. An explanation could be derived from studies on aging that have shown that organisms with large mass-specific metabolic rate, such as mice, have a relative high RONS production and a weak capacity to maintain homeostasis compared with humans.[45] This difference in homeostatic capacity may explain differences in the role of oxidative damage between species as well. Thus, it can be suggested that endogenous antioxidant systems in humans are sufficiently equipped to handle the excess RONS load during clinical reperfusion, thus preventing damage. A finding that is supported by abundant SOD and peroxidases activities before and after I/R.

Whether oxidative damage is indeed prevented in human I/R injury was assessed in this study. RONS have a transient nature and are difficult to assess *in vitro* and *in vivo*.[22] Consequently, we measured more stable oxidation products caused by RONS to determine the extent of oxidative damage in I/R injury. By combining multiple biomarkers, a footprint of oxidative and nitrosative damage could be reconstructed. Arteriovenous concentration differences of various established oxidative and nitrosative damage biomarkers were sequentially measured during reperfusion in kidney transplantation. To exclude organ- and procedure-specific findings, kidney results were compared with a different clinical setting, that is, myocardial I/R.

Arteriovenous measurements did not indicate release of oxidative or nitrosative damage biomarkers upon reperfusion of both the kidney and heart with the sole exception of a minimal and transient release of 15(*S*)-8-*iso*-PGF2α in a selection of patients. Although the arteriovenous measurements provide important information on local processes in the reperfused organ, sensitivity may be restricted by dilution of released biomarkers because of high flow rates. Therefore, accumulation of oxidative damage markers was measured in urine produced by the transplanted kidney (LD) during the first minutes of reperfusion. Urinary measurements were in accordance to plasma findings, showing no release of oxidative or nitrosative damage biomarkers, including 15(*S*)-8-*iso*-PGF2α.

In the absence of other markers, it can be questioned whether venous 15(*S*)-8-*iso*-PGF2α release observed in subgroups of patients in this study reflects oxidative damage

or alternatively reflects activation of inflammatory pathways. Since all other markers refute oxidative damage, it is highly unlikely that 15(*S*)-8-*iso*-PGF2α is the sole reflection of oxidative damage. All together, the multiple biomarkers of oxidative and nitrosative damage showed no consistent release upon reperfusion, denying RONS-mediated tissue damage.

Absence of venous release of oxidative and nitrosative damage markers may suggest that oxidative and nitrosative stress during I/R is less pronounced than commonly thought or may even be absent. We evaluated activation of Nrf2, the master regulator in cellular oxidative defense pathways, in a DNA-binding ELISA that exclusively measures the activated form of this transcription factor. Moreover, the activation of the conical downstream pathway of Nrf2 was evaluated through pathway analysis. Clear increases in Nrf2 activation and its downstream pathway show that oxidative stress does occur during I/R.

Evaluation of the XO activity, an often referred source of oxygen radicals in I/R injury, did not indicate XO activity in *human* kidney, although abundant XO was found in *rat* liver. These observations are in line with previous reports showing abundant XO activities in rodent tissue but not in human tissue.[46] XO therefore appears not to be a prominent source of RONS generation in human tissue.

Limitations

This is a clinical study, and as such, procedures and timing were dictated by the operative procedures. We did include two clinically distinct situations of I/R to identify universal mechanisms of free radical-mediated damage after reperfusion. In human kidney transplantation, I/R included extremes in both short and long cold ischemia duration, the use of antioxidant preservation fluids, corticosteroids, and other immunosuppressive drugs. In cardiac surgery, I/R involved a normothermic ischemic period, and no antioxidants, corticosteroids, or other immunosuppressive drugs were administered. Throughout this whole spectrum of clinical conditions, independent of antioxidant use or immunosuppression, results unequivocally showed no dominant role of oxidative and nitrosative damage in human I/R injury.

Sampling in our study was restricted to less than an hour post-reperfusion. This time window should be adequate as it is generally assumed that I/R injury is an acute process, initiated directly after reperfusion.[18] This notion is supported by spin trap experiments indicating free radical formation within 15 minutes after reperfusion.[37] Yet, we can not exclude that I/R injury is less acute than commonly thought and that oxidative damage occurs after an hour of reperfusion.

A second limitation of the study is that it largely relies on repetitive measurement of arteriovenous concentration differences. Although this methodology has the advantage of specificity, and has been proven effective in elaborating the inflammatory response after

I/R,[14, 47] the high flow rates over the organs may compromise the sensitivity. To compensate for this limitation, we considered an evaluation of the first urine produced after I/R and measurement of the classical redox response genes relevant.

Conclusion

The findings from this study do not indicate a dominant role for RONS-induced damage in the pathophysiology of early I/R injury. The *production* of RONS during I/R is explicitly not questioned by our findings. In fact, Nrf2 activation and the higher PGE_2 levels in post-transplantation urine indicate increased COX activity after reperfusion, suggesting that oxidative *stress* does occur during early reperfusion. Yet, the generated RONS apparently do not induce tissue *damage* in human I/R. These observations suggest an efficient antioxidant system, providing an explanation for the limited efficacy of antioxidant therapy in human I/R. All together, this study challenges the prevailing paradigm of prominent involvement of oxidative damage in the initiation of human I/R injury.

Innovation

I/R injury is a common clinical problem, complicating myocardial infarction, cardiovascular surgery, and organ transplantation. As such, it is of great importance to unravel the exact pathophysiological mechanisms leading to I/R injury. This comprehensive clinical study systematically assessed the putative role of oxidative stress in human I/R injury in two diverse clinical settings. Findings for all biomarkers studied were highly consistent and did not indicate release of markers of oxidative damage from the reperfused organs nor did we observe upregulation of redox-response genes. As such, this study questions the involvement of RONS in the pathophysiology of I/R injury in humans.

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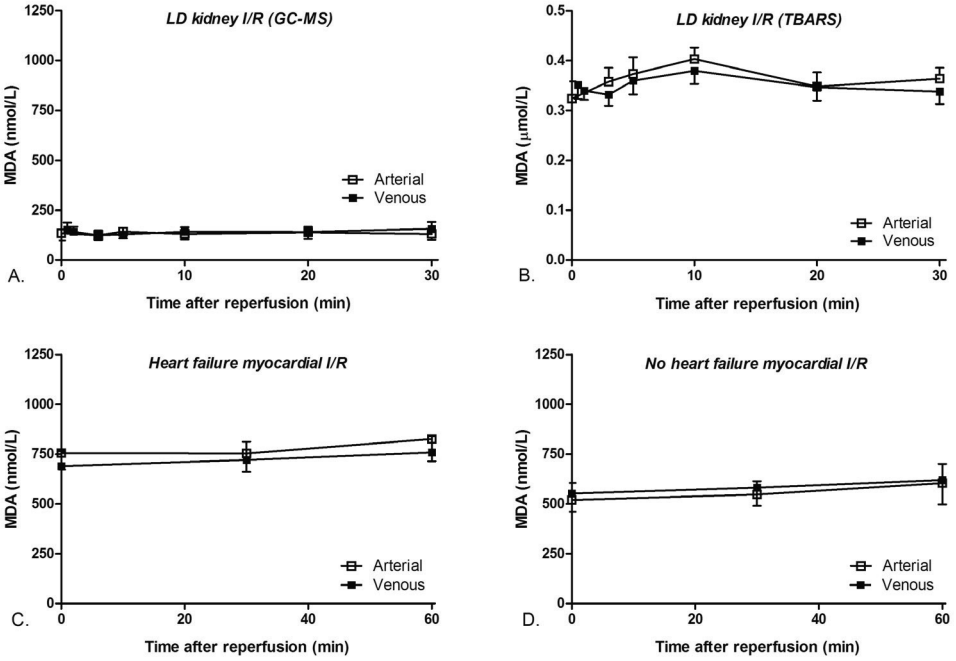
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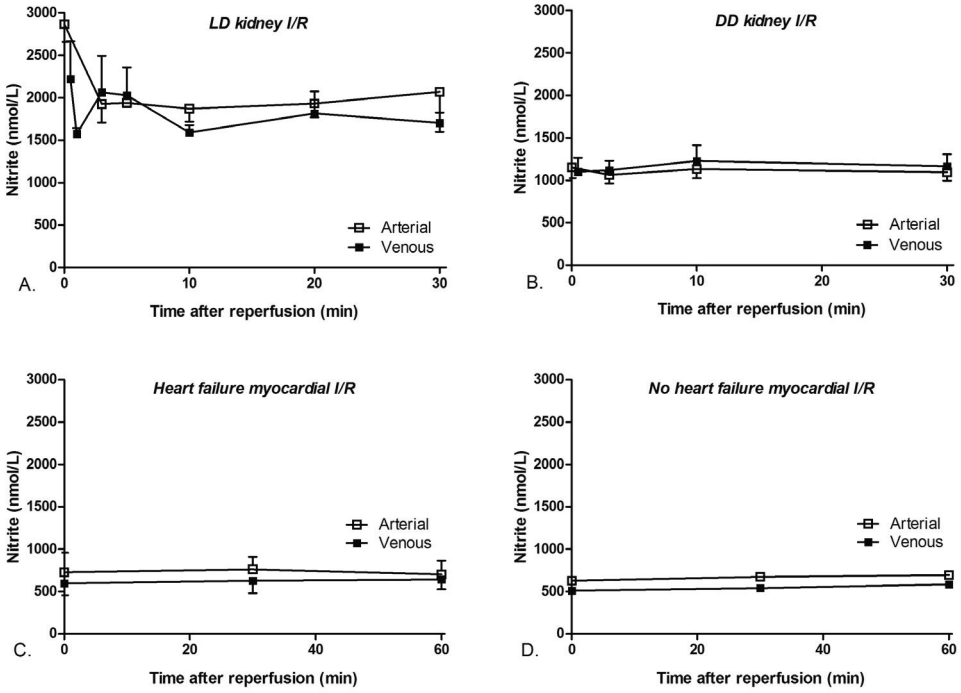
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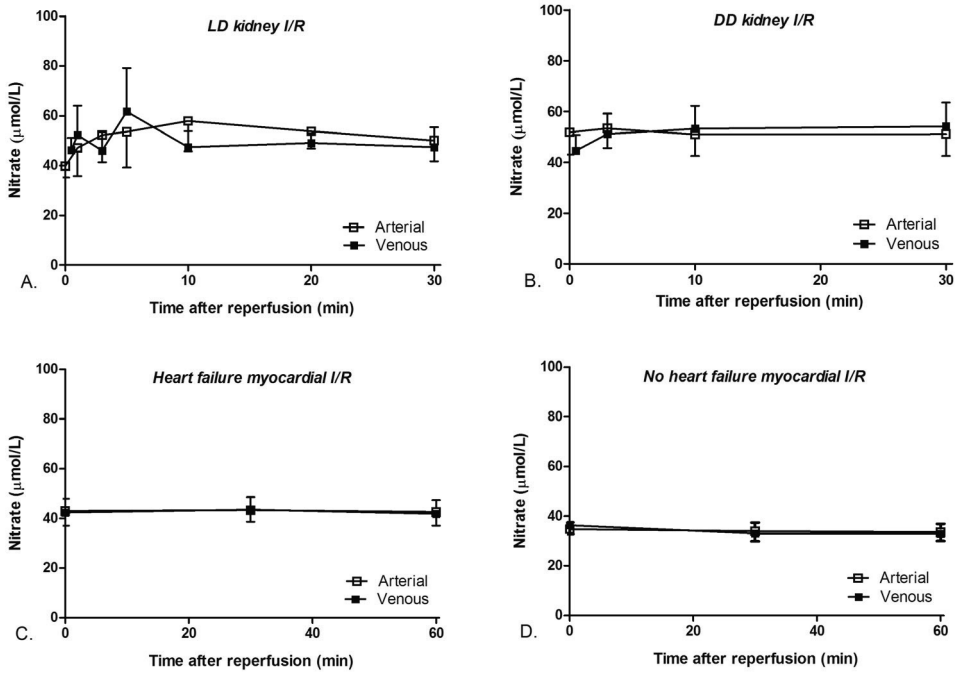
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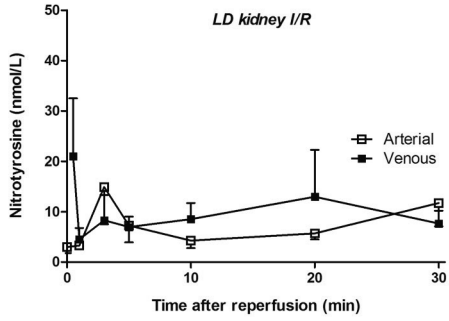
Supplementary data, Figure 1. Arterial and venous plasma concentrations of malondialdehyde.
(A) Arterial and renal venous plasma malondialdehyde (MDA) concentrations during the first 30 minutes of reperfusion in living donor (LD) kidney transplantation as determined by GC-MS/MS showed no MDA release ($P = 0.17$); (B) No MDA release was observed when determined by TBARS method ($P = 0.06$); (C) Arterial and myocardial venous plasma MDA concentrations during the first 60 minutes of reperfusion in patients with preexisting heart failure showed no release from the myocardium ($P = 0.05$). In fact, venous MDA levels tended to be lower than the arterial levels in patients with preexisting heart failure; (D) In patients without preexisting heart failure MDA was not released either ($P = 0.43$).



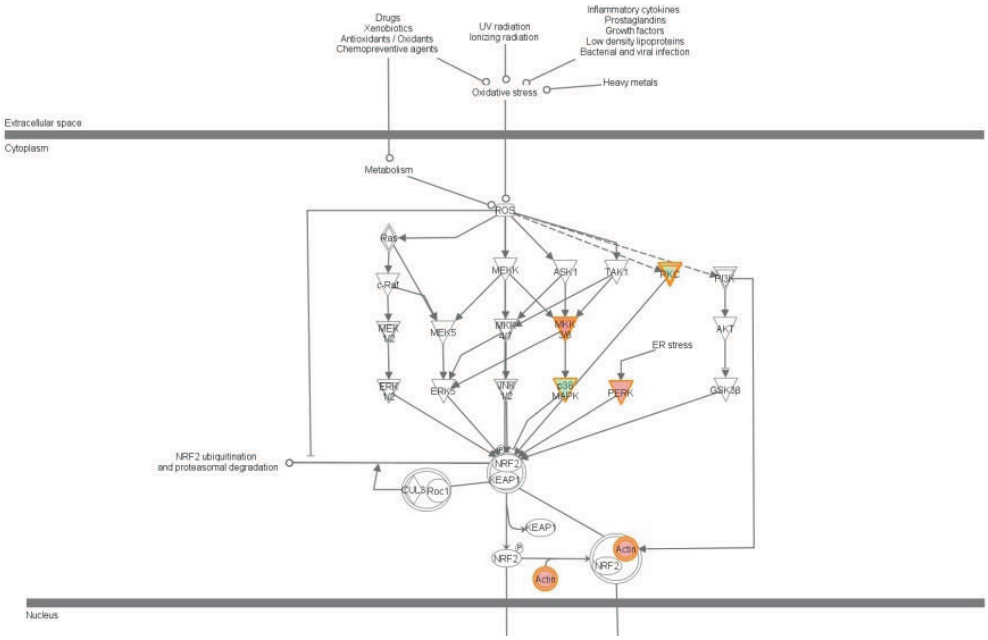
Supplementary data, Figure 2. Arterial and venous plasma concentrations of nitrite.
(A) Arterial and renal venous plasma nitrite concentrations during the first 30 minutes of reperfusion in living donor (LD) kidney transplantation showed no release from the kidney (P = 0.50); (B) In deceased donor (DD) kidney transplantation, nitrite was not released either during the first 30 minutes of reperfusion (P = 0.87); (C) Arterial and myocardial venous plasma nitrite concentrations during the first 60 minutes of reperfusion in patients with preexisting heart failure showed no myocardial release. However, a myocardial uptake was observed (P = 0.002); (D) Similarly, in patients without preexisting heart failure a myocardial nitrite uptake instead of a release was observed (P = 0.03).



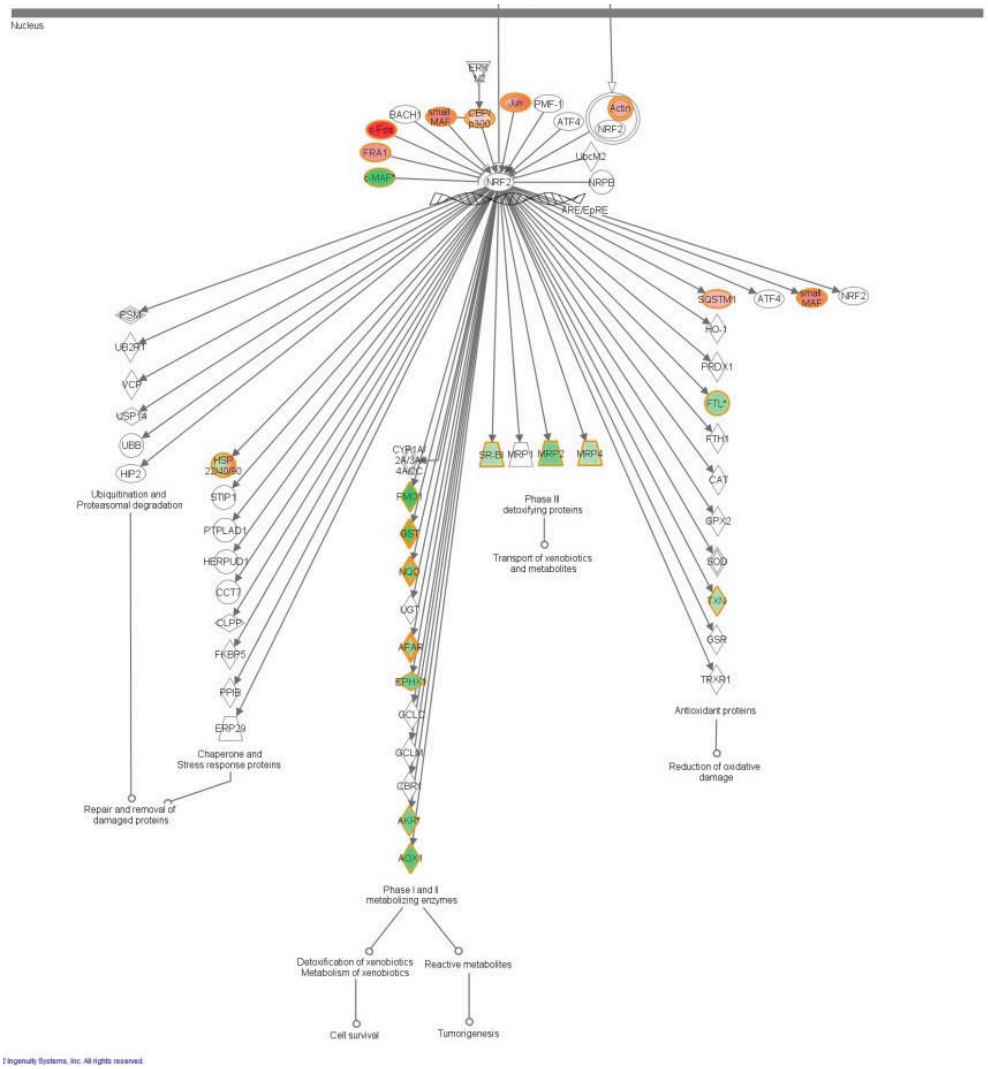
Supplementary data, Figure 3. Arterial and venous plasma concentrations of nitrate.
(A) Arterial and renal venous plasma nitrate concentrations during the first 30 minutes of reperfusion during living donor kidney (LD) transplantation showed no release from the kidney (*P* = 0.50); (B) During deceased donor (DD) kidney transplantation, nitrate was not released during the first 30 minutes of reperfusion (*P* = 0.98); (C) Arterial and myocardial venous plasma nitrate concentrations during the first 60 minutes of reperfusion in patients with preexisting heart failure showed no myocardial release (*P* = 0.53); (D) Similarly, in patients without preexisting heart failure no myocardial nitrate release was observed (*P* = 0.81).



Supplementary data, Figure 4. Arterial and venous plasma concentrations of nitrotyrosine.
These was no release of nitrotyrosine from living donor (LD) kidney grafts during early reperfusion (*P* = 0.93).

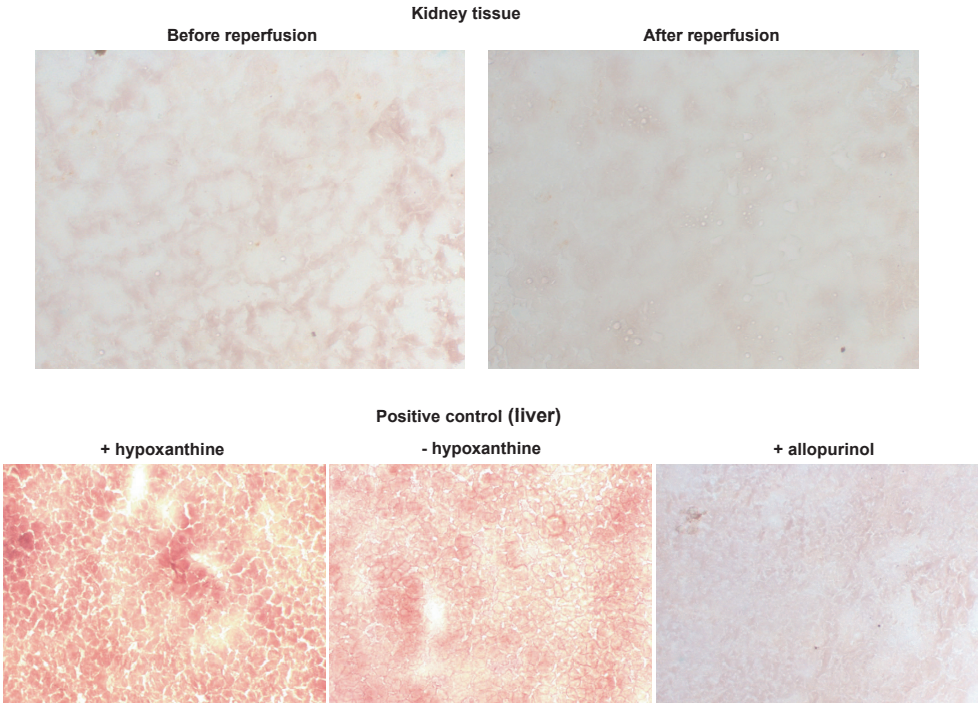


Supplementary data, Figure 5. Nuclear factor erythroid 2-related factor 2 downstream genes.
Pathway-based analysis of nuclear factor erythroid 2-related factor 2 (Nrf2) downstream genes showed highly significant upregulation of the canonical antioxidant responsive element (ARE) pathway in kidneys from living donors (LDs) ($P = 7.06*10^{-12}$).

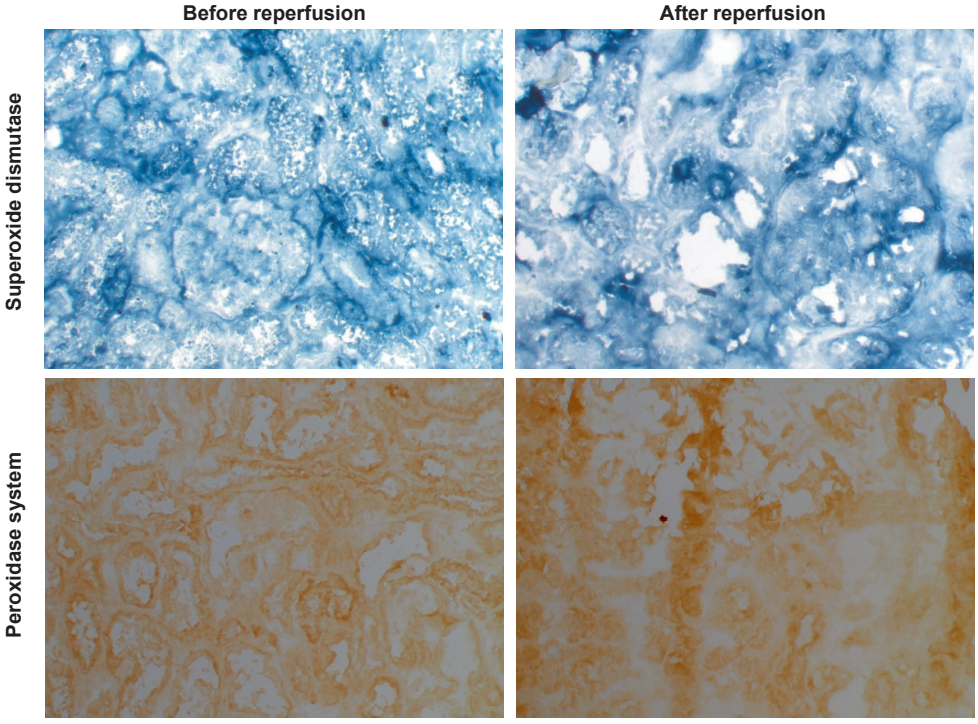


Supplementary data, Figure 5, continued. Nuclear factor erythroid 2-related factor 2 downstream genes.

Pathway-based analysis of nuclear factor erythroid 2-related factor 2 (Nrf2) downstream genes showed highly significant upregulation of the canonical antioxidant responsive element (ARE) pathway in kidneys from living donors (LDs) ($P = 7.06*10^{-12}$).



Supplementary data, Figure 6. Xanthine oxidase activity.
Histochemical analysis showing minimal xanthine oxidase (XO) activity in pre- and post-reperfusion kidney biopsies. Liver biopsies showing XO activity in the presence (left) and absence of hypoxanthine (middle) are included as a positive control. Staining in the absence of hypoxanthine implies endogenous hypoxanthine. Specificity of the analysis is shown by including the XO inhibitor allopurinol (right).



Supplementary data, Figure 7. Endogenous antioxidant enzymes.
Histochemical analysis showing that the endogenous antioxidant enzyme systems superoxide dismutase (SOD), and peroxidases (catalase and glutathione peroxidase), are active in pre- and post-reperfusion kidney biopsies.

CHAPTER FOUR

Eur J Cardiothorac Surg 2012;41:117-125

No prominent role for terminal complement activation in the early myocardial reperfusion phase following cardiac surgery

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Carla C **BAAN**, Cees **VAN KOOTEN**, Robert JM **KLAUTZ**

ABSTRACT

Background: Complement activation is considered an important mediator of myocardial ischemia/reperfusion (I/R) injury. Although complement inhibitors are highly effective in animals, clinical trials fail to show a substantial benefit in humans. This raises questions on the role of complement activation in human myocardial I/R injury.

Methods: Soluble C5b-9, i.e. terminal complement complex, and C5a were assessed in patients with non-ischemic (n = 10) and ischemic heart failure (n = 10), and patients without heart failure (n = 10) undergoing cardiac surgery. To study the pathophysiology of human I/R injury, a model of arteriovenous measurements over the reperfused heart was applied at consecutive time points during the early reperfusion phase. Furthermore, C3d and C5b-9 depositions in pre-reperfusion myocardial and endomyocardial tissue were evaluated and compared with pre-transplantation tissue from myocardial allografts.

Results: Simultaneous assessment of soluble C5b-9 and C5a in systemical and myocardial venous blood samples revealed the absence of net release from the reperfused heart in all three patient groups. Biopsies of patients with non-ischemic heart failure showed the most abundant myocardial depositions of C3d and C5b-9: 4.8 times more C3d (P = 0.008) and 4.7 times more C5b-9 (P = 0.004) than donor tissue. Also C3d was abundantly present in endomyocardial tissue of both heart failure groups compared with donors (both P = 0.02).

Conclusions: No evidence was obtained that terminal complement activation is involved in the acute phase following myocardial reperfusion. Since complement deposition was already present before reperfusion, human complement inhibition might be more beneficial in the preoperative phase than during reperfusion.

INTRODUCTION

Ischemia/reperfusion (I/R) injury is considered a major contributor to reversible but also to irreversible cellular damage in multiple clinical situations such as heart transplantation, myocardial infarction and cardiovascular surgery. The pathophysiology of I/R injury is complex and incompletely understood. The innate immune system has been suggested to provide short-term adaptive responses to tissue injury. If the innate immune signaling becomes sustained or excessive, the activation of these beneficial homeostatic pathways is abolished by deleterious effects of inflammatory signaling.[1]

The complement system is one of the basal defense mechanisms of the innate immune system consisting of a complex cascade of plasma proteins. The complement system is activated via three distinct pathways: the classical, alternative and lectin pathway. The end-result is activation of C5b-9, the cell-lysing membrane attack complex or terminal complement complex. When well-balanced and controlled, complement activation supports the defense against infections and facilitates wound healing. However, if the complement cascade is excessively activated it contributes to organ dysfunction leading to increased morbidity and mortality.[2]

Complement activation is considered one of the key mediators of myocardial I/R-induced tissue damage.[3-7] During cardiac surgery, the complement cascade is activated as a result of the surgical trauma, use of cardiopulmonary bypass (CPB) and I/R injury.

Complement inhibitors are highly effective in reducing tissue damage in animal studies of myocardial I/R injury.[4, 8, 9] However, anti-complement therapy in human myocardial I/R injury has lead to differential results without clinical relevant benefits.[10-12] Whether the pathophysiology of human myocardial I/R injury is based on complement activation may therefore be questioned. The limited human studies concerning complement activation in myocardial I/R injury have shown that there is an increased systemical activation of complement components the first hours after reperfusion.[5, 6] Those systemic levels show a biphasic pattern with inhibitory mechanisms from eight hours postoperatively onward.[7] Those previous studies have used systemic values as read-out of local myocardial complement activation.

In this clinical study, an integrative approach was used to measure simultaneously activated complement components in coronary sinus and radial artery blood for up to 24 hours after reperfusion. This set-up allows to specifically measure the myocardial uptake or release of circulating complement components such as C5a and soluble (s)C5b-9 instead of only measuring systemic values. In addition to the simultaneous collected blood samples, tissue samples were taken from the left ventricle during surgery and stained for C3d and C5b-9. The purpose of the present study was to examine the role of complement activation in the early myocardial reperfusion phase following cardiac surgery.

METHODS

Patient characteristics

To investigate the role of local complement activation in early I/R injury, 30 patients scheduled for elective cardiac valve surgery with use of CPB were studied. Ten patients with idiopathic non-ischemic heart failure were scheduled for an external constraint device. In addition, ten patients with ischemic heart failure were scheduled for a ventricular reconstruction after a large infarction (n = 5) or a coronary artery bypass grafting procedure (n = 5). Ten patients without heart failure undergoing cardiac surgery were also included. Their valve regurgitation was caused by organic pathology, with a predominance of prolapse disease. Heart failure was defined as an inadequate pump function of the heart with an echocardiographically estimated ejection fraction biplane below 35%[13] and the presence of one or more clinical symptoms such as dyspnea – reflected by their NYHA class[14] –, edema and fatigue existing for more than three months. All patients without heart failure had a moderate to normal pump function of the heart. These patients had no clinical signs of heart failure by physical examination and did not use high doses of diuretics. Exclusion criteria were perioperative corticosteroid treatment, minimal invasive surgical procedures, emergency operations and previous cardiac surgery. All included patients did not receive heparin, anti-complement therapy or immunosuppressive drugs before surgery, known to influence the measurements of complement activation markers. This single center study was approved by our local ethics committee and all patients provided written informed consent.

Anesthesia and surgical procedures

All participating patients received standardized anesthetic procedures, according to a fast-track protocol. Patients were premedicated with oral lorazepam 1-2 mg the evening and morning before surgery. The anesthetic regimen consisted of a continuous target controlled infusion of propofol with targets adjusted between 1.6 and 2 µg/mL to maintain a bispectral index value between 40 and 60. Sufentanil was infused at 0.1 µg/kg/hour and remifentanil target controlled infusion was guided by hemodynamic response on stimuli. If necessary, inotropics – dobutamine, norepinephrine, enoximone or a combination – were used. Cardiac surgery was performed according to the local standardized protocol. All surgical procedures were performed via a midline sternotomy under normothermic CPB (Jostra Maquet, Maquet, Hirrlingen, Germany) with intermittent antegrade warm-blood cardioplegia. The CPB system was coated with a heparin softline coating. Exclusion criteria related to the use of this CPB system were autologous priming and ultrafiltration, influencing the measurements of inflammatory mediators. In all patients, a mitral valve annuloplasty –

repair – procedure was performed. There is the possibility of introducing a coronary sinus catheter during this surgical procedure, since the transseptal approach of the mitral valve is the standard procedure in our hospital.

Biopsies

Five patients were selected from each patient group to obtain at two time points pre-reperfusion biopsies from the left ventricle (Figure 1). These patients were selected by a blinded student based on their aortic cross-clamp time and on the time-frame between the collection of the biopsies and start of aortic cross-clamping. This method was applied to create comparability for the immunohistochemical analyses between the groups. In specific, the ischemic heart failure group contained two biopsies of patients scheduled for a ventricular reconstruction procedure and three biopsies of patients scheduled for a coronary artery bypass procedure. The early pre-reperfusion biopsies were taken through the mitral valve early after aortic cross-clamping. In other words, these biopsies were taken directly after opening of the heart and are therefore a good measure for preoperative involvement of complement activation. The late pre-reperfusion biopsy was obtained from the same location just before releasing the aortic cross-clamp, the moment of reperfusion. Only pre-reperfusion biopsies could be collected since most of the included heart failure patients were hemodynamically instable in the early reperfusion phase. Lifting the heart, to take a post-reperfusion biopsy from the left ventricle, was considered unsafe. Biopsies were immediately snap frozen in liquid nitrogen and stored at -70°C until analysis. To compare the tissue of our pre-reperfusion biopsies, pre-transplantation tissue was collected from myocardial allografts of four brain-death donors.

Arteriovenous measurements

Arterial (radial artery, 10 mL) and myocardial venous blood samples (coronary sinus, 10 mL) were collected over the reperfused heart in all 30 patients (Figure 1). An arterial catheter was routinely placed in the radial artery. To collect the myocardial venous blood, a 5 French indwelling jugular vein catheter (PICC, Arrow International Inc, PA, USA) was inserted in the right atrium and was later, under direct vision of the surgeon, placed in the coronary sinus. This specific set-up allowed us to assess locally activated complement components in the early reperfusion phase. Simultaneous obtained arterial and myocardial venous blood samples were collected during the early reperfusion phase: 0, 15, 30, and 60 minutes after reperfusion. Two additional blood samples were drawn by venapunction the day before surgery (baseline) and 24 hours after reperfusion from the brachial vein (both 10 mL) and arterial catheter, respectively. All samples were collected in pre-cooled tubes containing

ethylenediaminetetraacetic acid (BD Vacutainer, Plymouth, UK) and immediately placed on melting ice. Blood samples were centrifuged (1.550 *g*, 10 min, 4°C) and plasma was re-centrifuged (10.000 *g*, 4 min, 4°C) to obtain leukocyte and thrombocyte free plasma. Aliquots were stored at -70°C until analysis.

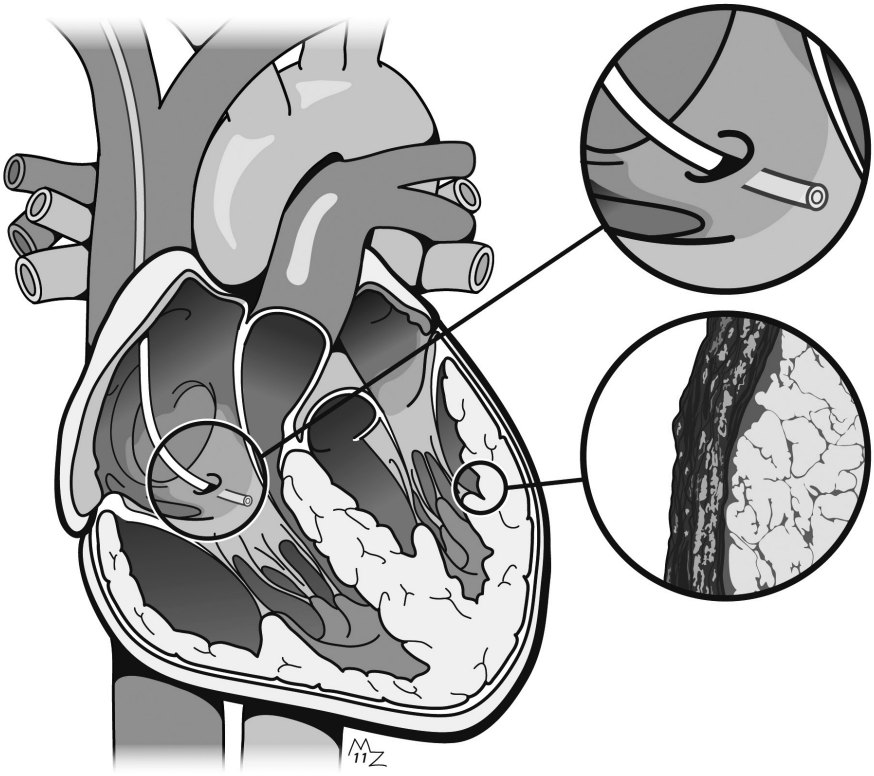


Figure 1. Schematic representation of the coronary sinus catheter and location of the biopsy. This figure shows the position of the coronary sinus catheter in situ to collect myocardial venous blood samples. Moreover, the location of the left ventricular biopsy is depicted in this illustration showing endomyocardial and myocardial tissue.

Immunohistochemistry

Sections (5 µm) of snap-frozen biopsies were air dried and acetone-fixed for 10 min. C3d and C5b-9 deposition was assessed using a monoclonal antibody to a neo-epitope on C3d (Quidel, San Diego, CA, USA) or C5b-9 (aE11; Hycult Biotechnology, Uden, The Netherlands), respectively. Antibody binding was detected with horseradish peroxidase (HRP)-labeled goat antimouse Ig (DAKO, Glostrum, Germany). After washing, sections

were incubated with tyramide-fluorescein isothiocyanate in tyramide buffer (NENTM Life Science Products, Boston, MA, USA), washed and incubated with HRP-labelled rabbit anti-fluorescein isothiocyanate (DAKO, Glostrum, Germany) and developed with DAB (Sigma, St Louis, MO, USA). Sections were counterstained with hematoxylin (Merck, Darmstadt, Germany) and mounted with imsol (Klinipath, Duiven, the Netherlands). Quantification of immunohistochemistry was performed in a blinded manner by assessing ten consecutive high power fields (magnification, ×200) of myocardial and endomyocardial tissue on each section. Using Image J software®, the positive area in each image expressed in pixels was determined.

Plasma measurements

sC5b-9 and C5a levels were assessed by sandwich ELISA. In brief, 96-well ELISA plates (Nunc Bioscience, Belgium) were coated with a monoclonal antibody to a neo-epitope on C5b-9 (aE11; Hycult Biotechnology, Uden, The Netherlands) or monoclonal antibody to C5a (HM2079b; Hycult Biotechnology, Uden, The Netherlands), respectively. Plasma was incubated in the coated well and bound sC5b-9 or C5a was detected with a biotin-labeled monoclonal anti-C6 antibody (9C4; Hycult Biotechnology, Uden, The Netherlands) or anti-C5a antibody (CL.561; Hycult Biotechnology, Uden, The Netherlands), respectively, followed by detection with streptavidin–poly-HRP (Sanquin, Amsterdam, The Netherlands). Enzyme activity was detected using 2,2-azino-bis3-ethylbenzothiazoline-6-sulphonic acid (Sigma Chemical Co., St. Louis, MO, USA). The optical density was measured at 415 nm using a microplate reader (Model 680; Biorad, Philadelphia, PA, USA). The detection limit for C5a was 1.95 ng/mL and for C5b-9 0.01 U/mL.

Statistical analysis

Differences between patient groups were analyzed using the Kruskal-Wallis test with *post-hoc* analyses with Mann-Whitney *U*-tests whenever appropriate. Categorical variables were compared using the Chi-square test. Comparisons between baseline values and values at 24 hours after reperfusion were analyzed with a Wilcoxon signed ranks test. The area under the curve (AUC) was calculated for the arterial and venous curves of the plasma measurements for the early (0-60 minutes) reperfusion phase. A delta AUC was calculated (venous minus arterial) and the null-hypothesis (delta AUC is 0) was tested by a paired *t*-test. Statistical analysis was performed using SPSS statistical analysis software version 17.0 (SPSS Inc, Chicago, IL, USA). A *P* value less than 0.05 was considered significant (two-sided).

RESULTS

Patient population

Patient characteristics and perioperative details are summarized in Table 1. Two patients died in hospital: one patient with non-ischemic heart failure died eight days after surgery as a result of ventricular fibrillation and the other patient with ischemic heart failure died 41 days after surgery as a result of therapy-resistant heart failure after a ventricular reconstruction.

Immunohistochemistry

To evaluate whether local complement deposition plays a role in myocardial I/R injury, presence and localization of C3d and C5b-9 were studied in human left ventricular tissue collected in the early phase of the ischemic period of the heart, i.e. the pre-reperfusion phase. No differences in deposition of C3d and C5b-9 were observed between the early and late pre-reperfusion biopsies (Supplementary data, Figure 1). Therefore, only the early pre-reperfusion biopsies were used, as read-out for preoperative involvement of complement activation. Immunohistochemical stainings showed deposition of C3d and C5b-9 in the vascular wall of small arteries in all pre-reperfusion biopsies (Figure 2).

C3d and C5b-9 deposition in myocardial tissue

Patients with non-ischemic heart failure showed the most abundant myocardial depositions of C3d and C5b-9: 4.8 times more C3d (P = 0.008) and 4.7 times more C5b-9 (P = 0.004) than donors. C3d deposition was accompanied by extensive C5b-9 deposition in patients with non-ischemic heart failure, which was less pronounced in patients with ischemic heart failure and patients without heart failure (Figure 3).

Table 1. Baseline, surgical and outcome characteristics per patient group.

	Median (interquartile range)			Kruskal-Wallis, *P* value	Mann-Whitney, *P* value		
	NIHF (n = 10)	IHF (n = 10)	NHF (n = 10)		NIHF vs. IHF	IHF vs. NHF	NIHF vs. NHF
Age (years)	64.26 (55.89-69.66)	68.34 (57.44-75.42)	65.78 (62.07-78.13)	0.35	-	-	-
NYHA class	3.00 (3.00-3.00)	3.00 (2.88-3.00)	2.00 (1.38-2.50)	< 0.001	NS	< 0.001*	< 0.001*
Baseline CRP (mg/L)	4.00 (1.50-7.25)	2.75 (1.50-4.25)	1.50 (1.50-2.13)	0.10	-	-	-
Baseline creatinine (µmol/L)	78.00 (70.50-101.50)	100.50 (83.75-120.50)	88.00 (72.25-105.00)	0.20	-	-	-
AoX time (min)	88.00 (60.50-96.50)	178.50 (144.50-191.50)	110.00 (91.25-130.50)	< 0.001	< 0.001*	0.01*	0.03*
CPB time (min)	133.50 (122.25-169.00)	233.00 (180.25-264.50)	151.50 (137.50-178.50)	0.008	0.008*	0.02*	NS
Ventilation time (hours)	10.50 (7.00-15.50)	16.50 (7.25-30.25)	8.50 (5.75-13.25)	0.18	-	-	-
Creatinine day after surgery (µmol/L)	70.50 (59.75-117.75)	103.00 (78.00-143.50)	77.50 (63-100.50)	0.17	-	-	-
Total stay in ICU (hours)	45.00 (21.00-75.75)	57.00 (28.75-120.75)	23.00 (21.38-24.25)	0.12	-	-	-
Total stay in hospital (days)	13.00 (9.75-23.25)	17.00 (10.75-32.25)	14.00 (10.75-19.00)	0.75	-	-	-

* $P < 0.05$ was considered significant.

Abbreviations: AoX, Aortic cross-clamping; CPB, Cardiopulmonary Bypass; CRP, C-Reactive Protein; ICU, Intensive Care Unit; IHF, Ischemic Heart Failure; NHF, Non-Heart Failure; NIHF, Non-Ischemic Heart Failure; NYHA, New York Heart Association.

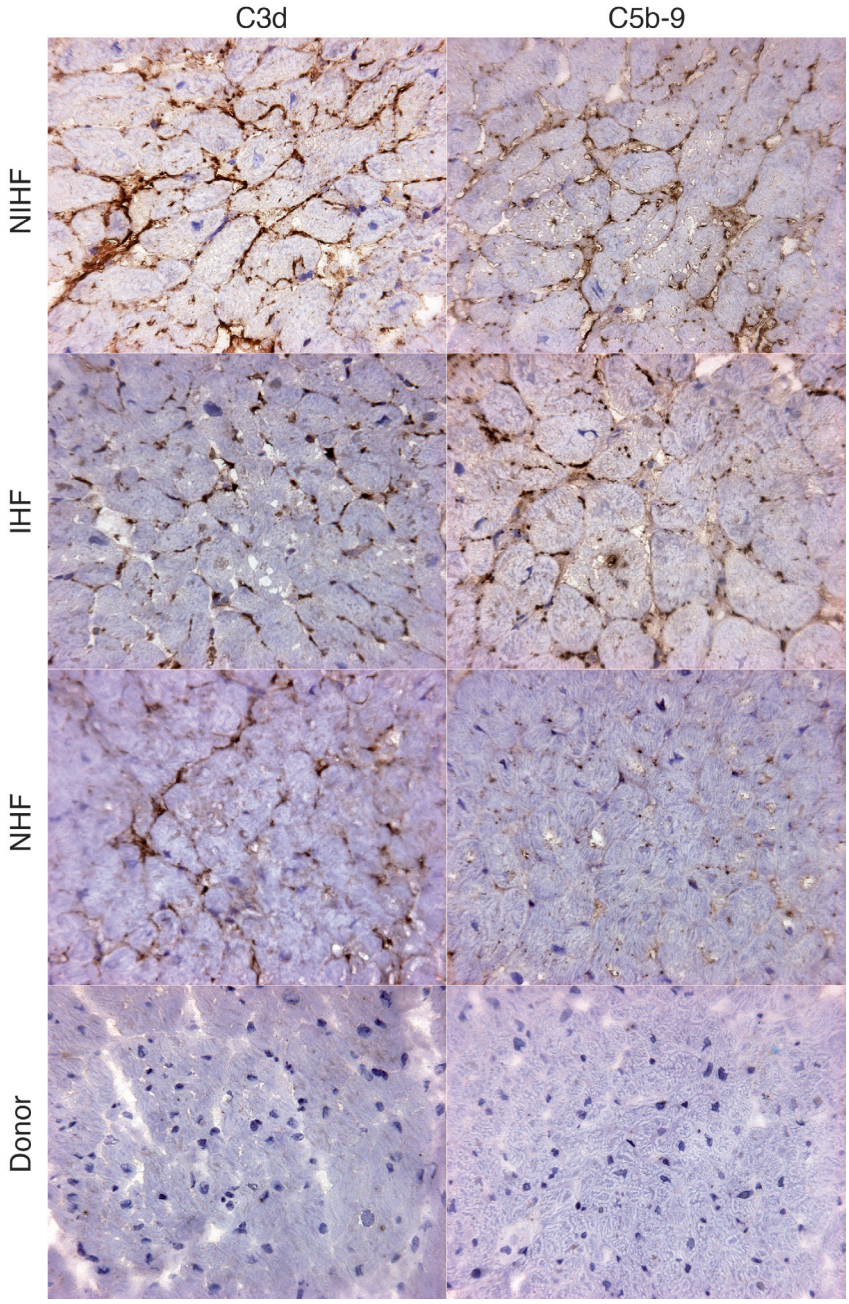


Figure 2. C3d and C5b-9 depositions in pre-reperfusion myocardial tissue.
Early ischemic myocardial tissue of patients with non-ischemic heart failure (NIHF), ischemic heart failure (IHF), non-heart failure patients (NHF) and donors were stained for C3d and C5b-9. Representative images are shown. Original magnifications x200.

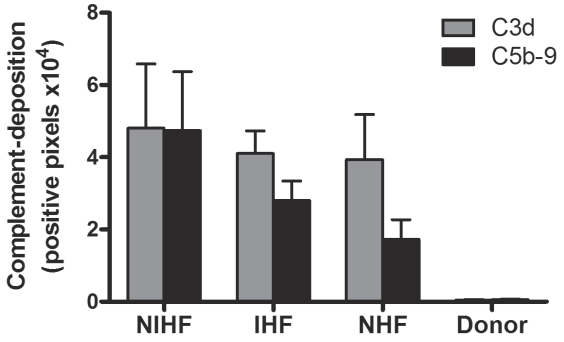


Figure 3. Quantification of complement deposition in myocardial tissue.
The amount of C3d was significantly different between patients with non-ischemic heart failure and donors (P = 0.008). Also C5b-9 was significantly higher in tissue of patients with non-ischemic heart failure compared with donor tissue (P = 0.004). Graph error bars represent mean ± SEM.
Abbreviations: IHF, ischemic heart failure; NHF, non-heart failure; NIHF, non-ischemic heart failure.

C3d and C5b-9 deposition in endomyocardial tissue

Unexpectedly, a distinct difference in C3d deposition was observed in the pre-reperfusion endomyocardial tissue of the various patient groups (Figure 4). C3d deposition was abundantly present in endomyocardial tissue of both patients with non-ischemic and ischemic heart failure in comparison to donor biopsies (both P = 0.02, Figure 5). This C3d deposition was, however, not correlated to preoperative NT-proBNP values (data not shown). No significant differences in endomyocardial C5b-9 deposition were observed between the groups (P = 0.06). Overall, endomyocardial tissue showed less C5b-9 deposition in all groups compared with myocardial tissue.

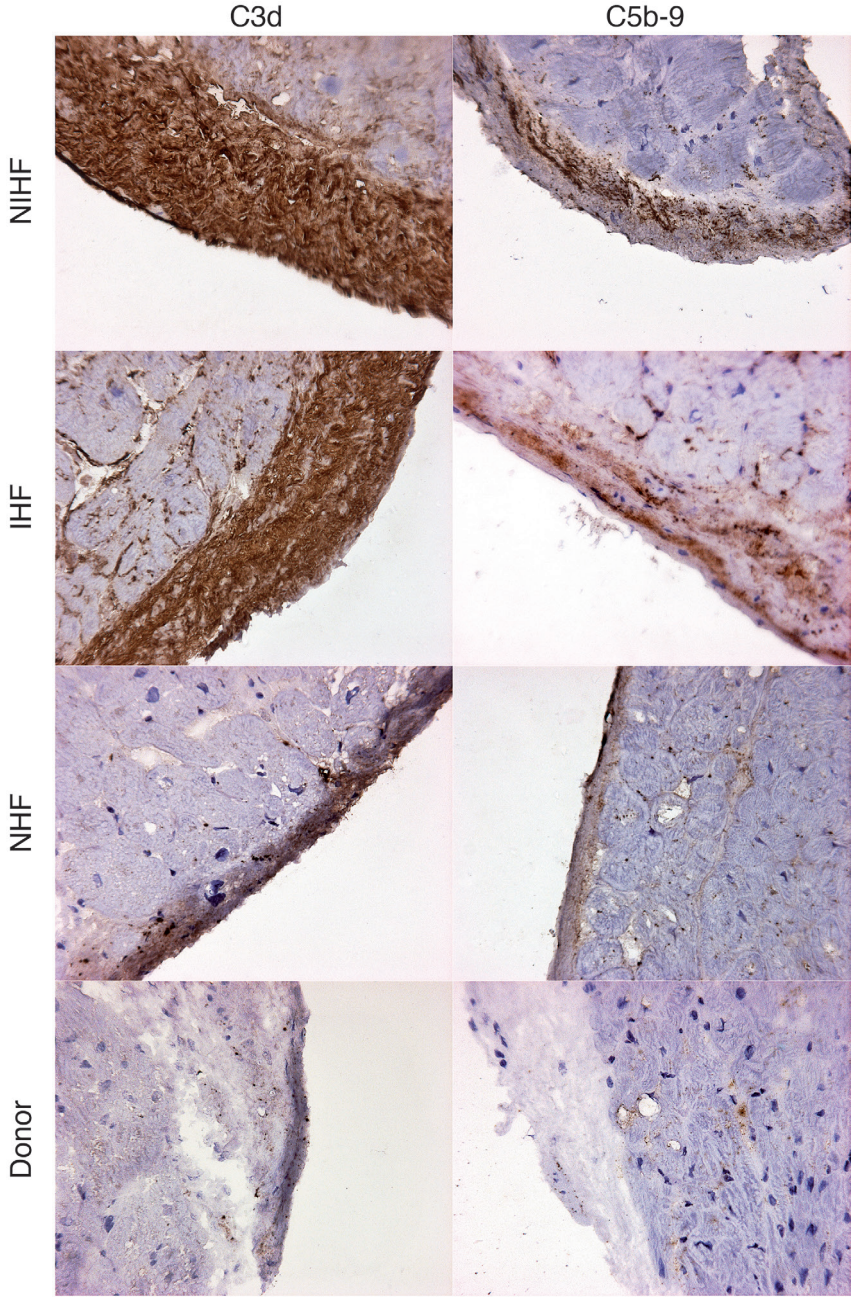


Figure 4. C3d and C5b-9 deposition in pre-reperfusion endomyocardial tissue.
Early ischemic endomyocardial tissue of patients with non-ischemic heart failure (NIHF), ischemic heart failure (IHF), non-heart failure patients (NHF), and donors were stained for C3d and C5b-9. Representative images are shown. Original magnifications x200.

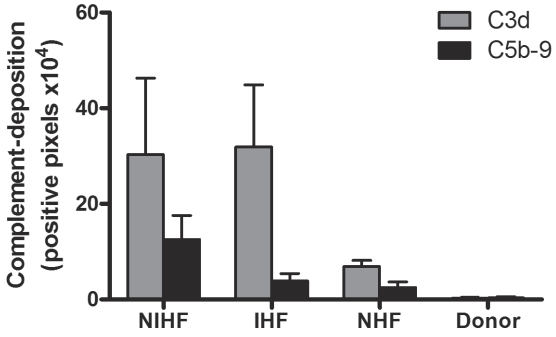


Figure 5. Quantification of complement deposition in endomyocardial tissue.
C3d was abundantly present in tissue of both patients with non-ischemic (NIHF) and ischemic heart failure (IHF) compared with donor tissue (both $P = 0.02$). Similar endomyocardial C5b-9 deposition was observed between the groups ($P = 0.06$). Graph error bars represent mean ± SEM.
Abbreviations: IHF, ischemic heart failure; NHF, non-heart failure; NIHF, non-ischemic heart failure.

Plasma measurements

Assessment of complement depositions of C3d and C5b-9 in pre-reperfusion biopsies indicated clearly that the complement system was activated in the very early stages of ischemia. To assess also plasma complement activation in the reperfusion phase, terminal complement complex activation was evaluated by measuring the release of sC5b-9 from the heart. This sC5b-9 release was assessed in the simultaneously collected systemical and myocardial venous blood samples. In addition, C5a was measured to assess whether the more upstream complement cascade is activated without causing the formation of the sC5b-9 complex (Figure 6).

No myocardial release or uptake of C5a or sC5b-9 in the early reperfusion phase
No significant differences in sC5b-9 or C5a were measured over the heart, indicating the absence of net release of both complement activation markers from the reperfused heart during the first 60 minutes of reperfusion (Table 2). This suggests that the observed complement deposition in myocardial tissue was generated already in the preoperative phase.

Similar levels of C5a and sC5b-9 at baseline and 24 hours after reperfusion
Not only arteriovenous concentration differences were studied. Similar baseline systemic levels of C5a ($P = 0.31$) and sC5b-9 ($P = 0.75$) were observed between the three patient groups the day before surgery. Also at 24 hours after reperfusion, similar systemic levels of C5a ($P = 0.20$) and sC5b-9 ($P = 0.92$) were observed between the three patient groups. C5a

and sC5b-9 returned even to baseline levels within 24 hours after reperfusion in all patient groups with the exception of slightly lower sC5b-9 values in patients with ischemic heart failure ($P = 0.03$).

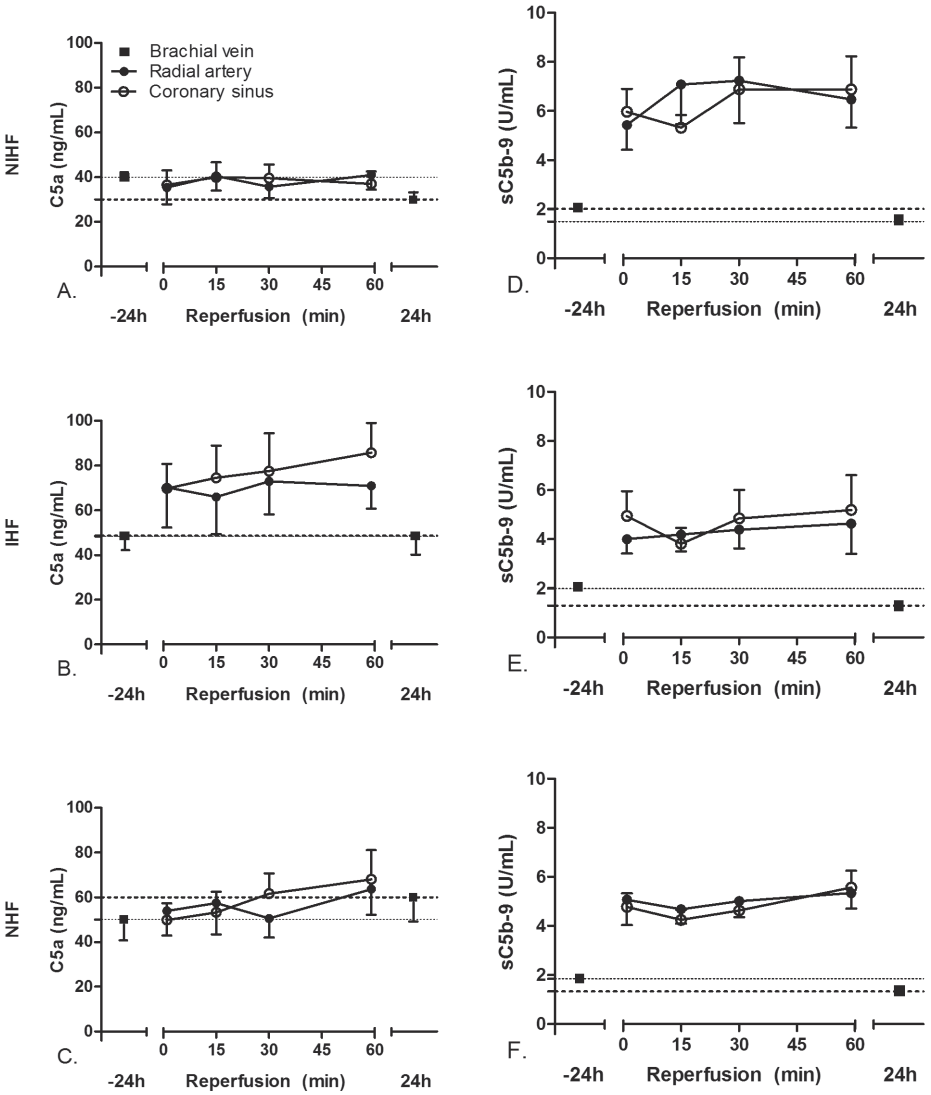


Figure 6. No local C5a and sC5b-9 release or uptake during the early myocardial reperfusion phase. No arteriovenous differences, expressed as mean ± SEM, were observed in all three patient groups: patients with non-ischemic heart failure (NIHF) [(A) = C5a, $P = 0.20$; (D) = sC5b-9, $P = 0.30$] and ischemic heart failure (IHF) [(B) = C5a, $P = 0.051$; (E) = sC5b-9, $P = 0.08$], and non-heart failure patients (NHF) [(C) = C5a, $P = 0.12$; (F) = sC5b-9, $P = 0.51$].

Table 2. No arteriovenous concentration differences in all three patient groups.

		Radial artery AUC	Coronary sinus AUC	Delta AUC	*P* value
NIHF	**C5a (µg/mL*min)**	2.20 (1.35-3.15)	2.29 (1.39-3.12)	0.09	0.20
	C5b-9 (U/mL*min)	407.47 (242.44-536.83)	406.85 (181.22-621.05)	-0.62	0.30
IHF	**C5a (µg/mL*min)**	4.83 (3.20-6.24)	4.35 (2.84-6.90)	-0.48	0.051
	C5b-9 (U/mL*min)	271.32 (138.71-410.24)	269.24 (126.65-390.05)	-2.08	0.08
NHF	**C5a (µg/mL*min)**	3.31 (2.30-3.65)	3.32 (2.03-4.19)	0.01	0.12
	C5b-9 (U/mL*min)	287.18 (247.76-332.85)	300.92 (222.07-355.85)	13.74	0.51

No release by the myocardium was observed for C5a or C5b-9 in patients with non-ischemic heart failure (NIHF), ischemic heart failure (IHF), and non-heart failure patients (NHF). A positive delta area under the curve (AUC) indicates a myocardial release of C5a or C5b-9, whereas a negative delta AUC represents a myocardial uptake. Means of the AUCs and interquartile ranges are shown.

DISCUSSION

It is generally accepted that complement plays a major role in myocardial I/R injury. However, since clinical trials consistently fail to show clinical relevant benefits of complement inhibitors, the role of complement activation in human myocardial I/R injury should be considered carefully.

The present clinical study shows that myocardial C3d and C5b-9 depositions are already present in early left ventricular pre-reperfusion biopsies. Most abundant deposition was observed in patients with non-ischemic heart failure. Endomyocardial biopsies, on the other hand, show clear depositions for C3d in both patients with non-ischemic and ischemic heart failure. These abundant early endomyocardial C3d depositions might be an underlying mechanism of heart failure, since these biopsies were used as read-out for preoperative involvement of complement activation. In addition, when comparing the heart failure groups with the donor group, also early endomyocardial C5b-9 deposition seems higher in the heart failure groups although not significantly different. Using our model of arteriovenous measurements, our results strongly suggest that terminal complement activation is not involved in the acute phase following myocardial reperfusion.

The observed myocardial and endomyocardial depositions of C3d and C5b-9 in our study are in line with previous human studies. However, these studies used autopsy or allograft tissue to investigate myocardial complement deposition. To our best knowledge, this is the first time that viable myocardial tissue is used to study myocardial and endomyocardial complement deposition. Nijmeijer et al. used autopsy tissue of patients who died as a result of an acute myocardial infarction.[15] Depositions of C3d and C5b-9 in patients treated with reperfusion or suffering from reinfarction were compared with depositions of both markers in patients who had no reperfusion or reinfarction. Patients with a reinfarction or with

reperfusion therapy showed more depositions of these two complement activation markers. Unfortunately, no comparisons between their and our study could be made for activation of complement components in plasma, since no plasma measurements were performed in addition to immunohistochemistry. In a study of Jenkins et al. myocardial tissue, obtained by autopsy, was stained for C4d and C9 after myocardial infarction.[16] Normal myocytes were non-reactive, whereas necrotic myocytes reacted strongly with the C4d and C9 antibodies. In our study, allograft tissue from donor hearts stained also negative for C3d and C5b-9.

As a result of the clear complement depositions in pre-reperfusion biopsies in our study, we were interested in the myocardial release of complement components upon myocardial reperfusion. By measuring arteriovenous differences over the reperfused organ, we were able to obtain data on local release of sC5b-9 and C5a from the heart. However, arteriovenous concentration differences did not indicate a myocardial release or uptake of C5a or sC5b-9.

Although no sC5b-9 or C5a release was observed in any of the tested groups, there was a remarkable increase in systemical sC5b-9 levels observed upon reperfusion in comparison to baseline, indicating activation of the immune system. This increase in complement components is in line with previous studies.[5, 17, 18] Remarkably, C5a levels were relatively high in patients with ischemic heart failure (e causa ignota). There might be a less effective uptake and removal of C5a from plasma by granulocytes via cellular receptors such as leucocyte C5a receptors. There also might be a difference in carboxypeptidase N activity between the patient groups. This enzyme cleaves the COOH-terminal arginine from C5a forming C5a des arginine. This C5a/C5a(desArg) complex has been measured with our C5a assay. The systemic increase of C5a and C5b-9 is not related to release of both activation markers from the heart, since our arteriovenous measurements over the heart did not show such a release. The systemic increase might be explained by the use of CPB and the surgical injury itself. However, the exact source remains to be unravelled. Baseline levels were similar between heart failure patients and non-heart failure patients in our study. This is in contrast to a study of Yasuda et al., which showed increased levels of C3 split-products and sC5b-9 in patients with ischemic heart failure.[19] The increased baseline levels in their study might be explained by the fact that their patients suffered an acute myocardial infarction before the plasma measurements. Our patients did not have an acute myocardial infarction prior to cardiac surgery and were classified as chronic heart failure patients.

Despite the fact that in animal studies of myocardial I/R injury beneficial effects are observed following complement inhibition[4, 8, 9], only few complement inhibitors reached clinical trials such as anti-C5 antibodies[20], soluble complement receptor 1 (TP10)[21] and C1-esterase inhibitor.[11] However, all consistently show minute and inconsistent effects of anti-complement therapy in humans. These disappointing results raise the question whether animal models are sufficiently comparable to the human situation with relation to innate

and adaptive immunity.[22] Another explanation might be that there is a narrow window of clinical opportunity in humans when treatments can be therapeutically effective[23], depending on timing, tissue penetration, patient characteristics, single versus multiple cycle administration and many others. Based on our results, it may be more beneficial to use complement inhibitors during the chronic state of heart failure by contributing to anti-inflammatory processes instead of during reperfusion. Furthermore, it might be that in humans complement is not really a key mediator of reperfusion injury. This may well explain the unsuccessful outcomes of anti-complement therapy during the early reperfusion phase so far.

Limitations

Our results show that terminal complement activation is not involved in the initiating phase of myocardial I/R injury. However, we only focused on the first 60 minutes of reperfusion and complement activation may result from other processes later in the cascade of I/R injury. This is confirmed in an animal study following renal I/R injury in which C3 deposition was present two hours after reperfusion although C6 and C9 depositions were present twelve hours after reperfusion.[24] Complement might therefore have a role in repair or removal of damaged cells in the late reperfusion phase instead of an initiating role in the acute reperfusion phase. This concept matches the observed depositions of C5a and C5b-9 in patients with heart failure in our present study, who are already exposed to damage before surgery. In this study, only ischemic pre-reperfusion biopsies could be collected since most of the included heart failure patients were hemodynamically instable in the early reperfusion phase. Myocardial tissue obtained during human autopsy is known for excessive deposition of C5b-9.[15] Therefore, biopsies of human myocardial allografts were preferred as control tissue. However, also in these biopsies some C5b-9 deposition might be observed as a result of the brain-death situation of the donor[25] but due to ethical concerns no left ventricular biopsies were taken in healthy people. Therefore, biopsies of patients without heart failure were valuable as additional controls, although we can not exclude cellular changes based on their valvular disease. As the goal of this study was to assess the basic pathophysiological role of complement activation in the process of I/R injury instead of correlating findings to clinical outcome, small patient numbers were sufficient. However, some results were close to significant values which might be related to the small sample size. At last, aortic cross-clamping times and CPB times were different between the patient groups, although no correlations were observed between these durations and the measured complement components (data not shown).

Conclusion

Our findings do not support a prominent role for terminal complement activation in the acute phase following myocardial reperfusion. This is of major importance for the focus on therapeutic interventions currently being explored for human myocardial I/R injury, since clinical anti-complement therapies have not led to appreciable clinical relevant beneficial effects so far. However, complement depositions of C3d and sC5b-9 were present before reperfusion in myocardial tissue, especially in patients with heart failure. This probably preoperative depositions might be an underlying mechanism of the syndrome of heart failure. Therefore, it may be more beneficial to use complement inhibitors during the chronic state of heart failure instead of during the acute phases of reperfusion.

Acknowledgements

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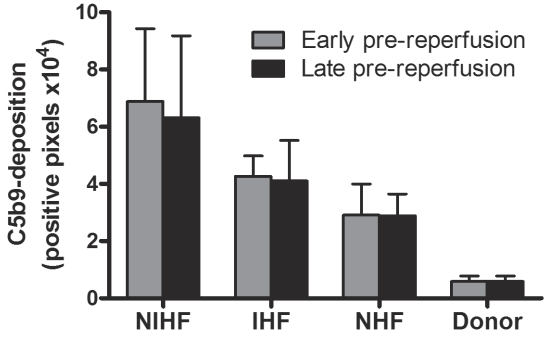
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Supplementary data, Figure 1. Quantification of C5b-9 depositions in early and late pre-reperfusion myocardial tissue.
The amount of C5b-9 was similar between early and late pre-reperfusion biopsies in all patient groups (Wilcoxon signed ranks test; non-ischemic heart failure (NIHF) *P* = 0.89, ischemic heart failure (IHF) *P* = 0.69, non-heart failure (NHF) *P* = 0.89, and donors *P* = 1.00). Graph error bars represent mean ± SEM.

CHAPTER FIVE

Submitted

Platelets: culprits in clinical myocardial ischemia/reperfusion injury?

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ABSTRACT

Background: The pathophysiology of ischemia/reperfusion (I/R) injury is complex and poorly understood. Preclinical studies implicate platelets in the inflammatory response upon reperfusion. However, their role in clinical myocardial I/R is unknown.

Methods: Patients undergoing scheduled I/R during mitral valve surgery were included. Paired arterial and myocardial venous blood samples, collected at timed intervals during the first hour post-reperfusion, allowed for cardiac-selective evaluation in time. We evaluated platelet counts, and established circulating markers of platelet activation (soluble P-selectin, RANTES, CXC chemokine ligand-8, and beta-thromboglobulin), and tested the responsiveness of platelets**.**

Results: In total, 39 patients were included: 20 patients with and 19 patients without left ventricular (LV) dysfunction. Platelet count was reduced at the start of reperfusion ($P < 0.001$), mainly due to hemodilution. Remarkably, reperfusion was not associated with myocardial release of platelet activation markers, except for minimal release of CXC chemokine ligand-8 in patients with LV dysfunction ($P = 0.02$). Absent release of platelet activation markers can be accredited to a lack of platelet activation or to platelet desensibilisation. Therefore, a bedside platelet excitability assay was performed in which platelets were stimulated and their response measured, showing a reduced post-reperfusion responsiveness of platelets.

Conclusions: Our studies indicate that platelets are not activated in early myocardial I/R injury in both patients with or without LV dysfunction. These findings suggest that platelets do not initiate the inflammatory response that is associated with I/R, indicating that preclinical evidence of platelet activation upon reperfusion can not directly be translated to the human situation.

INTRODUCTION

Ischemia/reperfusion (I/R) injury is the paradoxical increase of tissue damage upon reperfusion of ischemic tissue. This injury is an inherent component of cardiac surgery with artificial arrest and subsequent myocardial reperfusion. The pathophysiology of myocardial I/R injury is complex and incompletely understood. I/R is dominated by inflammation[1, 2], but the mechanisms initiating these inflammatory responses are unknown.

In addition to their well-defined role in hemostasis, platelets also serve as specialized cells of the innate immune system and modulators of inflammatory responses. Upon activation, platelets secrete various mediators into the circulation by exocytosis[3, 4], such as leukotrienes, sP-selectin, beta-thromboglobulin (beta-TG), platelet-derived growth factor (PDGF), and RANTES (Regulated upon Activation, Normal T-cell Expressed and Secreted), which can all contribute to the inflammatory response upon reperfusion by recruitment and activation of leukocytes.[5] Currently, preclinical evidence implies the activation of platelets as a major contributor to ischemia/reperfusion (I/R) injury.[6-9]

In contrast to ample preclinical data, involvement of platelets as key mediators in human I/R in general, and more specifically in myocardial I/R injury, is unclear with the current data.[10-12] Given the lack of clinical data, we performed a prospective study that aimed to evaluate platelet activation during controlled human myocardial I/R. We used the approach of consecutive arteriovenous differences, which allowed for discrimination between systemic platelet activation (as consequence of the surgery and the use of cardiopulmonary bypass), and myocardial reperfusion-related activation of platelets in time. Results of these measurements do not support a dominant role of platelet activation in human myocardial I/R injury.

METHODS

Patient population

Myocardial I/R was studied in 39 patients with mitral regurgitation undergoing mitral valve surgery with aortic cross-clamping. Within this group, 20 patients had left ventricular (LV) dysfunction. These two clinical groups, patients with and without LV dysfunction, were studied separately, as I/R may be influenced by a preexisting heart condition. In patients without LV dysfunction (control subjects), the mitral regurgitation was caused by organic pathology, with a predominance of prolapse disease. All patients were scheduled for restrictive mitral annuloplasty (RMA) ring implantation, with or without additional procedures. Exclusion criteria were perioperative corticosteroid treatment, active infection,

minimal invasive surgical procedures, emergency operations, and previous cardiac surgery. This study was approved by the local ethics committee, and written informed consent was obtained from each patient.

Surgical procedures

All participating patients received standardized anesthetic procedures, according to a fast-track protocol.[13] Cardiac surgery was performed according to local standardized protocols. All surgical procedures were performed via a midline sternotomy under normothermic CPB (Jostra Maquet, Maquet, Hirrlingen, Germany) with intermittent antegrade warm-blood cardioplegia and heparin softline coating. A uniform CPB protocol was designed, excluding autologous priming and ultrafiltration.

Arteriovenous measurements

Prior to surgery, a 5 French indwelling jugular vein catheter (PICC, Arrow International Inc., REF PS-01651, PA, USA) was inserted in the right atrium and placed in the coronary sinus by the surgeon. Since all patients underwent mitral valve surgery using a vertical transseptal incision, the coronary sinus could easily be cannulated during the surgical procedure. An arterial catheter was placed into the radial artery according to standard care. Arterial and myocardial venous blood samples were collected directly over the reperfused heart, that is from the radial artery (arterial) and coronary sinus (venous). These paired blood samples were obtained at 0, 15, 30, 45, and 60 minutes after reperfusion (after declamping of the aorta). Moreover, a systemic baseline sample was taken the day prior to surgery from the brachial vein. All samples were collected in pre-cooled tubes containing EDTA (BD Vacutainer, Plymouth, UK) and immediately placed in an ice water bath. In a pre-study evaluation, effects of storage conditions on platelet activation were analyzed. Blood samples were centrifuged (1,550 *g*, 10 min, 4°C) and the derived plasma was re-centrifuged (10,000 *g*, 4 min, 4°C) to obtain leukocyte and thrombocyte-free plasma. Aliquots were stored at -70°C until analysis.

Laboratory plasma measurements

Plasma measurements

Plasma levels of sP-selectin and RANTES were determined using a semi-automated enzyme-linked immunosorbent assay (ELISA) on a TECAN Freedom Evo robot. ELISA plates (384 wells, Maxisorp, cat no. 460372) were coated overnight (4°C) with coating buffer. Established human ELISA antibody duosets were used (sP-selectin, duoset DY137, R&D systems; anti-hCCL5/RANTES, MAB278 and AB-278-NA, R&D systems, Abingdon, UK). Plasma levels of

beta-TG were measured manually with commercially available antibodies (human beta-TG, MAB393, R&D systems, Abingdon, UK). Plasma levels of CXC chemokine ligand-8 (CXCL-8) were measured on the Bio-Plex system according to manufacturer's instructions (Biorad, Veenendaal, The Netherlands).

Platelet counts

Baseline platelet counts (day before surgery) and platelet counts for the arterial and coronary sinus samples collected at 0, 30, and 60 minutes after reperfusion were assessed (Sysmex XE-2100, Sysmex Corporation, Kobe, Japan).

Platelet activation and responsiveness

A novel bedside applicable assay was developed to explore more subtle changes in responsiveness of platelets. We determined the platelet responsiveness to agonists with serial dilutions of adenosine diphosphate (ADP), collagen-related peptide (C-RP), and trombin receptor-activating peptide (TRAP). Serial dilutions of ADP (125 µM, 31 µM, 8 µM, 2 µM, 500 nM, 125 nM, 31 nM, and 8 nM) were prepared in HEPES buffered saline (HBS; 10 mM HEPES, 150 mM NaCl, 1 mM MgSO4, 5 mM KCl, pH 7.4) with PE-labeled mouse anti-human P-selectin antibodies (diluted 1: 25). Similarly, serial dilutions of C-RP (2.5 µg/mL, 625 ng/mL, 156 ng/mL, 39 ng/mL 10 ng/mL, 2.5 ng/mL, 625 pg/mL, and 156 pg/mL) were prepared in HBS with PE-labeled mouse anti-human P-selectin antibodies (diluted 1:25). Furthermore, serial dilutions of TRAP (625 nM, 156 nM, 39 nM, 10 nM, 2.5 nM, 625 pM, 156 pM, and 156 pM) were prepared in HBS with PE labeled mouse anti-human P-selectin antibodies (diluted 1: 25).

The platelet activation test was initiated by addition of 5 µL fresh, citrate-anticoagulated, whole blood to each sample of the serial dilutions directly in the operating room. Samples as described above were used at various time points: baseline, and at 0, 30, and 60 minutes after reperfusion (both arterial and myocardial venous). After 20 minutes of incubation, the samples were fixed with 500 µL 0.2% formyl saline (0.2% formaldehyde in 0.9% NaCl, filtered through a 0.22 µm filter) and kept at 4°C until analyses. After an additional 1:4 dilution in 0.2% formyl saline, all samples were analyzed on a Beckman Coulter Cytomics FC500 flow cytometer (Beckman and Coulter, Krefeld, Germany) the same day after processing to assess the activation state and responsiveness of platelets. Samples were analyzed on GP1b positivity (all platelets) and P-selectin positivity (activated platelets). Analysis involved assessment of the maximum percentage of activated platelets (% P-selectin positive) and their intensity of activation (mean fluorescence index; MFI). Each serial dilution resulted in a typical sigmoid shaped curve. In other words, the percentage activated platelets increased with the concentration of the agonist in this sigmoid shaped curve. As a result, two different quantitative values were derived from these curves: (1) the area under the curve (AUC) and (2) the maximal median fluorescence intensity (max. MFI).

Statistical analysis

All statistical analyses were performed with the Statistical Package for the Social Sciences 20.0 (SPSS Inc., Chicago, IL, US). Clinical characteristics were compared using the Chi-Square test for the categorical data and the Mann-Whitney *U*-test for the numerical data, expressed as medians and interquartile ranges or frequencies (Table 1). Baseline plasma measurements were compared using a Mann-Whitney *U*-test, and expressed as medians and interquartile ranges (Table 2). Area under the curves (AUCs) were calculated for the arterial and coronary sinus blood levels of all three platelet activation markers. Arteriovenous AUC's and platelet counts were analyzed by a paired *t*-test, expressed as mean ± SEM. The excitability of platelets was evaluated in GraphPad Prism Software (Graphpad Software 5.01, Inc., San Diego, CA, USA) by the maximal MFI and AUC. The maximal MFI and AUC were analyzed by a Wilcoxon-signed rank test comparing baseline levels with levels early after reperfusion and arteriovenous differences at 0, 30, and 60 minutes after reperfusion. A P value < 0.05 was considered significant.

RESULTS

Patient population

This explorative study included 39 patients undergoing mitral valve surgery for severe mitral regurgitation. Clinical characteristics of patients with and without LV dysfunction are shown in Table 1. Two patients in the LV dysfunction group died: one patient died in hospital eight days after surgery as a result of ventricular fibrillation and the other patient died in hospital 42 days after surgery as a result of therapy-resistant heart failure.

Platelet activation and ischemia/reperfusion

No platelet activation in the reperfused heart

Patients with LV dysfunction show higher baseline values of sP-selectin (P = 0.03), and slightly higher values of RANTES (P = 0.06) as compared with patients without LV dysfunction, while the levels of CXCL-8 and the platelet-specific marker beta-TG were similar in the two groups (Table 2). Reperfusion was not associated with a myocardial release of sP-selectin, RANTES, or beta-TG in patients with LV dysfunction (P = 0.42, P = 0.51, and P = 0.67, respectively) as well as in patients without LV dysfunction (P = 0.07, P = 0.73, and P = 0.29, respectively; Figure 1). Myocardial release of CXCL-8 was observed in patients with LV dysfunction (P = 0.02), although not in the other patient group (P = 0.09).

Table 1. Clinical characteristics of patients with left ventricular dysfunction and controls.

	All patients n = 39	LV dysfunction n = 20	Controls n = 19	*P* value
Clinical characteristics				
Age (years)	65 (57-74)	65 (57-72)	65 (55-76)	0.78
Male gender (n)	24 (62%)	13 (65%)	11 (58%)	0.65
Body Mass Index (kg/m^2)	27 (24-29)	28 (25-30)	26 (23-28)	0.20
Systolic blood pressure (mmHg)	125 (115-140)	118 (105-130)	130 (120-144)	0.004*
Diastolic blood pressure (mmHg)	70 (65-80)	65 (60-80)	75 (70-80)	0.03*
Hemoglobin (mmol/L)	8.6 (8.1-9.2)	8.3 (7.8-9.0)	8.9 (8.5-9.5)	0.03*
Creatinine (µmol/L)	86 (74-103)	90 (74-109)	85 (74-98)	0.42
NT-proBNP (ng/L)	1242 (578-2295)	1954 (836-2610)	849 (90-1132)	0.003*
Logistic EuroSCORE (%)	5.97 (3.07-9.79)	8.66 (5.97-14.09)	3.07 (1.51-6.22)	< 0.001*
NYHA functional class	3 (2-3)	3 (3-3)	2 (1-2)	< 0.001*
Comorbidities				
Diabetes (n)	9 (23%)	7 (35%)	2 (11%)	0.07
COPD (n)	7 (18%)	5 (25%)	2 (11%)	0.24
AF (n)	11 (28%)	6 (30%)	5 (26%)	0.80
Medication				
ACE-inhibitors / ARBs (n, %)	27 (69%)	17 (85%)	10 (53%)	0.03*
Beta-blockers (n, %)	28 (72%)	19 (95%)	9 (47%)	0.001*
Statins (n, %)	22 (56%)	16 (80%)	6 (32%)	0.002*
Diuretics (n, %)	28 (72%)	19 (95%)	9 (47%)	0.001*
Digoxins (n, %)	7 (18%)	4 (20%)	3 (16%)	0.73
Echocardiography				
LVESV (mL)	92 (62-113)	110 (96-156)	62 (53-72)	< 0.001*
LVEDV (mL)	146 (135-194)	159 (139-218)	135 (128-154)	0.01*
LVEF (%)	39 (32-54)	32 (26-33)	54 (49-60)	< 0.001*
Surgical characteristics				
Aortic cross-clamp time (min)	120 (94-159)	112 (88-182)	125 (105-155)	0.52
CPB time (min)	178 (140-212)	197 (133-241)	176 (149-200)	0.54
Surgery time (min)	310 (255-352)	338 (277-389)	288 (247-319)	0.03*
Postoperative characteristics				
CPK directly in ICU (U/L)	613 (498-936)	656 (411-1425)	580 (500-899)	0.46
CPK 6 hours in ICU (U/L)	850 (594-1304)	923 (596-1621)	736 (594-1269)	0.22
CPK 12 hours in ICU (U/L)	971 (612-1519)	1117 (560-2101)	803 (630-1388)	0.25
Troponin T directly in ICU (ng/L)	1.07 (0.76-1.51)	1.07 (0.76-1.47)	1.07 (0.75-1.62)	0.67
Troponin T 6 hours in ICU (ng/L)	1.33 (0.82-1.78)	1.29 (0.83-1.91)	1.36 (0.76-1.81)	0.86
Troponin T 12 hours in ICU (ng/L)	0.91 (0.64-1.82)	0.91 (0.64-2.04)	0.92 (0.58-1.61)	0.51
Total ICU stay (hours)	24 (21-69)	57 (21-86)	22 (21-24)	0.01*
Total stay in hospital (days)	13 (10-19)	14 (10-26)	13 (10-17)	0.45

Medians and interquartile ranges are shown unless stated otherwise. * *P* values are given for the comparisons of patients with LV dysfunction and controls (patients without LV dysfunction).
Abbreviations: ACE, Angiotensin Converting Enzyme; AF, Atrial Fibrillation; ARB, Angiotensin Receptor Blocker; CPK, creatine phosphokinase; COPD, Chronic Obstructive Pulmonary Disease; CPB, Cardiopulmonary Bypass; ICU, Intensive Care Unit; LVEDV, Left Ventricular End-Diastolic Volume; LVEF, Left Ventricular Ejection Fraction; LVESV, Left Ventricular End-Systolic Volume; NT-proBNP, N-Terminal-proB-type Natriuretic Peptide; NYHA, New York Heart Association.

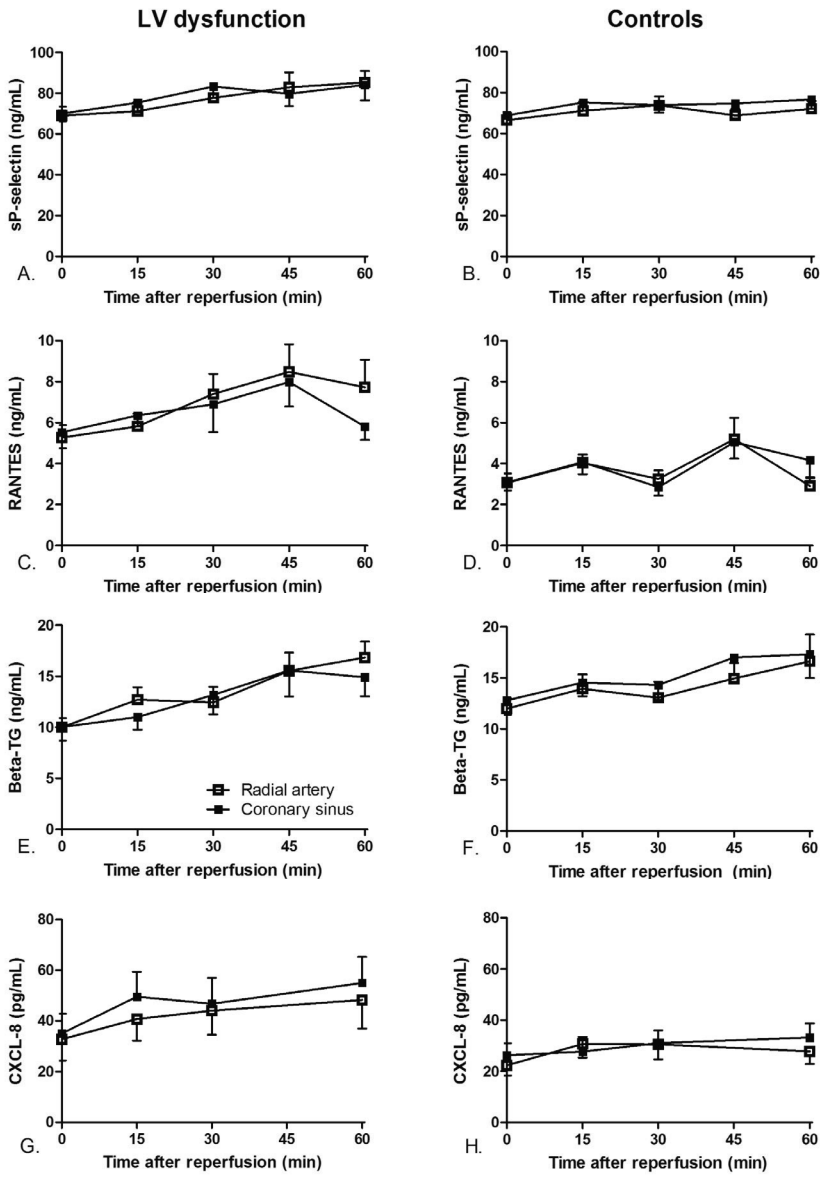


Figure 1. No myocardial release of platelet activation markers upon reperfusion.
Arteriovenous differences were used to determine the myocardial release of soluble P-selectin, RANTES (Regulated upon Activation, Normal T-cell Expressed and Secreted) and beta-thromboglobulin (beta-TG). The differences in area under the curve of the arterial and myocardial venous curves were assessed to quantify the net release from the myocardium. No arteriovenous differences were observed in both patient groups, despite a myocardial release of CXC chemokine ligand-8 in patients with LV dysfunction. Of note, time point zero is several seconds after the release of the aortic cross-clamp and not the baseline value (the day before surgery). Graph error bars indicate the standard error of the mean (SEM).

Table 2. Baseline values of patients with left ventricular dysfunction and controls.

	All patiens	LV dysfunction	Controls	*P* value
	n = 39	n = 20	n = 19	
Beta-TG (ng/mL)	6.87 (2.19-11.95)	6.87 (2.64-12.71)	6.63 (1.84-12.78)	0.87
RANTES (ng/mL)	3.98 (1.22-6.54)	4.82 (3.16-6.61)	1.87 (0.87-6.54)	0.06
sP-selectin (ng/mL)	62.71 (49.00-76.43)	73.78 (55.37-81.21)	54.59 (44.88-70.77)	0.03*
CXCL-8 (pg/mL)	8.44 (3.47-13.87)	8.94 (4.83-15.80)	8.07 (3.04-13.52)	0.47

Higher baseline values of soluble P-selectin (sP-selectin) were found in patients with LV dysfunction compared with controls (patients without LV dysfunction). Details are noted as medians (interquartile ranges). * indicates level of significance ($P < 0.05$) reached.
Abbreviations: Beta-TG, Beta-Thromboglobulin; CXCL-8, CXC Chemokine Ligand-8; LV, Left Ventricular; RANTES, Regulated upon Activation, Normal T-cell Expressed, and Secreted.

Thrombocytes are not retained in the heart

Thrombocyte counts of arterial and myocardial venous blood samples were determined, as differences in these levels could strongly influence the above described plasma measurements of sP-selectin, RANTES, beta-TG, and CXCL-8. To evaluate thrombocyte retention, arterial and myocardial venous number of thrombocytes were measured directly upon reperfusion, and at 30 and 60 minutes after reperfusion. The number of thrombocytes was found to be 50% reduced in comparison to the levels observed the day before surgery ($P < 0.001$). The present practice of CPB, which entails the addition of ≥ 1.5L of non-hematic fluids to the patient's blood volume, to prime the CPB circuit, frequently results in marked hemodilution. The reduction in number of thrombocytes can therefore be largely explained by hemodilution, since the hematocrit levels dropped with 36%. We did not observe differences in the number of arteriovenous thrombocytes within the first hour following reperfusion of the myocardium (Figure 2). In summary, thrombocyte counts were stable upon reperfusion and no evidence was found for thrombocyte sequestration in the heart.

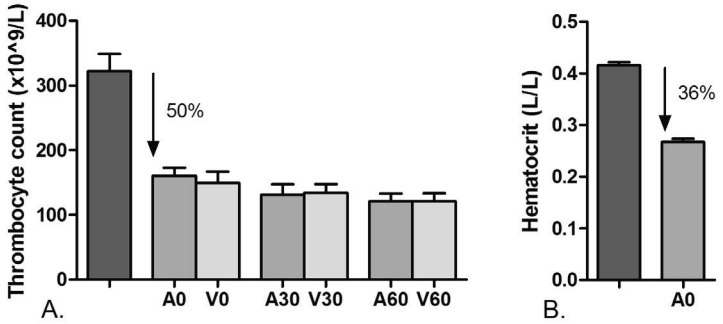


Figure 2. Thrombocytes are not retained in the heart.
(A) Thrombocyte counts were similar in arterial (A) and myocardial venous (V) blood collected at 0 (respectively A0 and V0, P = 0.38), 30 (respectively A30 and V30, P = 0.60), and 60 minutes (respectively A60 and V60, P = 0.96) after reperfusion. Upon reperfusion, the number of thrombocytes was significantly reduced in comparison to baseline (dark grey) as a result of hemodilution by use of cardiopulmonary bypass (P < 0.001). (B) Hematocrit levels decreased significantly after use of cardiopulmonary bypass compared with baseline (P < 0.001). Graph error bars indicate the standard error of the mean (SEM).

Platelet excitability assays

A novel bedside assay was used to evaluate the responsiveness of platelets in the presence of various agonists. With this method, more subtle changes in platelet responsiveness may be induced when platelets pass through the heart. These results were in line with our arteriovenous measurements of plasma markers, which revealed a lack of platelet activation during reperfusion. After in vitro stimulation of arterial and myocardial venous blood with the platelet activation agonists ADP, C-RP, and TRAP, the maximum total percentage of activated platelets (AUC) and the maximal mean fluorescence index (MFI) of activated platelets were measured. Reduced responsiveness of platelets upon reperfusion was observed, as indicated by attenuated P-selection expression on platelets, as evidenced by the AUC (Figure 3) and maximal MFI (Figure 4). Our approach of consecutive arteriovenous differences to assess the myocardial reperfusion-related activation of platelets did show similar responsiveness of platelets in arterial and coronary sinus blood.

Figure 3.

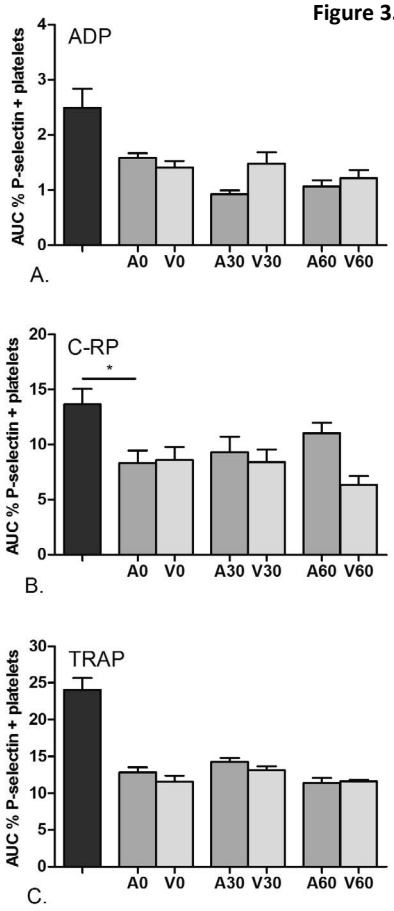


Figure 4.

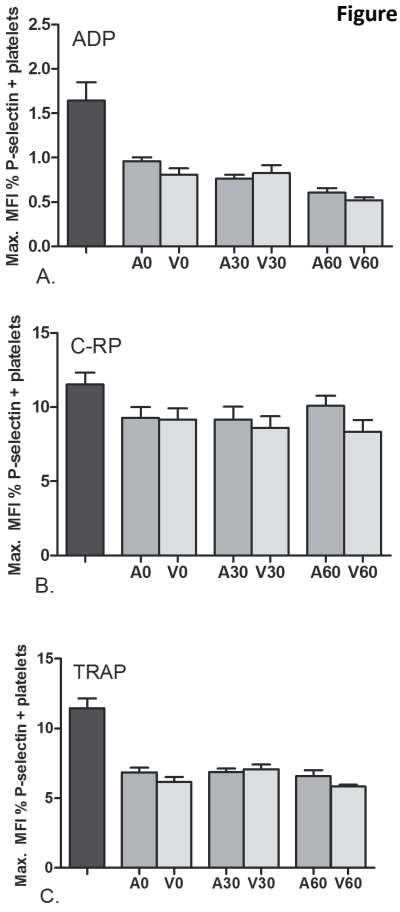


Figure 3. Reduced responsiveness of platelets upon reperfusion as evidenced by the area under the curves.

Graphs represent the area under the curves (AUCs) for the serial dilution series of (A) adenosine diphosphate (ADP), (B) collagen-related peptide (C-RP), and (C) trombin receptor-activating peptide (TRAP) per time point early after reperfusion. The P-selection expression on platelets was markedly reduced directly after reperfusion in comparison to baseline (dark grey). No arteriovenous differences were found for the AUC in coronary sinus blood (respectively V0, V30, and V60) as compared with arterial blood (respectively A0, A30, and A60) the first hour after reperfusion. * indicates level of significance ($P < 0.05$) reached. Error bars indicate the standard error of the mean (SEM).

Figure 4. Reduced responsiveness of platelets upon reperfusion as evidenced by the maximal median fluorescence intensity.

Graphs represent the maximal median fluorescence intensity (max. MFI) for the serial dilution series of (A) adenosine diphosphate (ADP), (B) collagen-related peptide (C-RP), and (C) trombin receptor-activating peptide (TRAP) per time point early after reperfusion. The max. MFI % P-selection expression on platelets was markedly reduced directly after reperfusion in comparison to baseline (dark grey). No arteriovenous differences were found for the max. MFI in coronary sinus blood (respectively V0, V30, and V60) as compared with arterial blood (respectively A0, A30, and A60) the first hour after reperfusion. * indicates level of significance ($P < 0.05$) reached. Error bars indicate the standard error of the mean (SEM).

DISCUSSION

Preclinical evidence implies activated platelets as a contributing factor to I/R injury.[6-9] In contrast to these data, involvement of platelets as a factor in human I/R in general, and more specifically in myocardial I/R injury, currently remains unclear. Results of this clinical study show that platelet activation is minimal or even absent in myocardial I/R, in contrast to findings from preclinical models.[6-9] In addition, a reduced responsiveness of platelets was observed upon myocardial reperfusion.

Platelet activation upon myocardial reperfusion was studied in two separate patient groups, patients with and without LV dysfunction. This subdivision was made as patients with LV dysfunction may be more susceptible to myocardial I/R injury.[14] Platelet activation was therefore expected to be more pronounced in patients with LV dysfunction. We observed higher levels of sP-selectin and modestly increased levels of RANTES at baseline (the day before surgery), yet the platelet specific marker beta-TG was similar between the two patient groups. As such, it is more likely that these elevated levels reflect preoperative endothelial cell activation in patients with LV dysfunction.[15]

Cardiospecific platelet activation was measured by assessing arteriovenous differences of sP-selectin, RANTES, beta-TG, and CXCL-8. Of all measured factors, only CXCL-8 was found to be released from the reperfused heart in patients with LV dysfunction. Since the other platelet activation markers were not released by the reperfused heart, it is more likely that CXCL-8 is a component of an early pro-inflammatory response.[16] The principal cellular sources of CXCL-8 are monocytes, macrophages, fibroblasts and endothelial cells.[17] The absence of a detectable release of platelet activation markers in our study is in line with the study of Sbrana et al.[18] Using fluorescence-activated cell sorting, Sbrana et al. did not observe platelet activation after cardiac surgery in either coronary sinus or peripheral blood. It must be noted however, that our study was restricted to a sample collection time shortly after reperfusion. I/R injury is generally considered an acute process, one that is initiated directly after reperfusion. We can not exclude the possibility of platelet activation during the later phases of myocardial I/R injury, nor in other organs.

The absence of platelet activation upon early reperfusion can potentially be accredited to either the absence of activation signals or to the desensibilisation of the platelets. Here, we detail excitability assays that were designed to evaluate the latter possibility, which revealed markedly reduced P-selectin expression on platelets directly upon reperfusion. These results suggest that activated platelets remain in the CPB, which could be an explanation for our inability to detect differences in arteriovenous concentrations of beta-TG, RANTES and sP-selectin. Of note, total number of platelets were equal in arterial and venous blood, indicating no adherence of platelets in the heart. One would then expect a measurable myocardial release of platelet activation markers as well.

Limitations

The absence of subgroup analyses for our studies due to the small sample size is a limitation of our study. Moreover, the observed reductions in thrombocyte counts were greater than could be explained by hemodilution alone, indicating a potential role for other mechanistic players in this process, such as an early mechanical disruption, and sequestration in organs.[19, 20] The two patient groups were different at baseline, reflecting a more vulnerable population of patients with LV dysfunction. The universal use of CPB during on-pump cardiac surgery likely also influences the assessment of factors that indicate the degree of platelet activation, for example by hemodilution and the use of heparin.[21] However, our results are in line with findings for kidney I/R in which the absence of platelet activation in early reperfusion injury was shown in both living and deceased donor kidney transplantation (personal communication, D.K. de Vries et al.).

Conclusion

This study identifies that platelets are not activated in human myocardial I/R injury after on-pump cardiac surgery. This suggests that platelets do not play a prominent role in the initiation of the acute inflammatory responses upon myocardial reperfusion. As a result, preclinical evidence can not directly be translated to the human situation in the context of platelet involvement.

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CHAPTER SIX

Submitted

Preexisting endothelial cell activation predicts post-cardiac surgery vasoplegia

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ABSTRACT

Background*:* Post-cardiac surgery vasoplegia is a frequent complication of cardiac surgery. The pathophysiological mechanisms underlying this syndrome are currently unknown. We hypothesized that this vasoplegia reflects endothelial dysfunction, either as preexisting condition or as consequence of the surgical procedure (i.e. ischemia/reperfusion injury and surgical injury).

Methods: To address these mechanisms, six established and distinct markers of endothelial cell activation were measured preoperatively and at various time points upon myocardial reperfusion. Post-cardiac surgery vasoplegia was defined as a mean arterial blood pressure < 60 mmHg with a cardiac index ≥ 2.2 L/min/m^2 treated with continuous intravenous administration of norepinephrine.

Results: Development of postoperative vasoplegia was associated with elevated baseline von Willebrand Factor propeptide levels (above 11.9 nM) as well as with baseline sP-selectin levels (above 64.4 ng/mL). In patients with vasoplegia (n = 15), no myocardial release of endothelial cell activation markers was observed upon reperfusion. In contrast, in patients without vasoplegia (n = 24), a myocardial release of von Willebrand Factor propeptide (P < 0.001), osteoprotegerin (P = 0.02), and interleukin-8 (P = 0.009) was demonstrated. Similar systemic levels of all markers were found upon reperfusion in both groups, except for higher sP-selectin levels in vasoplegia patients.

Conclusions: Preexisting low-grade endothelial cell activation, reflected by higher baseline von Willebrand Factor propeptide and sP-selectin levels, is a predisposing factor for post-cardiac surgery vasoplegia. Myocardial release of various endothelial cell activation markers in patients without this vasoplegia may appear counterintuitive, but may reflect the more prominent ability of the endothelium to respond to injury in this patient group.

INTRODUCTION

Vasoplegic syndrome occurs in 9 to 44% of the patients after cardiac surgery.[1-4] This syndrome is characterized by profound loss of systemic vascular resistance, resulting in severe hypotension, high cardiac output, and metabolic acidosis reflecting inadequate tissue perfusion.[5, 6] High levels of vasopressive agents are required to maintain an adequate blood pressure postoperatively. In a substantial proportion of the patients the vasoplegia appears refractory to these agents.[7] It is hypothesized that the surgical trauma and use of cardiopulmonary bypass (CPB) activate vasoactive inflammatory mediators, neurohumoral factors, and the coagulation system. This activation results in a disbalance in the regulation of the vascular tone.[1] Prolonged hypotension and the accompanying hypoperfusion subsequently lead to end-organ dysfunction. Consequently, this syndrome is associated with increased morbidity and mortality.[1, 8]

The pathophysiologic basis of this vasoplegia is unknown, and preventive or treatment strategies are lacking. We hypothesized that development of this syndrome could be caused by each or a combination of the following factors: preexisting endothelial dysfunction, endothelial dysfunction caused by myocardial ischemia/reperfusion (I/R) that occurs during surgery, and endothelial dysfunction caused by surgical injury including the use of CPB.[9-11] To address these issues, we performed an explorative study and collected serial blood samples in patients scheduled for mitral valve surgery. This patient population was specifically selected since patients with and without left ventricular dysfunction both undergo this homogeneous procedure. Arteriovenous concentration differences were obtained over the reperfused heart by selective cannulation of the coronary sinus, the venous pool of the heart. In parallel, we collected arterial samples reflecting systemic changes. Endothelial cell activation was assessed with measurement of six established markers, and the results were linked to clinical outcome.

METHODS

Patient population

In this 16 months prospective study, 40 patients undergoing cardiac surgery were included of which 20 patients had left ventricular dysfunction. All patients were scheduled for restrictive mitral annuloplasty (RMA) ring implantation with or without additional procedures. Exclusion criteria were perioperative corticosteroid treatment, active infection, minimal invasive surgical procedures, emergency operations, and previous cardiac surgery. Although there is no consensus in the literature about the definition of post-cardiac surgery vasoplegia, we

used the definition of Colson et al.[12] In the intensive care unit (ICU), dobutamine was used in order to obtain a cardiac index ≥ 2.2 l/min/m^2 as assessed by thermodilution (pulmonary artery catheter). A mean arterial blood pressure < 60 mmHg with a cardiac index ≥ 2.2 L/min/m^2 defined post-cardiac surgery vasoplegia the first 12 hours at the ICU, and was treated with continuous intravenous administration of norepinephrine.

Although post-cardiac surgery vasoplegia occurs more often in patients with low ejection fraction, it can also develop in patients with normal systolic function.[13] In daily clinical practice, vasoplegia is frequently observed in patients with prolonged aortic cross-clamp time, such as valve repair. Therefore, it is essential to unravel the underlying pathophysiological mechanisms to prevent it in all patients undergoing cardiac valve surgery, with or without systolic dysfunction. This study was approved by our local ethics committee, and written informed consent was obtained from each patient.

Anesthesia and surgical procedures

All participating patients received standardized anesthetic procedures, according to a fast-track protocol. A 5 French indwelling jugular vein catheter (PICC, Arrow International Inc., REF PS-01651, PA, USA) was inserted into the right atrium and placed in the coronary sinus by the surgeon. Since all patients underwent mitral valve surgery using a vertical transseptal incision, the coronary sinus could easily be cannulated during the surgical procedure. An arterial catheter was routinely placed in the radial artery. Cardiac surgery was performed according to local standardized protocols. All surgical procedures were performed via a midline sternotomy under normothermic CPB (Jostra Maquet, Maquet, Hirrlingen, Germany) with intermittent antegrade warm-blood cardioplegia and heparin softline coating. A uniform CPB protocol was designed, excluding autologous priming and ultrafiltration.

Plasma measurements

Arteriovenous measurements

Arterial (radial artery) and myocardial venous blood samples (coronary sinus) were collected simultaneously over the reperfused heart as described before.[14, 15] With this approach, accurate and specific measurements of locally ongoing processes were assessed the first hour after reperfusion. These paired blood samples were obtained during the early reperfusion phase: 0, 15, 30, 45, and 60 minutes after start of reperfusion, i.e. after removing the aortic cross-clamp. Two additional samples were collected: at baseline (the day before surgery from the brachial vein) and one day post-reperfusion (radial artery). Samples were collected in pre-cooled EDTA-tubes, immediately placed on melting ice, centrifuged twice, and stored at -70°C until analysis.

Measurement of endothelial cell activation markers

Plasma levels of the following endothelial cell activation markers, released by Weibel-Palade bodies, were assessed: von Willebrand factor (vWF), von Willebrand Factor propeptide (vWFpp), osteoprotegerin (OPG), and angiopoietin-2 (Ang-2). These markers were selected as they are released from prestored pools, and thus provide an immediate read-out of endothelial cell activation. vWF, vWFpp, and OPG were determined using a semi-automated enzyme-linked immunosorbent assay on a TECAN Freedom Evo robot using the following commercial antibody duosets: rabbit anti-human vWF and peroxidise-conjugated rabbit anti-human vWF, A00082 and P0226, Dako, Glostrup, Denmark; vWFpp rabbit-anti-propeptide and rabbit-anti-propeptide-biotine[16]; OPG, duoset DY805, R&D systems, Abingdon, UK. Plasma levels of Ang-2 were measured manually (human Ang-2, DY623, R&D systems, Abingdon, UK). Moreover, two other endothelial cell activation markers were measured: interleukin (IL)-8 and soluble P-selectin (sP-selectin).

Plasma levels of IL-8 were measured with a custom made X-plex assay according to instructions of the manufacturer (Biorad, Veenendaal, The Netherlands). Plasma levels of sP-selectin were determined using a semi-automated enzyme-linked immunosorbent assay (ELISA) on a TECAN Freedom Evo robot. ELISA plates (384 wells, Maxisorp, cat no. 460372) were coated overnight (4°C) with coating buffer. Established human ELISA antibody duosets were used (sP-selectin, duoset DY137, R&D systems, Abingdon, UK).

Statistical analysis

Continuous data are presented as medians with 25th and 75th percentiles. Categorical data are expressed as frequencies and percentages. Patients were divided into two subgroups based on the occurrence of post-cardiac surgery vasoplegia during follow-up. Perioperative data were evaluated between these groups with a Mann Whitney *U*-test or Chi-square test whenever appropriate. In addition, receiver operating characteristic (ROC) curve analysis was performed to determine the sensitivity and specificity for various cut-off values of baseline log-transformed vWFpp and sP-selectin levels for the prediction of post-cardiac surgery vasoplegia. Area under the curve (AUC) was calculated for the measurements in arterial and myocardial venous blood samples the first hour after reperfusion, and compared using a paired *t*-test. Arterial AUCs were compared between the two patient groups with a unpaired *t*-test. All statistical analyses were performed with the Statistical Package for the Social Sciences 20.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

This explorative study included 40 patients undergoing mitral valve surgery for severe mitral regurgitation. One patient without preexisting heart failure was excluded since the left ventricular function was perioperatively worse than estimated on the preoperative echocardiogram. In the remaining population of 39 patients, 15 (38.5%) patients developed a post-cardiac surgery vasoplegia. In four of these patients the vasoplegia persisted up to 24 hours after surgery. Two of these patients developed a more prolonged vasoplegia of more than 24 hours post-surgery. One patient in the vasoplegia group died 41 days after surgery as a result of therapy-resistant heart failure. One patient in the non-vasoplegia group, with a poor left ventricular function, died eight days after surgery as a result of ventricular fibrillation. Perioperative characteristics of the patient population are summarized in Table 1. Detailed information about the surgical procedures is provided in the Supplementary data, Table 1.

Association between vasoplegia, preoperative von Willebrand Factor propeptide, and soluble P-selectin levels

Baseline vWFpp levels (i.e. day before surgery) were elevated in patients with vasoplegia compared with non-vasoplegia patients (25.34 [IQR 14.85-48.37] vs 8.35 [IQR 6.57-11.09] nM, $P = < 0.001$). A correlation was observed between baseline vWFpp levels and vasoplegia (Spearman's rho correlation coefficient; r = 0.63, $P = < 0.001$). According to ROC curve analysis, a cut-off value of 11.9 nM showed a good accuracy to identify vasoplegia (Figure 1). Baseline sP-selectin levels were also higher in patients with post-cardiac surgery vasoplegia compared with non-vasoplegia patients (75.95 [IQR 62.12-81.56] vs 54.04 [IQR 43.77-72.70] ng/mL, $P = 0.006$, respectively). A correlation was observed between baseline sP-selectin levels and vasoplegia (Spearman's rho correlation coefficient; r = 0.45, $P = 0.004$). According to ROC curve analysis, a cut-off value of 64.4 ng/mL showed a good accuracy to identify vasoplegia (Figure 1). Baseline levels of vWF, OPG, Ang-2 and IL-8 were similar between the two groups (Table 2).

Table 1. Perioperative patient characteristics.

	All patients n = 39	Vasoplegia n = 15	No vasoplegia n = 24	*P value*
Clinical characteristics				
Age (years)	65 (57-74)	71 (57-77)	64 (56-71)	0.36
Male gender (n)	24 (62%)	11 (73%)	13 (54%)	0.23
Body Mass Index (kg/m²)	27 (24-29)	27 (25-30)	27 (24-29)	0.48
Systolic blood pressure (mmHg)	125 (115-140)	120 (105-130)	128 (120-140)	0.12
Diastolic blood pressure (mmHg)	70 (65-80)	70 (60-80)	73 (65-80)	0.59
Hemoglobin (mmol/L)	8.6 (8.1-9.2)	8.3 (7.8-9.2)	8.6 (8.3-9.3)	0.32
Creatinine (µmol/L)	86 (74-103)	93 (83-109)	81 (73-99)	0.19
NT-proBNP (ng/L)	1242 (578-2295)	1598 (660-2449)	898 (364-2257)	0.11
Logistic EuroSCORE (%)	5.97 (3.07-9.79)	6.96 (5.58-14.20)	4.29 (2.08-7.75)	0.02*
NYHA functional class	3 (2-3)	3 (3-3)	3 (2-3)	0.01*
Comorbidities				
Diabetes (n)	9 (23%)	4 (27%)	5 (21%)	0.44
COPD (n)	7 (18%)	3 (20%)	4 (17%)	0.79
AF (n)	11 (28%)	6 (40%)	5 (21%)	0.20
Medication				
ACE-inhibitors / ARBs (n, %)	27 (69%)	12 (80%)	15 (63%)	0.25
Beta-blockers (n, %)	28 (72%)	14 (93%)	14 (58%)	0.02*
Statins (n, %)	22 (56%)	11 (73%)	11 (46%)	0.09
Diuretics (n, %)	28 (72%)	13 (87%)	15 (63%)	0.10
Digoxins (n, %)	7 (18%)	2 (13%)	5 (21%)	0.55
Echocardiography				
LVESV (mL)	92 (62-113)	100 (92-120)	71 (57-102)	0.10
LVEDV (mL)	146 (135-194)	156 (135-194)	143 (133-191)	0.57
LVEF (%)	39 (32-54)	33 (30-38)	50 (32-59)	0.06
Surgical characteristics				
Aortic cross-clamp time (min)	120 (94-159)	147 (115-186)	108 (89-151)	0.04*
CPB time (min)	178 (140-212)	205 (154-252)	162 (135-198)	0.01*
Surgery time (min)	310 (255-352)	376 (275-406)	292 (226-322)	0.003*
Postoperative characteristics				
Norepinephrine (µg/kg/min)				
0-12 hours at the ICU	0.03 (0.01-0.10)	0.10 (0.07-0.20)	0.02 (0.00-0.05)	< 0.001*
12-24 hours at the ICU	0.00 (0.00-0.02)	0.02 (0.01-0.08)	0.00 (0.00-0.02)	0.004*
Dobutamine (µg/kg/min)				
0-12 hours at the ICU	3 (0-5)	5 (3-8)	2 (0-4)	0.004*
12-24 hours at the ICU	2 (0-5)	4 (2-7)	0 (0-4)	0.005*
Creatinine 24 hours after surgery (µmol/L)	79 (64-105)	96 (70-140)	77 (58-96)	0.06
Total stay in ICU (hours)	24 (21-69)	47 (21-93)	22 (21-25)	0.07
Total stay in hospital (days)	13 (10-19)	15 (11-21)	13 (10-19)	0.35

Medians and interquartile ranges are shown unless stated otherwise. * *P* values are given for the comparisons of patients with and without post-cardiac surgery vasoplegia.

Abbreviations: ACE, Angiotensin Converting Enzyme; AF, Atrial Fibrillation; ARB, Angiotensin Receptor Blocker; COPD, Chronic Obstructive Pulmonary Disease; CPB, Cardiopulmonary Bypass; ICU, Intensive Care Unit; LVEDV, Left Ventricular End-Diastolic Volume; LVEF, Left Ventricular Ejection Fraction; LVESV, Left Ventricular End-Systolic Volume; NT-proBNP, N-Terminal pro-Brain Natriuretic Peptide; NYHA, New York Heart Association.

Table 2. Baseline endothelial cell activation markers.

	All patients n = 39	Vasoplegia n = 15	No vasoplegia n = 24	*P value*
vWF (nM)	45.62 (32.09-64.05)	47.54 (36.88-57.28)	43.09 (25.63-66.40)	0.77
vWFpp (nM)	10.11 (7.20-24.48)	25.34 (14.85-48.37)	8.35 (6.57-11.09)	< 0.001*
OPG (pg/mL)	36.16 (19.09-53.49)	26.32 (15.53-45.99)	38.89 (24.57-60.11)	0.09
Ang-2 (ng/mL)	2.38 (2.08-3.07)	2.52 (2.06-3.54)	2.36 (2.05-2.83)	0.57
IL-8 (pg/mL)	8.44 (3.47-13.87)	8.33 (5.61-17.00)	8.44 (2.88-13.32)	0.45
sP-selectin (ng/mL)	62.71 (49.00-76.43)	75.95 (62.12-81.56)	54.04 (43.77-72.70)	0.006*

Medians and interquartile ranges are shown**.** * *P* values are given for the comparisons of patients with and without post-cardiac surgery vasoplegia.
Abbreviations: Ang-2, Angiopoeitin-2; IL-8, Interleukin-8; OPG, Osteoprotegerin; sP-selectin, soluble P-selectin; vWF, von Willebrand Factor; vWFpp, von Willebrand Factor propeptide.

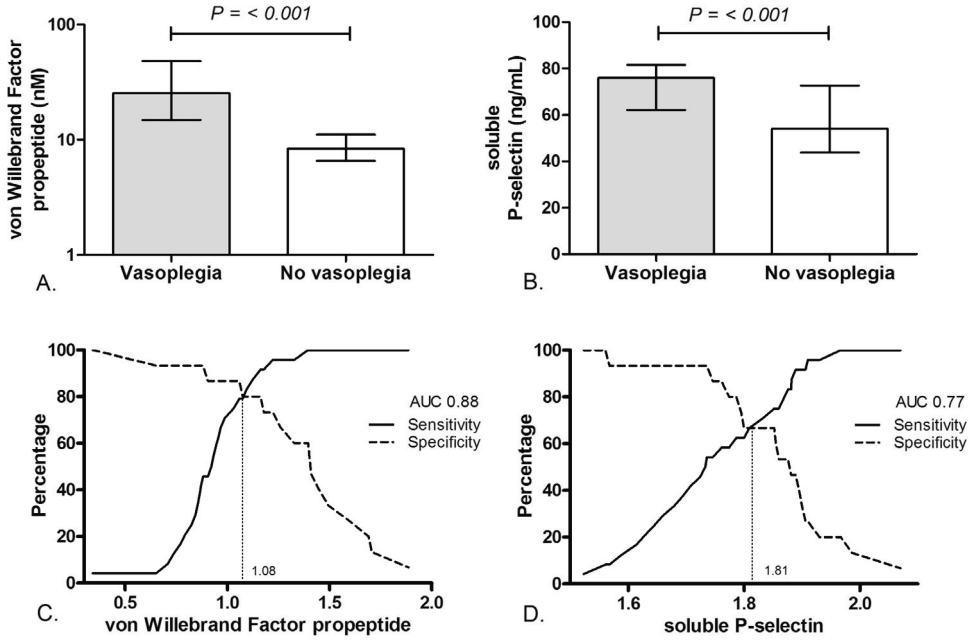


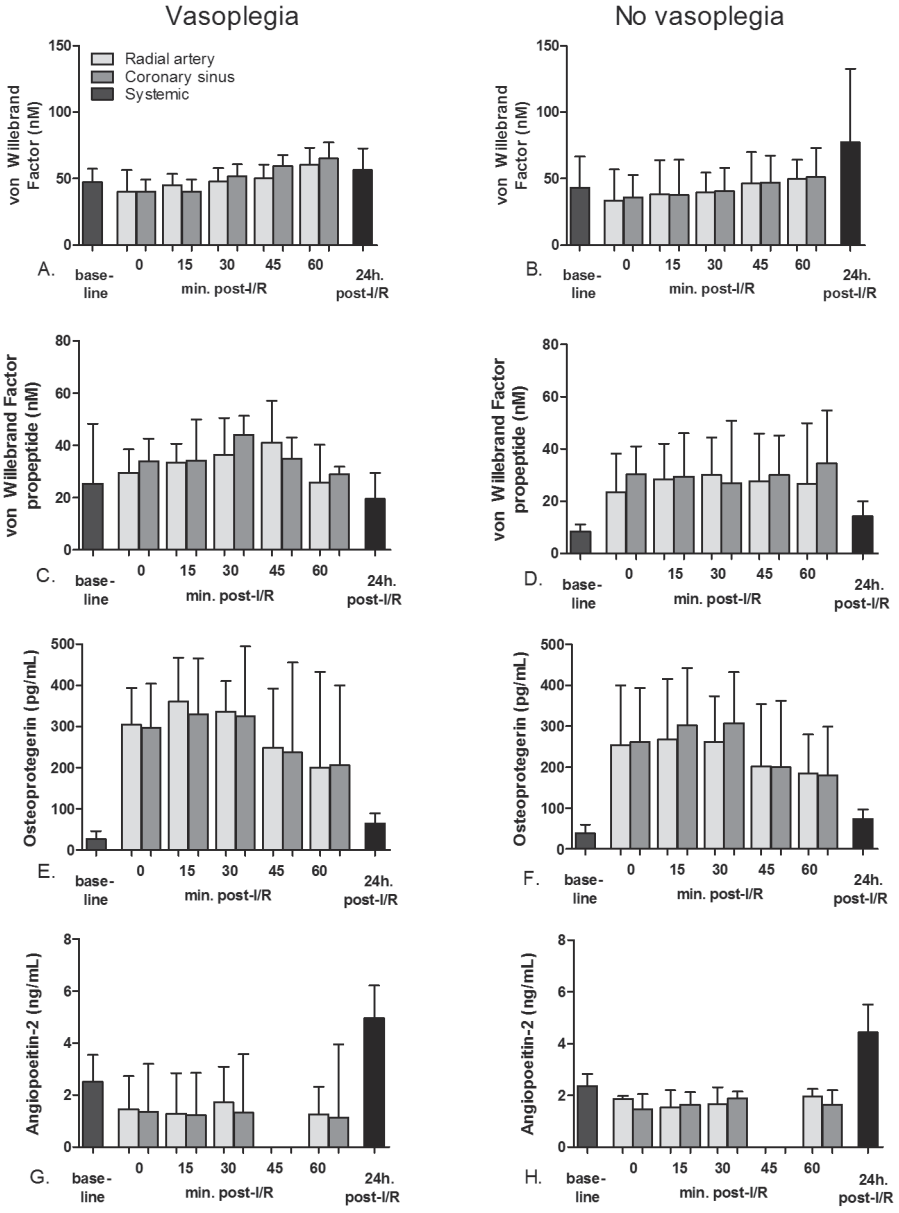
Figure 1. von Willebrand Factor propeptide and soluble P-selectin in patients with and without post-cardiac surgery vasoplegia.
Median and interquartile range concentrations of baseline (A) von Willebrand Factor propeptide (vWFpp) and (B) soluble P-selectin are shown in patients with and without vasoplegia. (C) Receiver operating characteristic (ROC) curve analysis demonstrated a sensitivity of 79% with a specificity of 80% to predict vasoplegia following cardiac surgery at a cut-off of log-transformed von Willebrand Factor propeptide of 1.08 at baseline (geometrical mean of 11.9 nM). (D) ROC curve analysis demonstrated a sensitivity of 67% with a specificity of 67% to predict vasoplegia following cardiac surgery at a cut-off of log-transformed soluble P-selectin of 1.81 at baseline (geometrical mean of 64.4 ng/mL).

Myocardial release of von Willebrand Factor propeptide, osteoprotegerin, and interleukin-8 upon reperfusion

Arteriovenous differences between blood collected from the radial artery and coronary sinus were analyzed in patients with and without post-cardiac surgery vasoplegia. In patients with vasoplegia, no myocardial release of vWF, vWFpp, OPG, Ang-2, IL-8 or sP-selectin was observed (P = 0.46, P = 0.86, P = 0.18, P = 0.16, P = 0.22, and P = 0.53, respectively). In contrast, in patients without vasoplegia, a myocardial release of vWFpp (P < 0.001), OPG (P = 0.02), and IL-8 (P = 0.009) was observed. No myocardial release of vWF, Ang-2 or sP-selectin was observed in this group (P = 0.60, P = 0.27, and P = 0.09, respectively).

Systemical levels of endothelial cell activation markers

In contrast to the preexisting differences of baseline vWFpp and sP-selectin between patients with and without vasoplegia, no differences were observed up to one hour after reperfusion comparing both patient groups for systemic levels of vWF (P = 0.96), vWFpp (P = 0.84), OPG (P = 0.80), Ang-2 (P = 0.81), and IL-8 (P = 0.16). However, sP-selectin levels were higher in patients who experienced post-cardiac surgery vasoplegia as compared with patients without this vasoplegia upon reperfusion (P = 0.03). Similar levels of all markers were found in both groups one day post-reperfusion: vWF (P = 0.52), vWFpp (P = 0.18), OPG (P = 0.51), Ang-2 (P = 0.40), IL-8 (P = 0.47), and sP-selectin (P = 0.16). Overall, reperfusion was characterized by a decrease in plasma vWF and Ang-2 levels, and increased levels of vWFpp, OPG, and IL-8. sP-selectin levels were relatively stable upon reperfusion as compared with baseline levels. Moreover, a steep increase of Ang-2 was observed one day post-reperfusion (Figure 2).


Vasoplegia
No vasoplegia
Radial artery
Coronary sinus
Systemic
von Willebrand Factor (nM)
von Willebrand Factor propeptide (nM)
Osteoprotegerin (pg/mL)
Angiopoeitin-2 (ng/mL)
A.
B.
C.
D.
E.
F.
G.
H.
base-line
0
15
30
45
60
24h. post-I/R
min. post-I/R

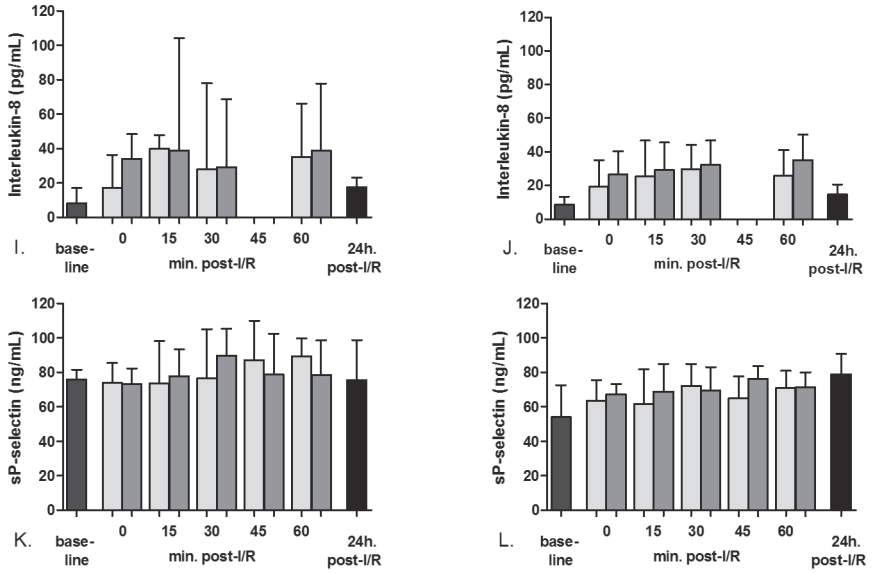


Figure 2. Systemic and myocardial venous plasma concentrations of various endothelial cell activation markers.
(A;B) von Willebrand Factor, (C;D) von Willebrand Factor propeptide, (E;F) osteoprotegerin, (G;H) angiopoeitin-2, (I;J) interleukin-8, and (K;L) sP-selectin at baseline, the first hour after reperfusion, and one day post-reperfusion in respectively patients with and without vasoplegia. Graph error bars indicate medians with interquartile ranges.

DISCUSSION

Our results show that preexisting low-grade endothelial cell activation is a predisposing factor for post-cardiac surgery vasoplegia, identified with elevated baseline vWFpp and sP-selectin levels. Effective treatment strategies for this vasoplegic syndrome are currently lacking although these markers may be of prognostic value. Moreover, these markers may be used as opportunity to develop preoperative interventions aimed at stabilizing the vascular tone. No myocardial release of endothelial cell activation markers was observed upon reperfusion in patients with vasoplegia. In contrast, patients without vasoplegia showed a myocardial release of vWFpp, OPG, and IL-8. This myocardial release of endothelial cell activation markers post-surgery in non-vasoplegia patients may appear counterintuitive, but may reflect the more prominent ability of the endothelium to respond to injury. The preexisting long-term demand of continuous systemic as well as myocardial endothelial cell activation in patients with vasoplegia may explain the absence of this myocardial release after surgery.

As indicated, elevated baseline levels of vWFpp were observed in patients who later developed post-cardiac surgery vasoplegia. vWF and vWFpp are stored in and released from activated Weibel Palade bodies, and are therefore markers of acute endothelial cell injury.[17] vWF, in contrast to vWFpp, is a substrate for platelet aggregation.[18] Moreover, the multimeric vWF is cleaved by a specific metalloproteinase (ADAMTS13, disintegrin-like and metalloproteinase with thrombospondin type I motifs 13), resulting in consumption of vWF.[18, 19] As a result, plasma vWFpp levels reflect more directly vascular endothelial cell injury than that of vWF.[18]

Another difference between vWF and vWFpp was observed with measurement of arteriovenous concentrations differences upon reperfusion, to discriminate between myocardial and systemic release of endothelial cell activation markers. Our results indicate a myocardial release of vWFpp, but not vWF upon reperfusion in patients without post-cardiac surgery vasoplegia. A possible hypothesis for this discrepancy is that the larger vWF molecules remain inside the heart, whereas vWFpp is systemically released. In line with our study, no myocardial release of vWF was observed in previous studies.[20, 21] Moreover, differences in plasma kinetics between vWF and vWFpp were observed: vWF levels increase much slower but are more sustained compared with vWFpp. Our results regarding these biokinetic properties are in line with a previous in vivo study of experimental endotoxemia.[16] Under *physiological* conditions, the molar concentration of vWFpp in normal plasma is about one tenth of the concentration of mature vWF. Yet, under *pathophysiological* conditions the molar ratio of vWFpp to mature vWF can increase four- to fivefold compared with baseline, presumably due to compartmentalization and/or catabolism of the mature vWF.

Limitations

This explorative study has some limitations. The small sample size might have limited the detection of minor differences, like a trend towards significance for elevated Ang-2 levels one day post-reperfusion in patients with vasoplegia (type II error). Moreover, no subgroup analysis could be performed neither correction for hemodilution was applied as a result of the small sample size. The differences at baseline between the groups, for example the higher logistic EuroSCORE and New York Heart Association (NYHA) class in patients with vasoplegia, reflect a more vulnerable patient population. Moreover, the mortality rate was low and not significantly different between patients with and without vasoplegia. This limits the potential for conclusions regarding the prognostic value of plasma concentrations of vWFpp and sP-selectin for clinical outcome. In addition, we decided to use a recent definition of Colson et al., although there is no consensus regarding the definition of post-cardiac surgery vasoplegia in the current literature.[12] Sun et al. described postoperative vasoplegic syndrome with a mean arterial pressure ≤ 70 mmHg, indexed systemic vascular

resistance < 1400 dyne/s/cm^5/m^2, cardiac index ≥ 2.5 L/min/m^2, and central venous pressure ≥ 10 mmHg.[22] Levin et al. used the amount of vasopressor support required to separate from the CPB as surrogate marker of vasoplegia.[6]

Conclusion

Elevated baseline vWFpp and sP-selectin levels were measured in patients who develop post-cardiac surgery vasoplegia in a later stage, reflecting a preexisting low-grade endothelial cell activation. These markers may be useful for the identification of patients at increased risk for developing vasoplegia postoperatively. In contrast to the preoperatively measured effects, no myocardial release of endothelial cell activation markers was observed upon reperfusion in patients with vasoplegia. In contrast, patients without vasoplegia showed a myocardial release of vWFpp, OPG, and IL-8. This myocardial release may appear counterintuitive, but may reflect the more prominent ability of the endothelium to respond to injury in this patient group. The preexisting long-term demand of continuous endothelial cell activation in patients with vasoplegia may result in no (additional) myocardial release of endothelial cell activation markers after surgery.

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Supplementary data, Table 1. Surgical procedures.

Surgical procedures (no.)	All patients n = 39	Vasoplegia n = 15	No vasoplegia n = 24
CorCap®, MVP, TVP, LV-lead +/- AF-ablation	6	1	5
CorCap®, MVP, LV-lead	3	1	2
CorCap®, CABG, MVP, LV-lead	1	1	0
LV-reconstruction, CABG, MVP, TVP, VT-ablation	1	1	0
LV-reconstruction, CABG, MVP, LV-lead	1	1	0
LV-reconstruction, MVP, TVP, VT- and AF ablation	2	2	0
LV-reconstruction, MVP, TVP, LV-lead	1	1	0
CABG, MVP, TVP, AF-ablation	1	1	0
CABG, MVP +/- LV lead	6	4	2
MVP/R, AVR	2	1	1
MVP/R, TVP +/- AF-ablation	9	1	8
MVP +/- AF-ablation	6	0	6

Abbreviations: AF, Atrial Fibrillation; CABG, Coronary Artery Bypass Grafting; LV, Left Ventricle/Ventricular; MVP/R, Mitral Valve Annuloplasty/Replacement; TVP, Tricuspid Valve Annuloplasty; VT, Ventricular Tachycardia.

CHAPTER SEVEN

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Heart failure determines the myocardial inflammatory response to injury

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ABSTRACT

Background: Systemic complications after cardiac surgery are common in heart failure patients. However, the pathophysiological mechanisms, such as a different local inflammatory response of failing hearts, remain in question. This study examines whether failing hearts respond differently to cardioplegic arrest and reperfusion compared with non-failing hearts (controls).

Methods and results: The inflammatory response was evaluated in samples collected simultaneously from the radial artery and coronary sinus, and in myocardial tissue of 62 patients undergoing cardiac surgery. No myocardial release of inflammatory mediators was observed upon reperfusion in controls (n = 19). In contrast, in patients with heart failure, reperfusion was characterized by a myocardial release of several cytokines. Myocardial interleukin-6 was 115% increased in non-ischemic heart failure patients (n = 18, *P* = 0.002) as compared with a 117% increase in patients with ischemic heart failure (n = 25, *P* = 0.01). Furthermore, a myocardial release of monocyte chemoattractant protein-1 was observed in both patient groups: a 109% (*P* = 0.001) and 114% (*P* = 0.01) increase in patients with non-ischemic heart failure and ischemic heart failure, respectively. Postoperative myocardial damage, expression of inflammatory mediators, and p65-nuclear factor-kappa B activity were similar in all patient groups. Inflammatory cell content was increased in early ischemic myocardial tissue in both heart failure groups compared with controls.

Conclusions: Heart failure patients show a clear myocardial inflammatory response upon reperfusion, probably explained by degranulation of infiltrated inflammatory cells. Results in controls indicate that they better withstand cardioplegic arrest and reperfusion without an explicit myocardial inflammatory response.

INTRODUCTION

Systemic complications after cardiac surgery, such as vasoplegia and infections, are common in patients with preexisting heart failure. These complications are a major contributor to morbidity and mortality. A remarkable observation is that these systemic complications appear to be independent of their pump failure itself.[1] They merely seem to reflect a different response of the body to the surgical procedure. This response can be due to systemic alterations in the setting of heart failure or may reflect a different response of the failing heart to cardioplegic arrest and reperfusion.

Ischemia/reperfusion (I/R) injury is the paradoxical increase of tissue damage upon reperfusion of ischemic tissue. This injury is an inherent component of cardiac surgery with artificial arrest and subsequent myocardial reperfusion. The pathophysiology of myocardial I/R injury is complex and not fully elucidated yet. Animal studies show that a complex cascade of events ultimately results in a myocardial inflammatory response upon reperfusion.[2-4] Whether this can be extrapolated to the more complex situation in humans is unknown.

In this clinical study, we evaluated whether failing hearts respond differently to cardioplegic arrest and reperfusion compared with non-failing hearts. The inflammatory response was studied in the two most common types of heart failure, non-ischemic and ischemic heart failure, and compared with patients without heart failure undergoing cardiac surgery. Release of various inflammatory mediators was sequentially measured after myocardial reperfusion in human cardiac surgery. Selective arteriovenous concentration differences could be obtained directly over the reperfused heart by cannulation of the coronary sinus (i.e. the venous pool of the heart). We evaluated which factors were released from the myocardium and whether failing hearts differ from non-failing hearts in their local inflammatory responses.

METHODS

Patient population

In this two-year prospective study, 62 patients were included: 43 patients with heart failure and 19 controls (Supplementary data, Figure 1). All heart failure patients were scheduled for restrictive mitral annuloplasty (RMA) ring implantation. Patients with non-ischemic heart failure (n = 18) also underwent a concomitant implantation of an external cardiac support device (CorCap cardiac support device, Acorn Cardiovascular, St. Paul, MN, USA). Patients with ischemic heart failure (n = 25) underwent coronary artery bypass surgery (n = 14) or

ventricular reconstruction surgery if they had a large area of anterior akinesia or dyskinesia (n = 11) in addition to RMA. Patients without heart failure, scheduled for reconstructive mitral valve surgery for degenerative, rheumatic, or cured endocarditis mitral valve pathology, were selected as controls. These controls were expected to have approximately the same aortic cross-clamp times as heart failure patients. Heart failure was preoperatively defined as an inadequate pump function of the heart with an echocardiographically estimated ejection fraction biplane below 35%[5] and the presence of one or more clinical symptoms as classified by the New York Heart Association (NYHA). All controls had a moderate to normal pump function of the heart (defined as an ejection fraction biplane above 45%), no clinical signs of heart failure by physical examination, and did not use high doses of diuretics. Exclusion criteria were perioperative corticosteroid treatment, active infection, minimal invasive surgical procedures, emergency operations, and previous cardiac surgery. Patients were followed during total hospital stay, including hospital stay after discharge to a referring hospital. This study was carried out in accordance with the Declaration of Helsinki and approved by the local ethics committee. All patients provided written informed consent.

Anesthesia and surgical procedures

All participating patients received standardized anesthetic procedures, according to a fast-track protocol. A 5 French indwelling jugular vein catheter (PICC, Arrow International Inc., REF PS-01651, PA, USA) was inserted into the right atrium and placed in the coronary sinus by the surgeon. Since all patients underwent mitral valve surgery using a vertical transseptal incision, the coronary sinus could easily be cannulated during the surgical procedure. An arterial catheter was routinely placed in the radial artery. Cardiac surgery was performed according to local standardized protocols. All surgical procedures were performed via a midline sternotomy under normothermic cardiopulmonary bypass (CPB; Jostra Maquet, Maquet, Hirrlingen, Germany) with intermittent antegrade warm-blood cardioplegia and heparin softline coating. A uniform CPB protocol was designed, excluding autologous priming and ultrafiltration.

Plasma measurements

Arteriovenous measurements

Arterial (radial artery) and myocardial venous blood samples (coronary sinus) were collected simultaneously over the reperfused heart (Figure 1). With this approach, accurate and specific measurements of locally ongoing processes were assessed up to 24 hours after reperfusion. Arterial and myocardial venous blood samples were obtained during the early reperfusion phase: 0, 15, 30, and 60 minutes after start of reperfusion, i.e. after removing the

aortic cross-clamp (early phase), and at 2, 4, 8, and 24 hours after reperfusion (late phase). In all patients, a baseline sample was taken the day before surgery from the brachial vein. All samples were collected in pre-cooled tubes containing EDTA (BD Vacutainer, Plymouth, UK) and immediately placed on melting ice. Blood samples were centrifuged within one hour after collection (1.550 *g*, 10 min, 4°C) and the derived plasma was re-centrifuged (10.000 *g*, 4 min, 4°C) to obtain leukocyte and thrombocyte free plasma. Aliquots were stored at -70°C until analysis.

Measurement of inflammatory mediators

A pilot study was performed in five heart failure patients (three patients with ischemic heart failure and two patients with non-ischemic heart failure) and five controls to identify detectable inflammatory mediators with the sensitive human 27-plex panel (Biorad, Veenendaal, The Netherlands). Based on the results of this pilot study, the following 12 cytokines were measured in the complete cohort with a custom-made multiplex assay according to the instructions of the manufacturer (X-plex, Biorad, Veenendaal, The Netherlands): interleukin-1 receptor antagonist (IL-1ra), IL-6, IL-8, IL-9, IL-10, IL-16, IL-18, granulocyte colony-stimulating factor (G-CSF), interferon-inducible protein-10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), regulated upon activation, normal T cell expressed and secreted (RANTES), and vascular endothelial growth factor (VEGF). Underlying mechanisms for the myocardial release of inflammatory mediators were investigated in the complete cohort. First, the possibility of passive secretion from damaged myocytes was looked for by measurement of troponin T. Next, we investigated whether active production and secretion by myocardial cells might be responsible for the release of inflammatory mediators by means of mRNA expression of various inflammatory mediators. Moreover, cellular activation was examined by assessing transcriptionally active nuclear factor-kappa B (NF-κB). Eventually, the possibility of release by infiltrating inflammatory cells was investigated.

Myocardial damage

To assess myocardial damage, troponin T values were measured by a routine laboratory assay (Roche Diagnostics, Mannheim, Germany). Dissimilarity in myocardial damage between the groups could explain differences in the myocardial release of inflammatory mediators. Troponin T values were measured 45 minutes after reperfusion in coronary sinus blood. This time point lies within the range of the early reperfusion phase, where the most dominant myocardial release of inflammatory mediators was observed. After cardiac valve surgery, troponin T values below 2.0 µg/L are considered normal.

Myocardial tissue samples

Through the mitral valve, an endomyocardial biopsy was obtained from a standardized location at the posterior wall of the left ventricle between the two papillary muscles, early after aortic cross-clamping. The late endomyocardial biopsy was collected on average 70 minutes later from the same location just before release of the aortic cross-clamp (Figure 1). Biopsies were divided and fixed in formalin or immediately snap-frozen in liquid nitrogen and stored at -70°C. Formalin-fixed, paraffin-embedded biopsies were cut into 5 µm thick sections with a microtome.

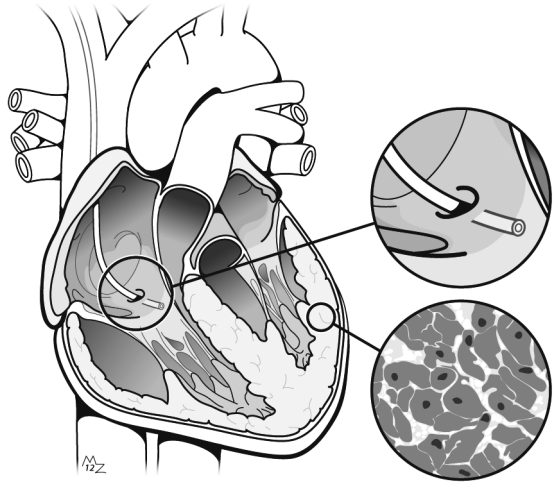


Figure 1. Schematic representation of the coronary sinus catheter and location of the biopsies.
This figure shows the position of the coronary sinus catheter and the location of the left ventricular biopsies.

RNA extraction and mRNA analysis

Total RNA was prepared from early and late endomyocardial biopsies. Total RNA extraction was performed using RNA-Bee (Campro Scientific, Veenendaal, The Netherlands) and glass beads. Subsequently, cDNA was prepared using a High Capacity RNA-to-cDNA Kit (Applied Biosystems Inc, Foster City, CA, USA). Taqman gene expression master mix, ABI 7500 Fast system and established primer/probe sets (Applied Biosystems Inc, Foster City, CA, USA) were used according to the manufacturer's instructions to quantify IL-1ra (Hs00893626_m1), IL-6 (Hs00174131_m1), IL-8 (Hs00174103_m1), IL-10 (Hs00961622_m1), tumor necrosis factor (TNF)-α (Hs00174128_m1), IL-1β (Hs00174097_m1), matrix metallopeptidase (MMP)-9 (Hs00957562_m1), cluster of differentiation (CD)68 (Hs00154355_m1) and MCP-1 (Hs00234140_m1). Glyceraldehyde phosphate dehydrogenase (GAPDH; VIC labelled) was used as housekeeping gene (Applied Biosystems Inc, Foster City, CA, USA).

Analysis of intramyocardial p65-nuclear factor-κB activity

To assess the role of NF-κB as transcription factor controlling the expression of genes involved in immune responses, p65-NF-κB activity was quantified in paired early and late endomyocardial biopsies of five patients of each patient group. Myocardial p65-NF-κB activity was determined as described previously by Kleemann et al.[6], using a TransAM transcription factor assay kit (no. 40097, Active Motif Europe, Rixensart, Belgium). The assays were performed according to the instructions of the manufacturer. Myocardial tissue homogenates were prepared using the Nuclear Extract Kit (no. 40010, Active Motif Europe, Rixensart, Belgium). Equal amounts of protein (6 µg/well) of these homogenates were used to determine the amount of active p65-NF-κB.

Immunohistochemical analysis of left ventricular tissue

Formalin-fixed, paraffin-embedded microtome sections were deparaffinized and rehydrated before staining for CD3, CD68, myeloperoxidase (MPO), and human leucocyte antigen (HLA)-DR. Some stainings required the use of a protein retrieval: an EDTA buffer solution for CD3 and CD68 (pH = 9.0), and a citrate buffer solution (pH = 6.0) for HLA-DR. Sections were incubated overnight with the primary antibody raised against CD3 (1:200, Abcam, Cambridge, MA, USA), CD68 (1:4000, DAKO, Glostrup, Denmark), MPO (1:5000, DAKO, Glostrup, Denmark), and HLA-DR (1:200, DAKO, Glostrup, Denmark) for staining of T-lymphocytes, macrophages, polymorphonuclear neutrophils, and activated leukocytes, respectively. Subsequently, envision and 3,3'-diaminobenzidine tetrahydrochloride as a chromogen for detection were applied. Slides were counterstained with Mayer's hematoxylin to allow morphological analysis. The number of positive cells was quantified in five views (original magnifications x200; AxioVision software, version 4.4.1.0 Carl Zeiss MicroImaging Inc, Gottingen, Germany). The number of cells over five calibrated grid areas per view was counted using ImageJ Cell Counter® by an independent blinded observer.

Statistical analysis

Continuous variables were analyzed using the Kruskal-Wallis test with post-hoc analyses with Mann-Whitney *U*-tests whenever appropriate. The significant Kruskal-Wallis offers protection against an inflated α-error rate when performing multiple comparisons. Furthermore, Benjamini-Hochberg correction was used to correct for multiple comparisons. If values were not significant after this correction, NS was placed in the text after the unadjusted *P* value. The area under the curve (AUC) was calculated for the arterial and venous curves of the plasma measurements for the early and late reperfusion phase. A delta AUC was calculated (venous minus arterial) and the null hypothesis (delta AUC is 0) was tested by a paired *t*-test. Histological stainings were analyzed with a one-way between-

groups analysis of variance (ANOVA). The Wilcoxon signed rank test was used to analyze differences between early and late ischemic biopsies for the mRNA analysis and NF-κB activity. All statistical tests were performed using SPSS statistical analysis software version 17.0 (SPSS Inc, Chicago, IL, USA). A P value < 0.05 was considered significant. Graph error bars represent the mean ± standard error of the mean (SEM) unless stated otherwise.

RESULTS

General findings

Patient characteristics are summarized in Table 1. A similar gender distribution was observed in all groups (P = 0.43). In two controls, a mitral valve replacement had to be done; a repair was not possible due to extensive calcifications. Moreover, one patient was scheduled for a mitral valve repair in combination with a left ventricular reconstruction procedure. Although included, this patient did not undergo this valve repair since there was no longer any mitral regurgitation after the reconstruction procedure. Two patients died during follow-up in hospital: one patient with non-ischemic heart failure died unexpectedly eight days after surgery as a result of ventricular fibrillation. The other patient, with ischemic heart failure, died 41 days after ventricular reconstruction surgery as a result of therapy-resistant heart failure. Baseline levels of all inflammatory mediators were comparable in all patient groups (Supplementary data, Table 1), of which controls generally showed the lowest baseline levels of pro-inflammatory mediators. Systemic levels of inflammatory mediators, i.e. concentrations in arterial blood samples, were similar during the first hour after reperfusion in all groups except for IL-1ra, IL-6, and G-CSF (Supplementary data, Figure 2).

Table 1. Baseline, surgical and outcome characteristics of patients with non-ischemic heart failure, ischemic heart failure, and controls.

	Median (interquartile range)			Kruskal-Wallis, *P* value	Mann-Whitney, *P* value		
	NIHF (n = 18)	IHF (n = 25)	Co (n = 19)		NIHF versus IHF	IHF versus Co	NIHF versus Co
Age (years)	64.56 (57.58-70.11)	66.86 (58.04-73.13)	64.28 (55.45-75.46)	0.55	—	—	—
NYHA class	3 (3-3)	3 (3-3)	2 (1-2)	< 0.001	0.28	< 0.001*	< 0.001*
Systolic blood pressure (mmHg)	108 (99-116)	120 (110-130)	125 (120-145)	0.001	0.01*	0.14	< 0.001*
Diastolic blood pressure (mmHg)	65 (60-70)	70 (65-85)	76 (70-80)	0.005	0.04 (NS)	0.22	0.001*
Body mass index (kg/m²)	27.91 (24.58-30.14)	27.16 (25.02-31.86)	26.22 (24.44-27.44)	0.18	—	—	—
Co-morbidities (n)							
Diabetes	8	3	1				
COPD	4	3	2				
Atrial fibrillation	5	5	5				
Logistic EuroSCORE (%)	10.70 (5.64-16.65)	6.96 (5.23-11.46)	3.07 (1.51-4.25)	< 0.001	0.47	< 0.001*	< 0.001*
Laboratory tests							
NTproBNP (ng/L)	1960.00 (876.68-2858.25)	1663.00 (517.65-2650.50)	853.40 (106.85-1159.5)	0.004	0.28	0.02*	0.002*
Hemoglobin (mmol/L)	8.8 (8.0-9.5)	8.6 (8.1-9.1)	9.0 (8.5-9.5)	0.37	—	—	—
Creatinine (µmol/L)	90 (73-113)	89 (74-111)	85 (77-108)	0.93	—	—	—
CRP (mg/L)	< 3 (< 3-5.3)	< 3 (< 3-4.0)	< 3 (< 3-3.5)	0.50	—	—	—
Echocardiography							
LVEF biplane (%)	30 (24-32)	31 (27-35)	53 (49-60)	< 0.001	0.19	< 0.001*	< 0.001*
LVESV (mL)	147 (114-176)	148 (107-156)	59 (48-71)	< 0.001	0.51	< 0.001*	< 0.001*
LVEDV (mL)	202 (167-245)	208 (167-223)	135 (117-146)	< 0.001	0.85	< 0.001*	< 0.001*
Use of medication (n)							
ACE-inhibitors / ARBs	16	23	11				
Beta-blockers	16	24	10				
Statins	14	20	6				
Diuretics	18	21	9				
Digoxin	5	1	1				
Aortic cross-clamp time (min)	88 (83-109)	151 (130-192)	125 (105-177)	< 0.001	< 0.001*	0.14	0.005*
Ventilation time (hours)	12 (7-24)	14 (8-24)	6 (6-11)	0.02	0.68	0.006*	0.045 (NS)
APACHE IV score	52 (46-61)	57 (48-66)	42 (28-57)	0.04 (N.S.)	0.58	0.02*	0.06
Total stay in ICU (hours)	67 (23-91)	42 (23-114)	22 (20-24)	0.002	0.89	0.001*	0.004*
Total stay in hospital (days)	14 (11-21)	15 (12-21)	13 (10-17)	0.39	—	—	—

* $P < 0.05$ was considered significant.

Abbreviations: ACE, Angiotensin Converting Enzyme; APACHE, Acute Physiology And Chronic Health Evaluation; ARB, Angiotensin Receptor Blocker; Co, Controls; COPD, Chronic Obstructive Pulmonary Disease; CRP, C-Reactive Protein; ICU, Intensive Care Unit; IHF, Ischemic Heart Failure; LVEDV, Left Ventricular End-Diastolic Volume; LVEF, Left Ventricular Ejection Fraction; LVESV, Left Vventricular End-Systolic Volume; NIHF, Non-Ischemic Heart Failure; NS, Non-Significant; NT-proBNP, N-Terminal pro-Brain Natriuretic Peptide; NYHA, New York Heart Association.

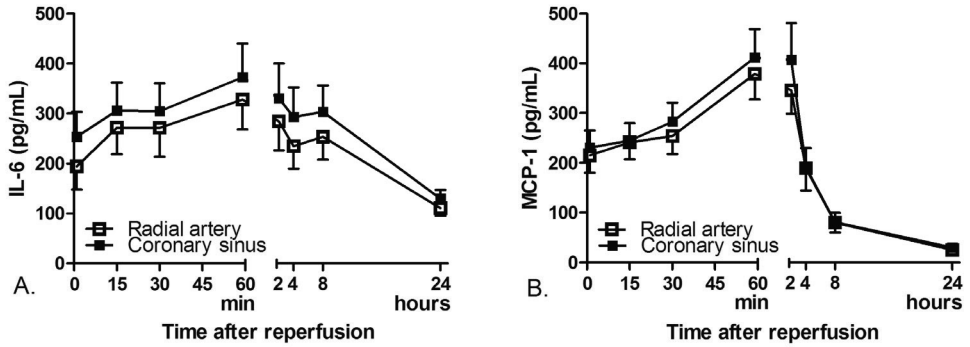


Figure 2. Illustration of our analysis method in patients with non-ischemic heart failure.
Arteriovenous differences were used to determine the release of inflammatory markers upon reperfusion. The difference in the area under the curve of the arterial and myocardial venous curves was assessed to quantify the net release from the myocardium. A significant myocardial release of interleukin-6 (IL-6) as well as monocyte chemoattractant protein-1 (MCP-1) was observed in patients with non-ischemic heart failure upon reperfusion. Graph error bars represent the mean ± SEM.

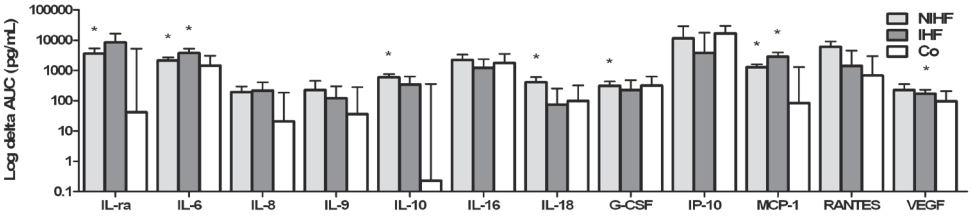


Figure 3. Myocardial release of inflammatory mediators in patients with heart failure.
Various inflammatory mediators were released by the myocardium in patients with non-ischemic and ischemic heart failure upon early reperfusion. No significant myocardial release was observed in controls. Graph error bars represent the mean ± SEM.
Abbreviations: Co, Controls; G-CSF, Granulocyte-Colony Stimulating Factor; IHF, Ischemic Heart Failure; IL, Interleukin; IL-1ra, Interleukin-1 receptor antagonist; IP-10, Interferon-inducible Protein-10; MCP-1, Monocyte Chemoattractant Protein-1; NIHF, Non-Ischemic Heart Failure; RANTES, Regulated upon Activation, Normal T cell Expressed and Secreted; VEGF, Vascular Endothelial Growth Factor.

Myocardial release of inflammatory mediators in patients with preexisting heart failure

Arteriovenous differences were used to determine the myocardial cytokine and chemokine release upon reperfusion. The differences in the AUC of the arterial and venous curves were assessed to quantify the net release from the myocardium. Our analysis method is illustrated in Figure 2. Both heart failure groups showed a significant myocardial release of various cytokines upon early reperfusion. As shown in Figure 3, early myocardial reperfusion of patients with non-ischemic heart failure was associated with a release of IL-1ra (P = 0.04, NS), IL-6 (P = 0.002), IL-10 (P = 0.002), G-CSF (P = 0.02), MCP-1 (P = 0.001) and IL-18

(P = 0.04, NS). In patients with ischemic heart failure, early reperfusion was characterized by a more limited number of inflammatory markers that were released: IL-6 (P = 0.01), MCP-1 (P = 0.01) and VEGF (P = 0.004). This inflammatory response was not observed during the late reperfusion phase. The early cytokine response was exclusive for heart failure patients, since controls did not show a significant myocardial release of any of the measured inflammatory mediators upon early and late reperfusion.

Similar postoperative myocardial damage

To assess whether variation in myocardial damage is the cause for the observed differences in cytokine release between the groups, troponin T values were measured 45 minutes after reperfusion. No differences were observed between the patient groups (P = 0.67). The median and interquartile range of troponin T was 1.24 µg/L (0.93 to 1.49) in patients with non-ischemic heart failure, 1.36 µg/L (0.83 to 2.49) in patients with ischemic heart failure, and 1.25 µg/L (0.83 to 1.60) in controls. A number of patients with ischemic heart failure underwent a ventriculotomy in the setting of their surgical ventricular reconstruction which might explain the higher range of troponin T values.

Similar expression of inflammatory mediators during ischemia

A subsequent analysis of inflammatory mediators in mRNA extracts of endomyocardial tissue showed no differences at baseline in all groups (early endomyocardial biopsies; Supplementary data, Table 2), except for a small difference in MMP-9 expression in patients with non-ischemic heart failure and controls (P = 0.01). More importantly, there were no differences in inflammatory gene expression in early versus late endomyocardial biopsies in all patient groups (Supplementary data, Table 3). This indicates that transcriptional changes are not responsible for the increased cytokine release in heart failure patients.

Similar p65-nuclear factor-kappa B activity during ischemia

Since transcription of cytokines itself did not change, NF-κB was measured as many of the myocardial released inflammatory mediators are under transcriptional control of this regulator. Myocardial NF-κB activity was compared between the groups using homogenates of paired endomyocardial tissue samples collected in the early and late ischemic period. Baseline values of NF-κB activity were similar between the groups (P = 1.00). No increase in NF-κB activity was observed in early versus late endomyocardial biopsies (P = 0.23, P = 0.14, and P = 0.89 for patients with non-ischemic heart failure, ischemic heart failure, and controls, respectively; Supplementary data, Figure 3).

More tissue macrophages and neutrophils in heart failure patients

Immunohistochemical stainings were performed to evaluate whether differences in inflammatory cell content caused differences in cytokine release between the groups. There was a significant difference in CD68-positive and MPO-positive cells between the groups in the early biopsies (Figure 4A). Post-hoc comparisons indicated that the amount of macrophages was 3.8 times higher in patients with non-ischemic heart failure relative to controls ($P = 0.002$; Figure 4B). Also the neutrophil content was 2.7 times higher in patients with non-ischemic heart failure compared with controls ($P = 0.006$; Figure 4C). T-lymphocytes (CD3) were sparsely present in all patient groups, with non-ischemic heart failure patients showing most T-lymphocytes ($P = 0.04$, NS). The content of activated leukocytes (HLA-DR) was 3.5 times higher in patients with non-ischemic heart failure as compared with both ischemic heart failure and control patients, although not significant ($P = 0.06$). No significant changes in macrophage or neutrophil content were observed between the early and late biopsies (Supplementary data, Figure 4).

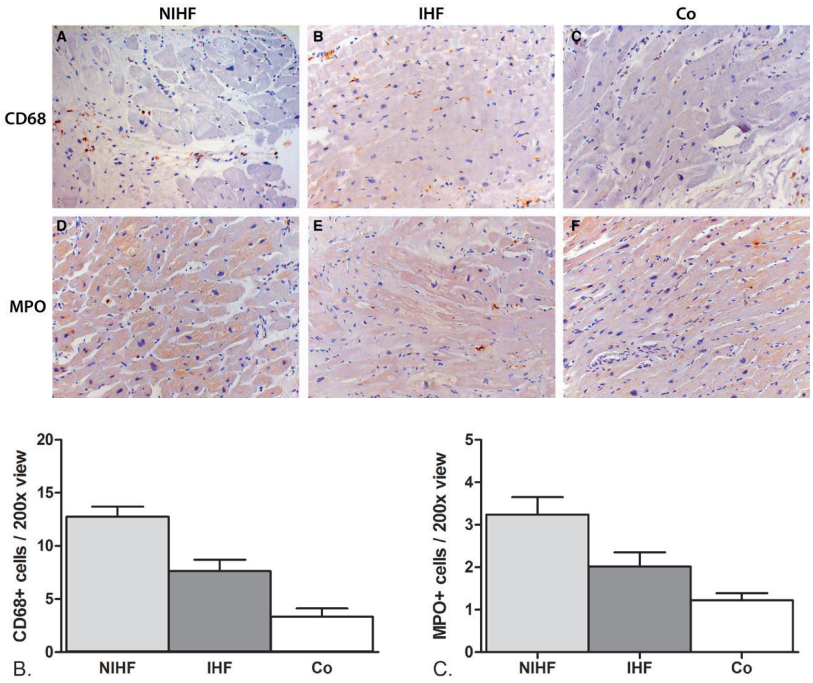


Figure 4. Macrophage and neutrophil contents.
(A) Early ischemic endomyocardial biopsies of patients with non-ischemic heart failure (NIHF) [A and D], ischemic heart failure (IHF) [B and E] and controls (Co) [C and F] were stained for cluster of differentiation 68 (CD68) and myeloperoxidase (MPO). Representative images are shown. Original magnifications x200. (B) Macrophage content was higher in patients with non-ischemic heart failure compared with controls, $P = 0.002$. (C) Neutrophil content was higher in patients with non-ischemic heart failure compared with controls, $P = 0.006$. Graph error bars represent the mean ± SEM.

DISCUSSION

Since heart failure patients experience various systemic complications postoperatively, we evaluated which inflammatory mediators are released by the myocardium and whether failing hearts differ from non-failing hearts in their local inflammatory responses. Besides the expected systemic inflammatory response in all patients, our results show that patients with preexisting heart failure have a different and more pronounced myocardial inflammatory response after cardiac surgery than controls. Myocardial IL-6 and MCP-1 were released in both non-ischemic and ischemic heart failure patients. Furthermore, IL-10, G-CSF, and IL-18 were released in patients with non-ischemic heart failure whereas patients with ischemic heart failure showed a release of VEGF. This myocardial release of various inflammatory mediators may cause systemic complications with a resultant increase in morbidity and mortality.

Release of inflammatory mediators

Baseline values of the inflammatory markers were not statistically different, although a trend was observed for higher levels in patients with preexisting heart failure. Various previous studies showed higher cytokine values in patients with heart failure.[7, 8] However, the opposite has been described as well by Vanderheyden et al.[9] They showed a higher TNF-α and IL-6 expression in patients with aortic stenosis with normal ejection fraction compared with patients with dilated cardiomyopathy.

In our study, a difference in the myocardial inflammatory response between the two most common types of heart failure was observed, comparing non-ischemic and ischemic heart failure patients. Patients with non-ischemic heart failure showed a more diverse and pronounced release of inflammatory mediators than patients with ischemic heart failure. The myocardial inflammatory response is, however, specific for patients with heart failure, as controls did not show a myocardial release of inflammatory mediators. This may indicate that controls are able to cope with the stress associated with tissue reperfusion.

Mechanisms of the myocardial inflammatory response

Mechanisms underlying the observed myocardial inflammatory response in patients with heart failure were analyzed in more detail. Since troponin T values were similar between the groups, passive secretion of inflammatory mediators via myocardial damage is unlikely to be a possible cause of the differences. Secondly, similar expression of several inflammatory mediators was observed during ischemia between the groups. This suggests that the release of inflammatory mediators from pre-stored pools or circulating cells may be responsible

for the myocardial response in heart failure patients. Moreover, no cellular activation by means of NF-κB activity was observed during ischemia. NF-κB activity was already high at baseline in patients with ischemic heart failure, which might explain why NF-κB activity could not increase further during cardioplegic arrest. A more likely explanation for the myocardial release of inflammatory mediators is the observed difference in tissue content of inflammatory cells. Macrophages and neutrophils can release inflammatory mediators such as IL-6, IL-10, IL-18, G-CSF and MCP-1,[10, 11] which were found to be elevated in our study. Since no changes in transcription of these cytokines were observed and that is likely to take longer, degranulation of pre-stored cytokine pools is the likeliest explanation.[12, 13] The post-reperfusion accumulation and activation of neutrophils in the post-ischemic myocardium in the first hours after initiation of myocardial reperfusion have been described in animals.[14] However, we observed increased amounts of infiltrated neutrophils and macrophages already during early ischemia. This may suggest pre-ischemic presence of higher amounts of inflammatory cells in the failing heart. During reperfusion, these tissue inflammatory cells and their released chemoattractants may amplify the influx of more inflammatory cells into the myocardium, thereby contributing to damage.

Clinical implications

It is evident that the clinical prognosis after cardiac surgery is influenced by the balance between pro- and anti-inflammatory mediators. Elevated levels of various pro-inflammatory cytokines are associated with adverse events after cardiac surgery.[15] For example, high circulating IL-6 levels correlate with hepatic and renal dysfunction.[16] It has been shown that IL-6, IL-8, and MCP-1 showed higher peaks and persisted longer in patients with a complicated course after cardiac surgery in comparison with patients with an uneventful recovery.[10] In our study, no significant correlations were found between any of the measured inflammatory mediators and intensive care unit or hospital stay.

The increased inflammatory cell content and release of inflammatory mediators in patients with heart failure stresses the potential of preoperative anti-inflammatory therapy to attenuate the inflammatory response postoperatively. This could contribute to fewer systemic complications and therefore to a more widespread use of cardiac surgical procedures in patients with heart failure. Yet, clinical trials of anti-inflammatory therapies have failed to show a benefit in patients with heart failure.[17] We should therefore be cautious when intervening with cytokine pathways. In addition to the detrimental role of inflammatory mediators, some also appear to have protective effects depending on the presence of responsive cells, the timing, and combination of released cytokines. For example, both receptors of TNF have disparate and opposing effects in mice with heart failure.[18] TNFR1 exacerbates and TNFR2 ameliorates NF-κB activation and inflammation. In renal I/R injury,

it has been shown that the role of IL-6 is not straightforward either.[19] A renal release of IL-6 was observed upon reperfusion in human kidney transplantation, but neutralization of IL-6 in mice undergoing renal I/R injury resulted in aggravation of tissue injury. Therefore, further clinical studies are needed to determine the possibilities of preoperative anti-inflammatory therapy in patients with heart failure undergoing cardiac surgery. To stress the multifactorial approach of treating patients with heart failure even more, bone marrow dysfunction due to inflammation[20], renal insufficiency, and hemodilution also plays a major role in addition to myocardial and systemic inflammatory responses.

Limitations

Since the aim of this study was to identify differences in the release of inflammatory mediators between the groups instead of correlating findings to clinical outcome, small patient numbers were sufficient. However, this small sample size might have limited the detection of minor differences (type II errors). The three patient groups are inherently heterogeneous regarding clinical characteristics. No adjustment for these inevitable differences were made. One might consider the collection of pre-reperfusion biopsies only as a limitation. However, a post-reperfusion biopsy could not be collected since most of the included heart failure patients were hemodynamically unstable during the early reperfusion phase. Lifting the heart, to take a biopsy, was considered unsafe. However, human vital myocardial tissue is not often studied since most previous studies used post-mortem biopsies. Moreover, there were also inter-group differences in aortic cross-clamp times. However, no strong relationships between aortic cross-clamp times and plasma levels of inflammatory mediators were observed. In addition, patients with non-ischemic heart failure had the shortest aortic cross-clamp times, but showed the most diverse release of inflammatory mediators upon reperfusion. Finally, our explorative study was not designed to evaluate inflammatory markers in relation to clinical endpoints. Larger, preferably multicenter, studies are therefore essential.

Conclusions

Patients with heart failure show a clear myocardial inflammatory response upon reperfusion. This can be explained by degranulation of preoperatively infiltrated inflammatory cells in myocardial tissue of failing hearts. In contrast, results in control patients indicate that they can better withstand cardioplegic arrest and subsequent reperfusion without a myocardial release of inflammatory mediators.

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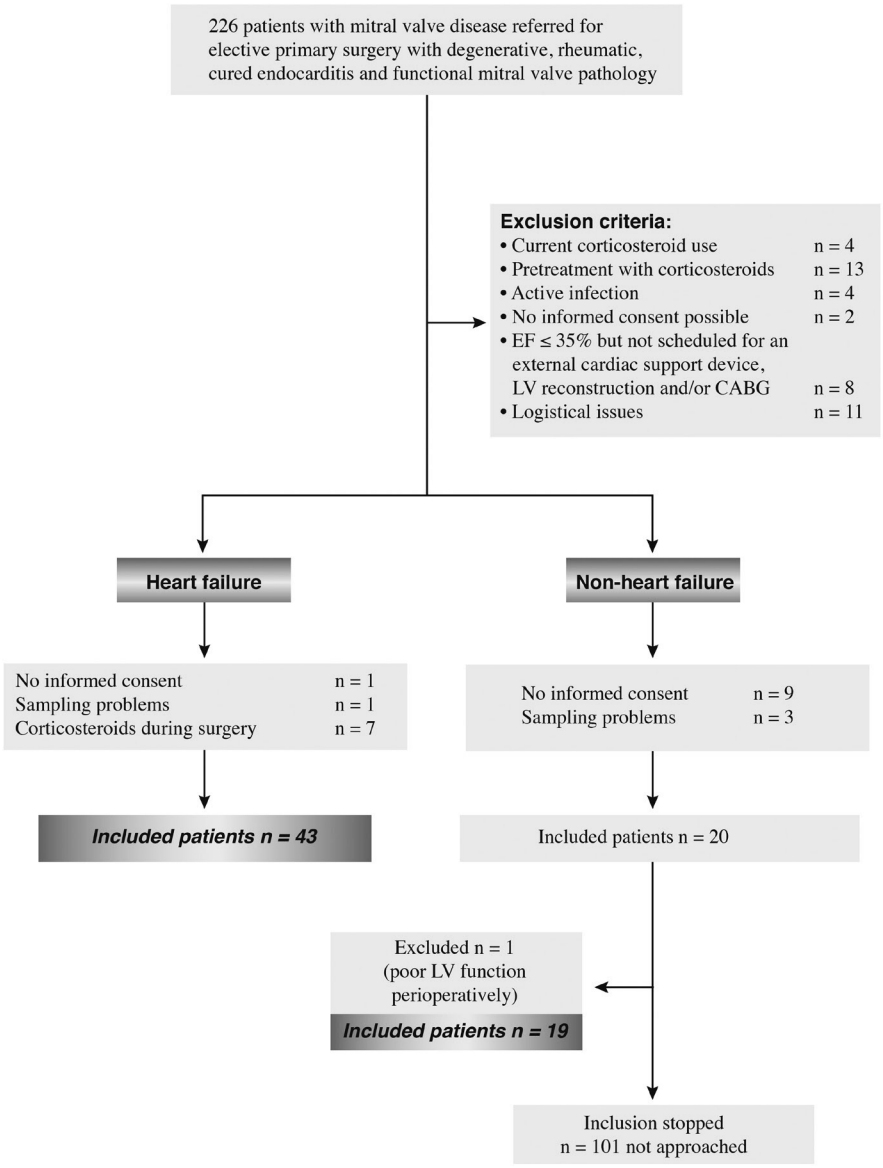
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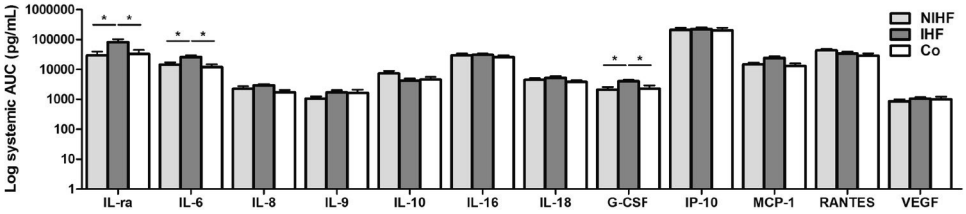
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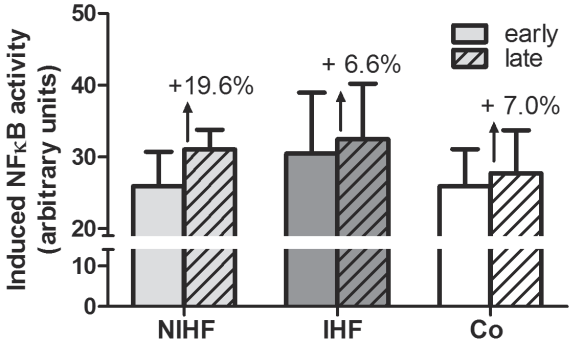
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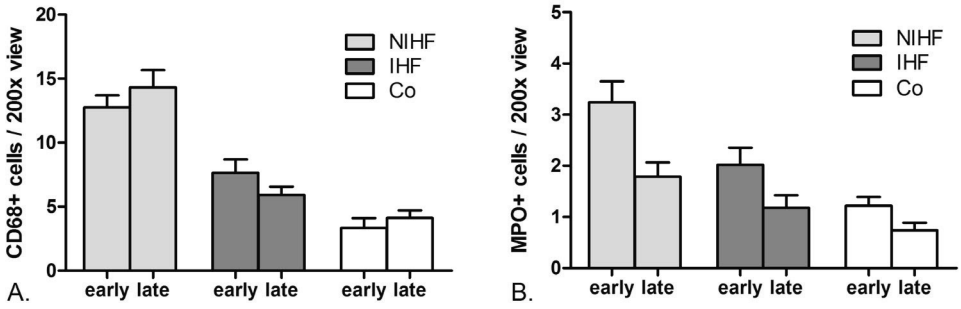
Supplementary data, Figure 1. Flowchart of patient inclusion.
Abbreviations: CABG, Coronary Artery Bypass Grafting; EF, Ejection Fraction; LV, Left Ventricle.



Supplementary data, Figure 2. Arterial levels of inflammatory mediators.
Logarithmic AUCs of systemic (that is, arterial) inflammatory mediators were similar between patients with non-ischemic heart failure, ischemic heart failure, and controls except for interleukin-1 receptor antagonist, interleukin-6, and granulocyte-colony stimulating factor during the first hour after reperfusion (IHF versus NIHF: IL-1ra $P = 0.03$; IL-6 $P = 0.006$; G-CSF $P = 0.005$; IHF versus Co: IL-1ra $P = 0.004$; IL-6 $P = 0.009$; G-CSF $P = 0.002$). Graph error bars represent the mean ± SEM.
Abbreviations: AUC, Area Under the Curve; Co, Controls; G-CSF, Granulocyte-Colony Stimulating Factor; IHF, Ischemic Heart Failure; IL, Interleukin; IL-1ra, Interleukin-1 receptor antagonist; IP-10, Interferon-inducible Protein-10; MCP-1, Monocyte Chemoattractant Protein-1; NIHF, Non-Ischemic Heart Failure; RANTES, Regulated upon Activation, Normal T cell Expressed and Secreted; VEGF, Vascular Endothelial Growth Factor.



Supplementary data, Figure 3. No differences in NF-κB activity during ischemia.
No significant increase in nuclear factor-kappa B (NF-κB) activity was observed in early versus late endomyocardial biopsies in all groups. Graph error bars represent the mean ± SEM.



Supplementary data, Figure 4. Macrophage and neutrophil contents.
Endomyocardial biopsies of patients with non-ischemic heart failure (NIHF), ischemic heart failure (IHF), and controls (Co) were stained for (A) cluster of differentiation 68 (CD68) and (B) myeloperoxidase (MPO). No significant differences in inflammatory cell content were observed between early and late pre-reperfusion biopsies. Graph error bars represent the mean ± SEM.

Supplementary data, Table 1. Baseline values in pg/mL of inflammatory plasma mediators.

	NIHF (n = 18)	IHF (n = 25)	Co (n = 19)	Kruskal-Wallis, *P* value
IL-1ra	69.85 (26.72-117.56)	95.84 (34.46-155.40)	45.00 (22.85-158.39)	0.72
IL-6	7.67 (4.97-13.98)	11.10 (4.68-17.15)	7.12 (1.52-11.76)	0.43
IL-8	9.19 (6.98-13.76)	9.58 (6.51-19.43)	6.77 (3.04-15.72)	0.45
IL-9	3.35 (0.44-8.25)	1.39 (0.91-14.19)	3.78 (1.60-21.32)	0.47
IL-10	3.69 (1.74-7.39)	2.29 (1.69-7.20)	3.05 (1.02-5.66)	0.94
IL-16	93.73 (54.35-146.46)	96.66 (41.35-170.83)	133.22 (21.36-237.52)	0.86
IL-18	75.98 (37.28-131.77)	61.86 (30.52-144.80)	60.65 (18.04-83.47)	0.26
G-CSF	34.58 (14.42-50.99)	38.33 (21.82-56.44)	24.21 (6.78-39.85)	0.21
IP-10	736.81 (308.66-1039.02)	581.81 (324.59-959.91)	456.10 (135.92-794.17)	0.28
MCP-1	19.80 (12.26-23.20)	20.36 (12.82-36.78)	19.96 (7.19-32.22)	0.95
RANTES	416.53 (157.88-787.20)	219.50 (121.65-728.99)	211.46 (64.07-1067.45)	0.52
VEGF	5.00 (3.63-13.07)	10.02 (5.00-19.85)	5.38 (3.14-23.69)	0.32

Baseline values of the inflammatory mediators were not statistically different in patients with non-ischemic heart failure, ischemic heart failure, and controls. Medians ± interquartile ranges are shown.
Abbreviations: Co, Controls; G-CSF, Granulocyte-Colony Stimulating Factor; IHF, Ischemic Heart Failure; IL, Interleukin; IL-1ra, Interleukin-1 receptor antagonist; IP-10, Interferon-inducible Protein-10; MCP-1, Monocyte Chemoattractant Protein-1; NIHF, Non-Ischemic Heart Failure; RANTES, Regulated upon Activation, Normal T cell Expressed and Secreted; VEGF, Vascular Endothelial Growth Factor.

Supplementary data, Table 2. Baseline myocardial gene expression of inflammatory markers.

	ΔCt (median, interquartile range)			Kruskal-Wallis
	NIHF (n = 7)	IHF (n = 8)	Co (n = 7)	*P* value
IL-1ra	8.85 (8.05 to 9.04)	4.80 (3.05 to 8.91)	6.16 (3.16 to 9.07)	0.20
IL-6	4.86 (2.99 to 6.83)	2.84 (-3.20 to 4.62)	1.28 (-2.32 to 2.97)	0.14
IL-8	3.84 (2.68 to 5.89)	2.76 (1.19 to 6.15)	4.68 (2.49 to 6.22)	0.88
IL-10	9.90 (4.95 to 11.75)	10.34 (6.59 to 11.78)	7.56 (5.06 to 10.17)	0.52
TNF-α	6.89 (6.63 to 8.25)	7.42 (3.94 to 8.84)	4.96 (4.00 to 5.74)	0.19
IL-1β	8.49 (6.25 to 8.53)	5.77 (4.83 to 8.68)	5.24 (4.55 to 8.65)	0.41
MMP-9	7.09 (6.24 to 8.55)	4.26 (2.80 to 7.08)	4.93 (3.38 to 6.05)	0.047*
CD68	4.89 (4.28 to 5.22)	2.81 (1.29 to 5.25)	2.49 (1.09 to 2.97)	0.13
MCP-1	6.11 (5.54 to 6.46)	2.91 (2.34 to 7.09)	3.25 (2.35 to 3.53)	0.16

mRNA expression was analyzed in endomyocardial tissue of patients with non-ischemic heart failure, ischemic heart failure, and controls. Levels of mRNA expression were determined using Ct values, i.e. number of cycles at which the fluorescent signal reaches a fixed threshold. ΔCt was calculated according to the formula: Ct target gene - Ct housekeeping gene. High ΔCt values reflect low mRNA expression levels. *Matrix metallopeptidase 9 expression was significantly different in early endomyocardial biopsies of patients with non-ischemic heart failure and controls ($P = 0.01$).

Abbreviations: CD68, Cluster of Differentiation 68; Co, Controls; IHF, Ischemic Heart Failure; IL, Interleukin; IL-1ra, Interleukin-1 receptor antagonist; MCP-1, Monocyte Chemoattractant Protein-1; MMP, Matrix Metallopeptidase; NIHF, Non-Ischemic Heart Failure; TNF, Tumor Necrosis Factor.

Supplementary data, Table 3. Log relative changes in gene expression of inflammatory markers.

	Log relative changes in gene expression (log $2^{-\Delta\Delta Ct}$) Median (interquartile range)			Kruskal-Wallis
	NIHF (n = 7)	IHF (n = 8)	Co (n = 7)	*P* value
IL-1ra	0.08 (-0.13 to 0.25)	0.74 (-0.98 to 1.84)	0.34 (-0.23 to 0.86)	0.63
IL-6	1.15 (0.07 to 1.88)	0.66 (-0.02 to 2.06)	0.45 (-0.09 to 1.27)	0.39
IL-8	0.45 (0.04 to 1.02)	1.52 (-0.67 to 1.99)	0.85 (-0.12 to 1.27)	0.57
IL-10	0.03 (-1.53 to 0.43)	0.49 (0.12 to 0.85)	0.40 (-0.47 to 0.90)	0.20
TNF-α	0.13 (-0.08 to 0.44)	0.03 (-0.08 to 0.42)	0.003 (-0.22 to 0.14)	0.23
IL-1β	-0.12 (-0.62 to 0.26)	0.28 (0.05 to 1.13)	0.33 (-0.48 to 0.54)	0.20
MMP-9	0.48 (0.39 to 0.61)	0.25 (-0.58 to 1.09)	0.18 (0.03 to 0.70)	0.41
CD68	0.05 (-0.15 to 0.21)	0.25 (-0.61 to 0.80)	-0.03 (-0.31 to 0.10)	0.48
MCP-1	0.30 (-0.12 to 0.46)	0.99 (-0.64 to 1.56)	0.21 (0.12 to 0.42)	0.85

Similar log relative changes in gene expression of inflammatory markers were observed in early versus late endomyocardial biopsies of patients with non-ischemic heart failure, ischemic heart failure, and controls. For each sample, ΔΔCt was calculated according to the formula: ΔCt target gene late biopsy − ΔCt target gene early biopsy.

Abbreviations: CD68, Cluster of Differentiation 68; Co, Controls; IHF, Ischemic Heart Failure; IL, Interleukin; IL-1ra, Interleukin-1 receptor antagonist; MCP-1, Monocyte Chemoattractant Protein-1; MMP, Matrix Metallopeptidase; NIHF, Non-Ischemic Heart Failure; TNF, Tumor Necrosis Factor.

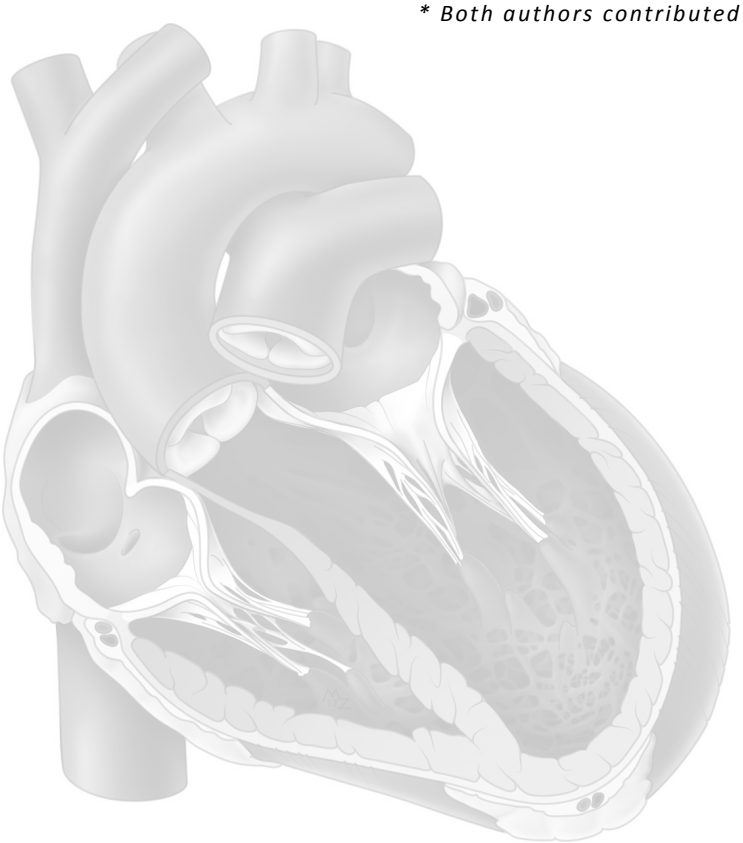
CHAPTER EIGHT

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Galectin-3 and left ventricular reverse remodeling after surgical mitral valve repair

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ABSTRACT

Background: Mitral valve repair in patients with functional mitral regurgitation (FMR) has been associated with beneficial left ventricular (LV) reverse remodeling. Recently, galectin-3 emerged as a marker of myocardial inflammation and fibrosis which may influence LV remodeling after surgery. The aim of the current study was to evaluate the association between preoperative galectin-3 levels, and LV reverse remodeling in heart failure patients with significant FMR who underwent mitral valve repair.

Methods and Results: In total, 42 heart failure patients (66 ± 10 years, 69% male) were evaluated. Plasma galectin-3 levels were assessed preoperatively. Two-dimensional echocardiographic parameters were measured at baseline, and at six and twelve months after surgery. LV reverse remodeling was defined as a decrease in LV end-systolic volume ≥ 15% at six months follow-up. In total, 57% of the patients showed LV reverse remodeling. Patients with LV reverse remodeling showed significantly lower preoperative galectin-3 levels (17.5 ± 5.6 vs. 23.7 ± 9.9 ng/mL, P = 0.009) compared with patients without LV reverse remodeling. In addition, patients with galectin-3 ≤ 18.2 ng/mL had a six-fold higher probability of showing LV reverse remodeling after surgery as compared with patients with levels > 18.2 ng/mL (odds ratio 6.58, 95% confidence interval 1.32 – 33.33, P = 0.02).

Conclusions: High preoperative plasma galectin-3 is independently associated with the absence of LV reverse remodeling after mitral valve repair. Galectin-3 may be useful to identify heart failure patients who will need additional treatment to obtain beneficial LV reverse remodeling.

INTRODUCTION

Surgical mitral valve repair in patients with heart failure, and moderate to severe functional mitral regurgitation (FMR) has been associated with improvement in clinical symptoms, left ventricular (LV) reverse remodeling and better prognosis.[1-4] In particular, the occurrence of LV reverse remodeling has important prognostic implications, since the absence of significant LV reverse remodeling or the presence of progressive LV dilatation after surgical mitral valve repair has been associated with recurrence of mitral regurgitation and worse prognosis.[2] The incidence of favorable LV reverse remodeling ranges between 40% and 52% of the patients, according to different series.[1, 3, 5] Preoperative LV dimensions have been associated with the extent of LV reverse remodeling after mitral valve repair.[2] In particular, in patients with LV end-diastolic dimension > 65 mm, the presence of LV reverse remodeling after mitral valve repair is less frequent as compared with patients with smaller LV dimensions.[2] This suggests that a severely dilated left ventricle may not show reverse remodeling after restoration of valvular function and volume load. Therefore, additional surgical procedures have been proposed to improve the outcomes of patients with FMR and severe LV dilatation undergoing mitral valve repair.[2] Detailed knowledge of the pathophysiological mechanisms underlying LV reverse remodeling after mitral valve repair may prompt the search for novel (surgical) therapies that lead to improved outcomes.

Recently, it has been demonstrated that galectin-3, a novel biomarker in patients with heart failure, plays a key role in myocardial fibrosis processes that may influence LV remodeling after surgery.[6-8] In animal models, intrapericardial infusion of galectin-3 has been associated with increased deposition of collagen I and induction of LV dysfunction.[9] However, whether galectin-3 is associated with LV reverse remodeling after mitral valve repair remains unexplored. Therefore, the aim of the present study was to evaluate the association between preoperative galectin-3 levels and LV reverse remodeling in non-ischemic and ischemic heart failure patients with moderate to severe FMR who underwent mitral valve repair.

METHODS

Patient population

In total, 42 patients were included to participate in this two-year prospective observational study: 18 patients with non-ischemic heart failure and 24 patients with ischemic heart failure. Patients with heart failure and significant FMR who underwent surgical mitral valve repair were included. Baseline clinical variables were registered, including age, gender, blood

pressure, New York Heart Association (NYHA) functional class, quality of life score (according to the Minnesota Living with Heart Failure Questionnaire), six-minute walk test (6-MWT), medical treatment, and co-morbidities. In addition, common laboratory parameters were assessed, including hemoglobin, C-reactive protein, and creatinine. Glomerular filtration rates (GFRs) were calculated according to the Modification of Diet in Renal Disease formula, and renal dysfunction was defined as recommended by the National Kidney Foundation and the Kidney Disease Outcomes Quality Initiative Guidelines (estimated GFR (eGFR) < 60 mL/min/1.73m^2).[10] Moreover, logistic EuroSCOREs were calculated in all patients to assess the peri-operative risk. All patients had NYHA function class II-IV heart failure symptoms, moderate to severe FMR, and reduced LV ejection fraction (LVEF < 60%).[11]

One day before surgery, venous galectin-3 levels were determined in resting conditions. Moreover, two-dimensional (2D) echocardiography was performed before surgery and repeated before hospital discharge, and six and twelve months after surgery. Clinical and echocardiographic data were collected at the departmental Cardiology Information System (EPD-Vision®, Leiden University Medical Centre) and the echocardiography database, respectively, and retrospectively analyzed.

This study complied with the Declaration of Helsinki, was approved by our local ethics committee, and written informed consent was obtained from all patients.

Echocardiography

Images were acquired at rest with the patient in the left lateral decubitus position using a commercially available system (Vivid 7 and E9, General Electric-Vingmed, Horten, Norway). Data acquisition was performed with a 3.5-MHz transducer in the parasternal and apical views. M-mode and 2D images were obtained and saved in cine-loop format. Data analysis was performed offline (EchoPAC version 111.0.0; General Electric-Vingmed, Horten, Norway). LV end-systolic volumes and end-diastolic volumes were measured in the apical 2- and 4-chamber views using the biplane Simpson's method and the LVEF was calculated.[12] LV volumes were indexed to body surface area. The intra-observer agreement for LV end-systolic and end-diastolic volume, and LVEF were 7.0 ± 10.1 mL, 7.4 ± 11.2 mL, and 1.9 ± 4.4%, respectively. The inter-observer agreement for LV end-systolic and end-diastolic volume, and LVEF were 11.3 ± 13.9 mL, 12.9 ± 14.7 mL, and 2.5 ± 4.9%, respectively.[13] LV reverse remodeling was defined as a reduction in LV end-systolic volume of ≥ 15% at six months follow-up (or at twelve months follow-up when not available).[3, 4] Baseline LV volumes and LVEF were measured in all patients. In patients who underwent concomitant LV reconstruction surgery, LV end-systolic volumes were also measured before hospital discharge and were used as a reference to evaluate LV reverse remodeling at follow-up.

Severity of FMR was assessed using a multiparametric approach, including the effective regurgitant orifice area and regurgitant volume calculated with the proximal isovelocity surface area method.[14] Color Doppler images were obtained using a Nyquist limit of 30-60 cm/s and a color gain that just removes random color speckle from non-moving areas.[14, 15]

In addition, LV sphericity index was calculated at end-systole and end-diastole as the ratio between the LV-short-axis diameter and the LV long-axis diameter in the apical 4-chamber view.[16, 17] Moreover, maximal left atrial volume was measured according to the biplane Simpson's technique in the apical 2-chamber and 4-chamber views and indexed to the body surface area, as recommended.[12]

Finally, systolic pulmonary artery pressure was assumed by adding the right ventricular pressure to the right atrial pressure. Right ventricular pressure was estimated by calculating the systolic pressure gradient between the right ventricle and right atrium by the maximum velocity of the regurgitant jet using the modified Bernoulli equation.[18] Right atrial pressure was estimated by measuring the diameter and the inspiratory collapse of the inferior vena cava, as recommended.[18]

Measurement of circulating levels of galectin-3

Plasma galectin-3 levels were measured in samples collected the day before surgery using a commercially available enzyme-linked immunosorbent assay (BG Medicine, Waltham, MA, USA). The lower detection limit was 1.13 ng/mL.[19] The assay has been optimized and standardized, and intra- and inter-assay variation have been reported.[19] In addition, no potential interference was found for commonly used cardiovascular medications with this galectin-3 assay.[20]

Measurement of N-terminal pro-brain natriuretic peptide

Plasma N-terminal pro-brain natriuretic peptide (NT-proBNP) levels were determined in samples collected the day before surgery by using proBNP and proBNP II kits from Roche Diagnostics on a Modular Analytics E170 analyser (Roche Diagnostics, Mannheim, Germany). A sandwich-type quantitative immunoassay was performed using antibodies against epitopes in the N-terminal part of proBNP. The lower detection limit was 5 ng/L. Total coefficients of variance at low concentrations (77 ng/L) and high concentrations (2,170 ng/L) were both 2.7%.

Surgical procedures

All surgical procedures were performed via a midline sternotomy under normothermic cardiopulmonary bypass with intermittent antegrade warm-blood cardioplegia and heparin softline coating. An external cardiac support device (CorCap cardiac support device, Acorn Cardiovascular, St. Paul, MN, USA) was placed in patients with non-ischemic heart failure and a LV end-diastolic diameter > 65 mm, measured on the preoperative echocardiographic examination. Patients with ischemic heart failure underwent a coronary artery bypass grafting procedure based on the preoperative coronary angiogram, and were considered eligible for ventricular reconstruction as described by Dor et al. if akinesia or dyskinesia was present in a large area of the anterior LV wall.[21, 22] Mitral valve repair was considered successful if there was no residual mitral regurgitation, and if a leaflet coaptation height of ≥ 8 mm at the A2-P2 level was achieved as measured directly after the surgical procedures.[23]

Statistical analysis

Continuous data are presented as the mean ± standard deviation or standard error of the mean, or median with 25th and 75th interquartile ranges, where appropriate. Categorical data are expressed as frequencies and percentages. Changes in LV volumes and EF during follow-up in the overall population were assessed by linear mixed models. Patients were divided into two subgroups based on the occurrence of LV reverse remodeling during follow-up. Clinical and echocardiographic differences at baseline were evaluated between these subgroups with the unpaired Student's *t*-test and χ^2 test. For this analysis, galectin-3, NT-proBNP, C-reactive protein, creatinine, and eGFR values were log-transformed to obtain a Gaussian distribution. Statistical tests that required normality (or rather symmetry) were applied on these transformed data. Thereafter, data were back-transformed to the original data scales, and expressed as means with confidence intervals (CIs). Subsequently, differences in changes in LV volumes and EF over time between patients with LV reverse remodeling, and patients without were analyzed with linear mixed models. Moreover, to assess the association between baseline log-transformed galectin-3 levels and changes in LV volumes during follow-up, correlation analysis was performed using Pearson correlation coefficients. Again, after performing the statistical tests, data were back-transformed to the original scales.

In addition, receiver operating characteristic (ROC) curve analysis was performed to determine the sensitivity and specificity for various cut-off values of baseline galectin-3 levels for the prediction of LV reverse remodeling. Finally, univariate and multivariate logistic regression analyses were performed to identify independent baseline clinical and echocardiographic determinants as outlined in Table 1 of LV reverse remodeling during

follow-up. All significant univariate variables ($P < 0.05$) were included in a multivariate logistic regression model. To avoid multicollinearity between univariate predictors, a correlation coefficient of < 0.7 was set.[24]

All statistical tests were two-sided, a P value < 0.05 was considered statistically significant. All statistical analyses were performed with SPSS software (version 20.0, SPSS Inc., Chicago, IL, USA).

RESULTS

Baseline characteristics

In total, 42 heart failure patients with moderate to severe FMR (mean age 66 ± 10 years, 69% men) were evaluated. Baseline characteristics of the patient population are summarized in Table 1. The mean galectin-3 level was 20.1 ± 7.9 ng/mL, which is comparable to previously published heart failure cohorts. All patients underwent surgical mitral valve repair; 18 patients also received an external cardiac support device and 24 patients underwent concomitant ventricular reconstruction surgery (n = 10), and/or coronary artery bypass surgery (n = 14). Tricuspid valve annuloplasty, aortic valve replacement, and ablation procedures were additionally performed if indicated (Table 2).

Surgical outcomes

There were no intra-operative deaths. Early mortality (30-day mortality and in-hospital mortality when length of stay after surgery was > 30 days) was 4.8% (two patients). One patient with non-ischemic heart failure died eight days after surgery as a result of ventricular fibrillation. The other patient with ischemic heart failure died 41 days after surgery as a result of refractory heart failure after ventricular reconstruction surgery.

Time course of New York Heart Association functional class, left ventricular volumes, and ejection fraction

Within the first six months after surgery, two patients died of a cardiac cause as discussed above. Moreover, 2D echocardiography was not available at six and twelve months follow-up in three patients. As a result, echocardiography at six and/or twelve months follow-up was available in 37 patients (88%). Of these patients, 28 patients had an echocardiogram at both six and twelve months follow-up, four patients only had an echocardiogram at 6 months follow-up, and five patients only had an echocardiogram at twelve months follow-up.

Table 1. Baseline characteristics.

	Total patient population n = 42	Reverse remodeling n = 21	No Reverse remodeling n = 16	*P* value
Clinical characteristics				
Age (years)	66 ± 10	64 ± 11	66 ± 8	0.50
Male gender	29 (69%)	16 (76%)	11 (69%)	0.61
Body surface area (m^2)	2.02 ± 0.21	2.08 ± 0.21	1.98 ± 0.20	0.16
Ischemic etiology	24 (57%)	12 (57%)	8 (50%)	0.67
Systolic blood pressure (mmHg)	117 ± 16	118 ± 15	114 ± 18	0.55
Diastolic blood pressure (mmHg)	70 ± 11	71 ± 10	71 ± 13	0.94
Logistic EuroSCORE (%)	10.0 ± 5.8	10.1 ± 6.4	8.8 ± 3.8	0.45
NYHA functional class	2.9 ± 0.3	2.9 ± 0.4	2.9 ± 0.3	0.45
QoL score	28 ± 15	26 ± 13	32 ±17	0.33
6MWT (m)	381 ± 109	395 ± 122	380 ± 76	0.73
Comorbidities				
Diabetes	11 (26%)	7 (33%)	3 (19%)	0.32
COPD	7 (17%)	3 (14%)	3 (19%)	0.72
AF	10 (24%)	4 (19%)	5 (31%)	0.39
Laboratory tests				
Galectin-3 (ng/mL)	18.9 [17.1 – 20.9]	16.8 [14.9 – 18.9]	22.4 [19.1 – 26.3]	0.009
NT-proBNP (pg/mL)	1,408 [1,054 – 1,882]	1,108 [750 – 1,636]	1,826 [1,164 – 2,864]	0.12
Hb (mmol/L)	8.7 ± 0.9	8.8 ± 0.8	8.7 ± 0.9	0.87
CRP (mg/L)	2.4 [0.3 – 17.6]	2.3 [1.8 – 3.1]	2.3 [1.8 – 3.1]	0.98
Creatinine (µmol/L)	65 [59 – 72]	86 [77 – 95]	105 [89 – 123]	0.04
eGFR (mL/min/1.73m^2)	92 [85 – 99]	74 [65 – 83]	56 [47 – 67]	0.01
eGFR < 60 mL/min/1.73m^2	14 (33%)	4 (19%)	9 (56%)	0.02
Medication				
ACE-inhibitors / ARBs	38 (91%)	19 (91%)	15 (94%)	0.72
Beta-blockers	39 (93%)	20 (95%)	15 (94%)	0.84
Statins	33 (78%)	17 (81%)	12 (75%)	0.66
Diuretics	38 (91%)	19 (91%)	15 (954%)	0.72
Digoxins	6 (14%)	2 (10%)	4 (25%)	0.21
Echocardiography				
LVESV (mL/m^2)	73 ± 26	156 ± 56	143 ± 41	0.44
LVEDV (mL/m^2)	102 ± 30	105 ± 31	105 ± 33	0.99
LVEF (%)	30 ± 6	29 ± 7	30 ± 6	0.59
LV sphericity index end-diastole	58 ± 8	58 ± 8	58 ± 9	0.39
LV sphericity index end-systole	53 ± 9	58 ± 9	58 ± 10	0.85
LAVI (mL/m^2)	41 ± 16	40 ± 12	44 ± 20	0.99
SPAP (mmHg)	33 ± 16	33 ± 16	34 ± 16	0.65
Mean MR grade	3.0 ± 0.4	3.1 ± 0.4	3.1 ± 0.4	0.82
EROA (cm^2)	0.27 ± 0.11	0.26 ± 0.09	0.29 ± 0.14	0.43
R Vol (mL/beat)	41 ± 14	39 ± 13	44 ± 17	0.32

Data are presented as mean ± standard deviation, mean [confidence interval] or as number (percentage). *P* values are given for the comparisons of patients with and without LV reverse remodeling.
Abbreviations: 6MWT, six-Minute Walk Test; ACE, Angiotensin Converting Enzyme; AF, Atrial Fibrillation; ARB, Angiotensin Receptor Blocker; COPD, Chronic Obstructive Pulmonary Disease; CRP, C-Reactive Protein; eGFR, estimated Glomerular Filtration Rate; EROA, Effective Regurgitation Orifice Area; Hb, Hemoglobin; IQR, Interquartile Range; LAVI, Left Atrial Volume Index; LV, Left Ventricular/Ventricle; LVEDV, Left Ventricular End-Diastolic Volume; LVEF, Left Ventricular Ejection Fraction; LVESV, Left Ventricular End-Systolic Volume; MR, Mitral Regurgitation; NT-proBNP, N-Terminal Pro-brain Natriuretic Peptide; NYHA, New York Heart Association; QoL, Quality of Life; R Vol, Regurgitant Volume; SPAP, Systolic Pulmonary Artery Pressure.

Echocardiographic characteristics of the total patient population at baseline, and at six and twelve months follow-up are summarized in Figure 1. A significant decrease in LV end-systolic (baseline: 73 ± 4 mL/m^2; six months: 55 ± 4 mL/m^2; twelve months: 54 ± 4 mL/m^2, $P < 0.001$), and end-diastolic volumes (baseline: 102 ± 5 mL/m^2; six months: 84 ± 5 mL/m^2; twelve months: 85 ± 5 mL/m^2, $P < 0.001$) was observed over time. These changes resulted in a significant improvement in LVEF from 30 ± 1% at baseline to 34 ± 1% at six months follow-up, and to 37 ± 2% at twelve months follow-up ($P = 0.005$ for trend over time). In patients with concomitant LV reconstruction or use of Corcap cardiac support device, the time evolution of LV volumes is provided in the Supplementary data, Tables 1 and 2.

Importantly, these LV volumetric and functional improvements were accompanied by improvements in NYHA functional class (baseline: 2.9 ± 0.0, six months: 1.9 ± 0.1, twelve months: 2.0 ± 0.1, $P < 0.001$).

Patients with left ventricular reverse remodeling vs. non-left ventricular reverse remodeling

Patients were divided into two subgroups based on the occurrence of LV reverse remodeling at six months follow-up (or, when not available, at twelve months follow-up). LV reverse remodeling (reduction in LV end-systolic volume ≥ 15%) was observed in 21 patients (57%).

The differences in baseline characteristics between patients with and without LV reverse remodeling are shown in Table 1. Patient groups were comparable in terms of clinical characteristics, comorbidities, medical treatment, and echocardiographic characteristics. However, patients who showed LV reverse remodeling also demonstrated a better preservation of renal function compared with patients who did not show LV reverse remodeling. In addition, patients showing LV reverse remodeling had significantly lower baseline galectin-3 levels compared with patients without LV reverse remodeling (16.8 [14.9 – 18.72] versus 22.4 [19.1 – 26.31] ng/mL, $P = 0.009$).

In both patient groups, significant improvements in NYHA functional class were observed, albeit that this improvement was more pronounced in patients with LV reverse remodeling: from 2.9 ± 0.1 at baseline to 1.6 ± 0.2 and 1.9 ± 0.2 ($P < 0.001$) at six and twelve months, respectively, in patients with LV reverse remodeling versus from 2.9 ± 0.1 to 2.2 ± 0.2 and 2.2 ± 0.2, respectively ($P = 0.003$), in patients without LV reverse remodeling.

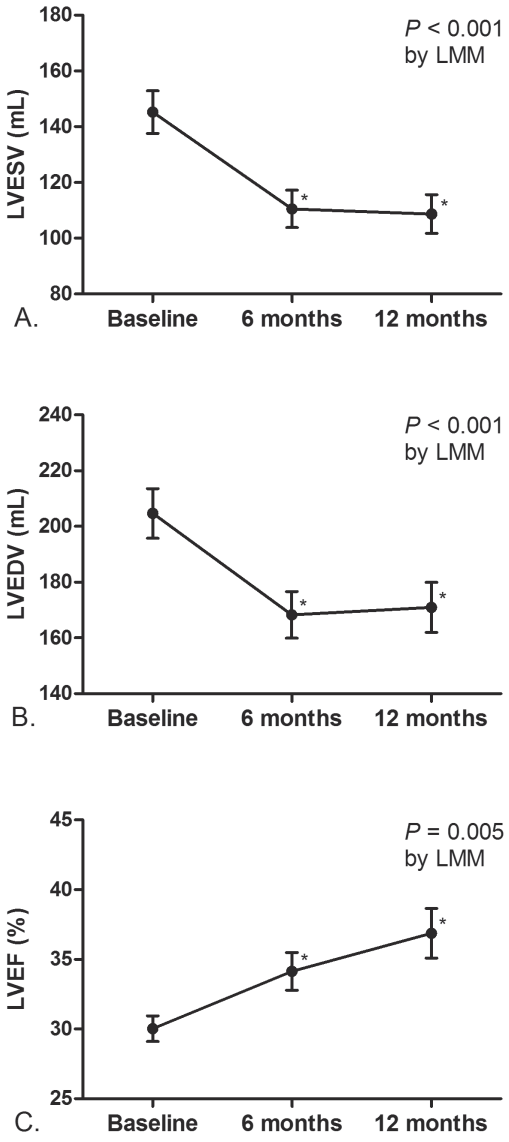


Figure 1. Echocardiographic data in heart failure patients.
Time course of (A) LV end-systolic volumes (LVESV), (B) LV end-diastolic volumes (LVEDV), and (C) LV ejection fractions (LVEF) in heart failure patients who underwent mitral valve repair. Data are presented as mean ± standard error of the mean. * $P < 0.05$ compared with baseline.
Abbreviation: LMM, linear mixed model.

Association between preoperative galectin-3 and LV reverse remodeling

A significant correlation was observed between baseline galectin-3 levels and changes in LV end-systolic volume after mitral valve repair (r = 0.47, *P* = 0.003, Figure 2).

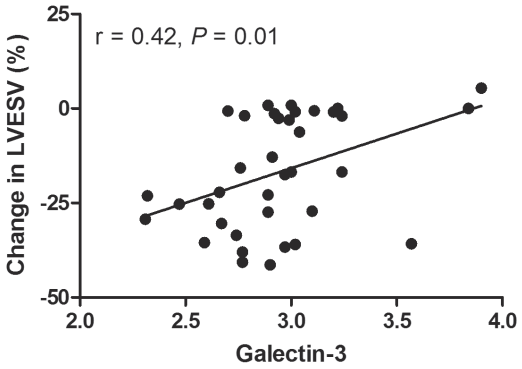


Figure 2. Correlation between baseline log-transformed galectin-3 levels and changes in LV end-systolic volume after mitral valve repair.
Five patients with high log-transformed galectin-3 levels (≥ 2.9; geometrical mean of ≥ 18.2 ng/mL) showed significant LV reverse remodeling (≥ 25% reduction): three patients underwent coronary artery bypass grafting and two patients received an external cardiac support device in addition to their mitral valve repair.

According to the ROC curve analysis, galectin-3 levels at baseline showed a modest accuracy to predict LV reverse remodeling, with an area under the curve (AUC) of 0.77. The highest sensitivity and specificity could be demonstrated at a cut-off value of galectin-3 of 18.2 ng/mL (Figure 3).

Univariate and multivariate logistic regression analyses were performed to evaluate the association between LV reverse remodeling and galectin-3 (Table 3). The final multivariate model included galectin-3 ≤ 18.2 ng/mL and eGFR < 60 mL/min/1.73 m^2. In this model, galectin-3 ≤ 18.2 ng/mL (odds ratio 6.58, 95% confidence interval 1.32 – 33.33, *P* = 0.02) was as an independent determinant of LV reverse remodeling at six months follow-up.

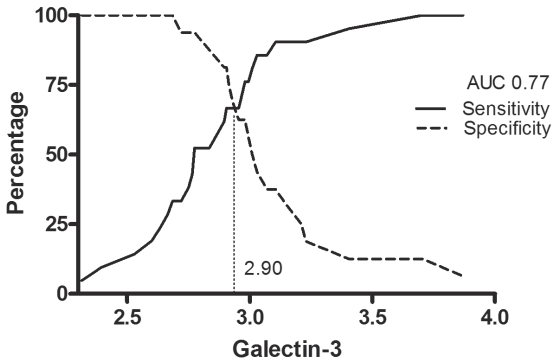


Figure 3. Prediction of LV reverse remodeling with galectin-3 levels.
Receiver operating characteristic (ROC) curve analysis demonstrated a sensitivity of 67% with a specificity of 81% to predict LV reverse remodeling following mitral valve repair at a cut-off of log-transformed galectin-3 of 2.9 at baseline. This level corresponds to a non log-transformed value of 18.2 ng/mL.

Table 2. Number of surgical procedures.

	Total patient population n = 42	Reverse remodeling n = 21	No Reverse remodeling n = 16
CorCap®	18	9	8
LV reconstruction	10	5	4
CABG (mean number of grafts)	24 (2.5)	13 (2.3)	7 (2.4)
Mitral valve annuloplasty	42	21	16
Tricuspid valve annuloplasty	22	12	9
Aortic valve replacement	2	0	2
AF ablation	8	4	4
VT ablation	5	1	3
CRT	27	15	10

Abbreviations: AF, Atrial Fibrillation; CABG, Coronary Artery Bypass Grafting; CRT: Cardiac Resynchronization Therapy; LV, Left Ventricular/Ventricle; VT, Ventricular Tachycardia.

Table 3. Determinants of left ventricular reverse remodeling after mitral valve repair.

	Univariate analysis			*Multivariate analysis*		
	OR	**95% CI**	***P value***	**OR**	**95% CI**	***P* value**
Galectin-3 ≤ 18.2 ng/mL	8.70	1.84 – 40.00	0.006	6.58	1.32 – 33.33	0.02
eGFR < 60 mL/min/1.73m²	0.18	0.04 – 0.80	0.02	0.28	0.06 – 1.37	0.12

Abbreviations: eGFR, estimated Glomerular Filtration Rate; CI, Confidence Interval; OR, Odds Ratio.

DISCUSSION

The present study demonstrates that lower preoperative galectin-3 levels are independently associated with LV reverse remodeling after mitral valve repair in heart failure patients with moderate to severe FMR. Heart failure patients with a galectin-3 level ≤ 18.2 ng/mL had a six-fold higher probability of showing LV reverse remodeling as compared with patients with a galectin-3 value > 18.2 ng/mL.

Left ventricular remodeling after mitral valve repair in heart failure patients

The incidence of LV reverse remodeling after mitral valve repair in patients with heart failure and FMR still remains relatively low.[1-4] Several pathophysiological factors are involved in the LV reverse remodeling process after surgical mitral valve repair. A previous study has shown that preoperative LV dimensions are predictive of LV reverse remodeling after mitral valve repair, suggesting that very distorted and diseased left ventricles may no longer recover after restoration of mitral valve function, and additional surgical procedures may be needed (such as CorCap® devices).[2] In addition, LV fibrosis and inflammation may also be important determinants of LV reverse remodeling after mitral valve repair.[8, 25, 26] In patients with mitral valve regurgitation, Oral et al. showed that preoperative levels of tumor necrosis factor-α were significantly associated with changes in LV volumes following surgery, emphasizing the role of inflammation in LV remodeling.[27] Reduction in LV dimensions after surgical mitral valve repair has been largely associated with improved outcome. Therefore, understanding the underlying pathophysiological determinants of LV remodeling may be an important clue to provide tailored surgical strategies to improve the outcome of heart failure patients with FMR. In particular, evaluation of biomarkers associated with LV remodeling, such as the β-galactoside-binding lectin galectin-3, may provide further insight into this field.

Galectin-3: a marker of inflammation, fibrosis, and cardiac remodeling

Recently, galectin-3 emerged as a novel biomarker in patients with heart failure.[6, 7] Galectin-3 is secreted by activated macrophages and involved in various physiological and pathological processes.[6, 7, 9] Preclinical studies provide evidence on the association between inflammation, fibrosis, and cardiac remodeling, with galectin-3 as the main underlying pathophysiological factor.[9] Patients with heart failure show increased levels of galectin-3 compared with the general population, and several studies demonstrated the prognostic value of elevated galectin-3 levels in this population.[19, 28-32] In addition, Lok et al. recently observed in 240 chronic heart failure patients that baseline galectin-3 values were independently correlated with changes in LV volumes three months after optimal medical treatment.[30] However, the

association between galectin-3 and occurrence of LV reverse remodeling after heart failure therapies (including surgical mitral valve repair) remains unknown.

Galectin-3: a marker of left ventricular reverse remodeling after mitral valve repair

The present study provides additional information on the association between baseline galectin-3 levels and LV reverse remodeling in patients with non-ischemic and ischemic heart failure, and moderate to severe FMR undergoing mitral valve repair. As hypothesized, one of the major findings was an independent association between preoperative galectin-3 levels and LV reverse remodeling during follow-up. We observed that a cut-off value of 18.2 ng/mL best predicted LV reverse remodeling. This value is very close to the 17.8 ng/mL cut-off value in the Food and Drug Administration (FDA) label, that was set to prognosticate stable heart failure patients. Patients with low preoperative values of galectin-3 more frequently showed LV reverse remodeling compared with patients with high preoperative levels of galectin-3. This suggests that patients without LV reverse remodeling may have had an enhanced inflammatory and pro-fibrotic status compared with patients who did show LV reverse remodeling. Therefore, galectin-3 levels may be an important marker of LV reverse remodeling after surgical mitral valve repair, possibly via modulation of fibrosis.

Current guidelines provide recommendations on medical, device-based, and surgical therapies of patients with heart failure.[33] In addition, identification of patients who will benefit from those therapies, specifically device-based and surgical therapies, is paramount. Evaluation of the pathological substrate (LV volumes and EF, extent and location of scar, and LV dyssynchrony) has been proposed to improve selection of patients for specific therapies. In this regard, heart failure patients undergoing surgical mitral valve repair and LV reconstruction surgery may benefit from a thorough pre-procedural evaluation to anticipate to the therapy outcomes. In this regard, the results of the present hypothesis-generating evaluation points towards the relevance of assessing LV fibrosis and inflammatory activity in these patients, by measuring, for example, galectin-3 levels. Moreover, measurement of galectin-3 levels may potentially be helpful in the selection of patients who will benefit from transcatheter valve repair in terms of LV reverse remodeling.[34, 35] The present results should be considered hypothesis-generating and must be confirmed in larger trials or registries before being implemented in daily clinical practice.

Limitations

Some limitations should be acknowledged. The present study has an observational design and has a relatively small sample size with various etiologies of heart failure and treatment procedures concomitant with mitral valve repair. The small sample size precluded us from

performing subgroup analyses and evaluating the impact of galectin-3 levels on long-term prognosis. Furthermore, no follow-up galectin-3 assessments were available, which could have clarified further the relationship between this biomarker and LV geometry. Finally, our echocardiographic follow-up period was limited to twelve months after surgery, although we can not exclude that the process of LV reverse remodeling takes longer.[3]

Conclusion

In conclusion, preoperative plasma galectin-3 is independently associated with LV reverse remodeling after mitral valve repair for FMR in heart failure patients. Therefore, measuring plasma galectin-3 may be useful for the identification of patients who will respond to additional (surgical) therapy with the aim of achieving beneficial LV reverse remodeling.

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Supplementary data, Table 1. Time course of left ventricular volumes and ejection fractions in patients who underwent left ventricular reconstruction surgery in addition to mitral valve repair (n = 10).

	Pre-surgery	Before discharge	6 months follow-up	12 months follow-up	*P* value
LVESV (mL/m²)	76 ± 12	45 ± 3	38 ± 3 †	39 ± 4 †	0.03
LVEDV (mL/m²)	105 ± 12	68 ± 4	61 ± 5 †‡	63 ± 6 †	0.002
LVEF (%)	30 ± 3	34 ± 3	37 ± 2	40 ± 4	0.32

Data are presented as mean ± standard error of the mean.
P values are given for the overall change over time.
† $P < 0.05$ compared with pre-surgery measurements (adjusted for multiple comparisons).
‡ $P < 0.05$ compared with before discharge measurements (adjusted for multiple comparisons).
Abbreviations: LVEDV, Left ventricular End-Diastolic Volume; LVEF, Left Ventricular Ejection Fraction; LVESV, Left Ventricular End-Systolic Volume.

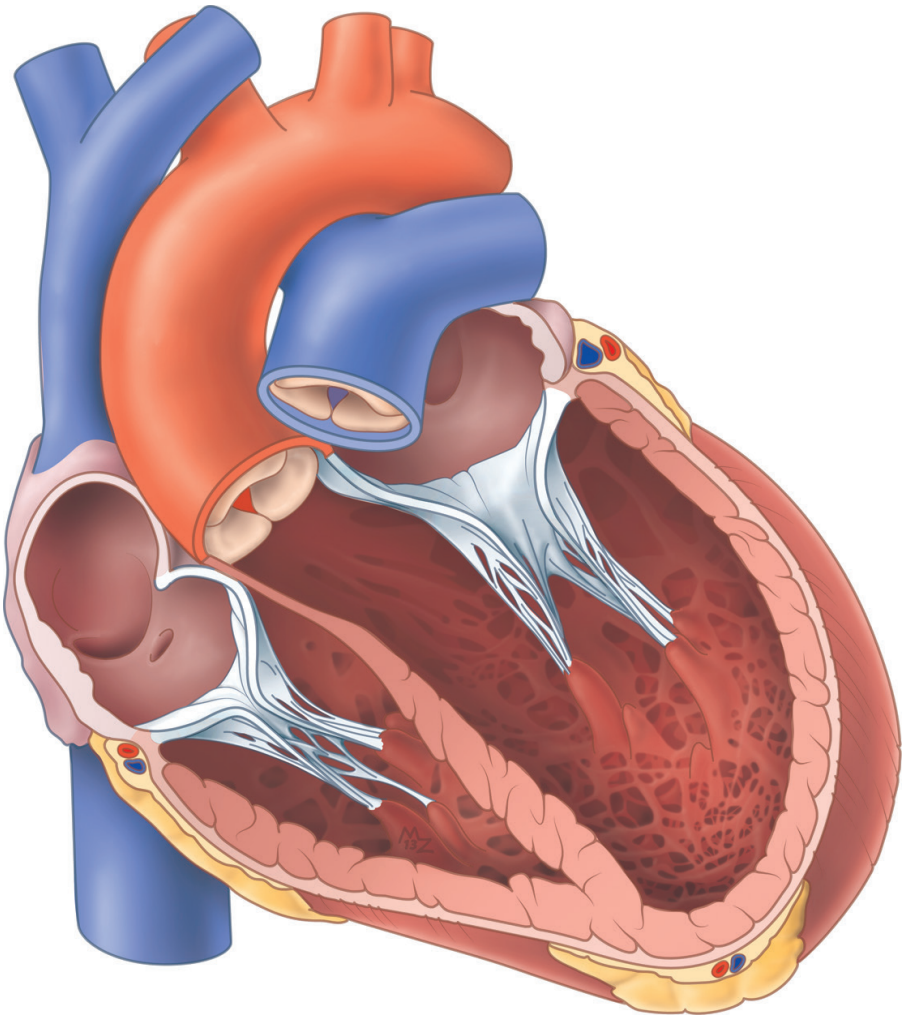
Supplementary data, Table 2. Time course of left ventricular volumes and ejection fractions in patients who underwent an external cardiac support device implantation in addition to mitral valve repair (n = 18).

	Pre-surgery	Before discharge	6 months follow-up	12 months follow-up	*P* value
LVESV (mL/m²)	76 ± 6	74 ± 6	66 ± 6 †‡	65 ± 7 †‡	0.005
LVEDV (mL/m²)	105 ± 8	105 ± 8	95 ± 8	96 ± 8	0.04
LVEF (%)	29 ± 1	30 ± 1	31 ± 2	32 ± 2	0.13

Data are presented as mean ± standard error of the mean.
P values are given for the overall change over time.
† $P < 0.05$ compared with pre-surgery measurements (adjusted for multiple comparisons).
‡ $P < 0.05$ compared with before discharge measurements (adjusted for multiple comparisons).
Abbreviations: LVEDV, Left ventricular End-Diastolic Volume; LVEF, Left Ventricular Ejection Fraction; LVESV, Left Ventricular End-Systolic Volume.

PART TWO

Reducing myocardial ischemia/reperfusion injury

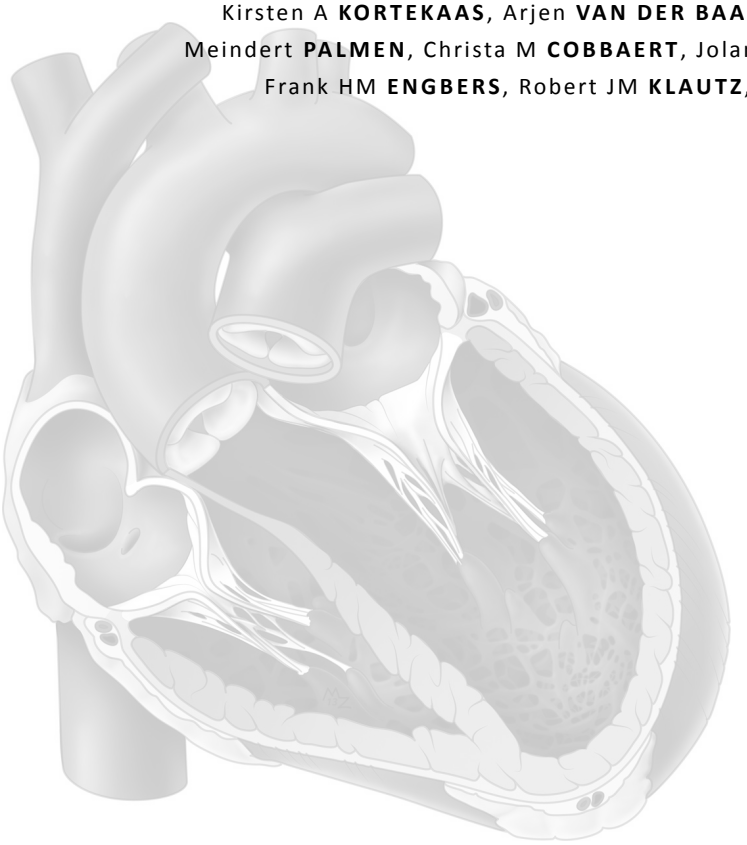


CHAPTER NINE

Submitted

Cardiospecific sevoflurane treatment quenches inflammation but does not provide cardiac protection: a study in patients undergoing mitral valve repair

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ABSTRACT

Background: Inflammation is considered a key mediator of complications after cardiac surgery. Sevoflurane has been shown to quench inflammation and to provide cardioprotection in preclinical studies. Clinical studies using sevoflurane confirm this effect on inflammation but fail to show a clinical benefit. This paradox may indicate that the contribution of inflammation to postoperative squalae is less than commonly thought or, alternatively, that doses used in clinical studies are too low. To test the latter we evaluated the effect of intramyocardial sevoflurane delivery.

Methods: Selective myocardial sevoflurane delivery was performed during aortic cross-clamping in patients undergoing mitral valve surgery (n = 11). Results were compared with a control group not receiving sevoflurane (n = 10). A reference group (n = 5) was added to evaluate the effects of systemic sevoflurane delivery. Paired arterial and myocardial venous blood samples were collected at various time points after reperfusion. Inflammatory mediators and myocardial cell damage were studied.

Results: Selective (myocardial) and systemic sevoflurane delivery effectively suppressed surgery-related inflammatory responses including postoperative C-reactive protein ($P = 0.01$ and $P = 0.04$, respectively). Intramyocardial delivery was superior to systemic delivery in attenuation of interleukin-6 and interleukin-8 (both $P = 0.001$). Sevoflurane treatment did not reduce postoperative myocardial damage as assessed by troponin T, creatine kinase, and creatine kinase-MB levels ($P = 0.87$, 0.38, and 0.17, respectively).

Conclusions: This clinical proof-of-concept study shows that intramyocardial delivery of sevoflurane is superior to its systemic delivery with respect to attenuation of the systemic inflammatory response. However, these findings are not followed by cardioprotective effects.

Introduction

A pronounced inflammatory response following on-pump cardiac surgery is linked to postoperative circulatory failure. Moreover, this response is considered a major contributor to postoperative morbidity and mortality.[1, 2] Consequently, it has been suggested that anti-inflammatory strategies, attenuating this response, are of benefit.

The hypothesized anti-inflammatory potential and tissue protective properties of sevoflurane, a frequently used volatile anesthetic, have been firmly established in preclinical studies.[3-6] The anti-inflammatory potential of sevofluane has been confirmed in several clinical studies, including patients undergoing cardiac surgery with the use of cardiopulmonary bypass (CPB).[7, 8] Yet, in contrast to animal studies, clinical studies do not show an overt cardioprotective or clinical benefit of volatile anesthetics such as sevoflurane.[9-13]

Absence of this clinical benefit in humans may suggest that the contribution of the inflammatory response to perioperative sequelae is less pronounced than commonly thought. Alternative explanations are apparent fundamental differences in the inflammatory response between rodent models and patients[14] or sevoflurane levels established in clinical studies are simply too low to show a clinical benefit. As such, it can not be excluded that clinical benefits are observed at higher concentrations. Although systemic toxicity limits the dosing, high local concentrations can be established during local, direct administration to an organ at risk. This approach is well achievable in the clinical context of cardiac surgery during which sevofluane can be directly delivered to the heart in the cardioplegia solution used during on-pump cardiac surgery.[15, 16]

In this proof-of-concept study, we tested whether sevoflurane-enriched warm blood cardioplegia solution attenuates postoperative inflammatory responses and myocardial cell damage. In order to differentiate between local and systemic effects we applied the technique of selective arteriovenous concentration differences over the reperfused heart. This set-up allows for independent evaluation of local and systemic effects.

METHODS

Patient characteristics

Twenty-one patients scheduled for elective mitral valve surgery were included in this prospective interventional study. Eleven patients received intramyocardial sevoflurane (experimental group) and ten patients were included as controls. Randomization was accomplished through use of sealed envelopes. Participating patients were blinded for patient group assignment until day five after surgery (that is, end of the study period).

Because of unanticipated systemic effects in the sevofluane treated group, we performed an additional reference experiment in five other patients in whom sevoflurane (0.5%) was delivered systemically. All patients had severe mitral regurgitation due to degenerative mitral valve disease. Exclusion criteria were left ventricular dysfunction (ejection fraction below 35%), minimal invasive or emergency procedures, previous cardiac surgery and the use of ketamine, aprotinin, corticosteroids, and volatile sevoflurane perioperatively. This single center study was approved by the ethics committee of the Leiden University Medical Center and each patient provided written informed consent. Moreover, this study was registered in the Nederlands Trial Register (NTR2089).

Anesthesia and surgical procedures

All patients received standardized anesthetic procedures according to a fast-track protocol. In detail, all patients were premedicated with oral lorazepam 1-2 mg the evening and morning before surgery. The anesthetic regimen consisted of a continuous target controlled infusion of propofol (with targets adjusted between 1.6 and 2 µg/mL to maintain a bispectral index value between 40-60), remifentanil (guided by hemodynamic responses on stimuli) and sufentanil 0.1 µg/kg/hour. If necessary, inotropes (dobutamine, norepinephrine, enoximone or in combination) were used. All patients underwent mitral valve repair according to standard operating techniques including an annuloplasty as part of their repair procedure, except for one patient in which a repair was not possible due to extensive calcifications. Surgery was executed via a median sternotomy with normothermic CPB (Jostra Maquet, Maquet, Hirrlingen, Germany) and intermittent antegrade warm-blood cardioplegia.

Delivery of sevoflurane

A standardized CPB protocol was used in all patients. After aortic and bicaval cannulation, normothermic CPB was initiated. The CPB system was coated with a heparin-free bioinert coating and a Kids D-100 oxygenator (Sorin group, Mirandola, Italia) was added to maintain *p*O2 and *p*CO2 concentrations within the normal range (15-20 kPa and 4.5-6.0 kPa, respectively). The CPB system was primed with 1300 mL priming volume consisting of 1000 mL Voluven® 6%, 100 mL Mannitol 20% and 200 mL Ringer solution. In addition, a loading dose of 5000 IU of heparin was added.

While administrating intermittently an antegrade warm-blood cardioplegia solution with a flow of approximately 450 mL/min, a gas flow of 1000 mL/min was given with an oxygen concentration of 21% and around 100 mL/min carbon dioxide. In the experimental group, sevoflurane 2% was administrated directly to the heart by addition of sevoflurane to this gas mixture. Cardioplegia was infused in the aortic root for the duration of two minutes

and repeated every 15-20 minutes. In the reference group, a time frame and concentration was chosen to approximate the systemic exposure of myocardial sevoflurane delivery: 0.5 L/min (cardioplegia flow) * 2% sevoflurane * 3 minutes (maximal duration of cardioplegia) = 4.5 L/min (blood flow) * 0.5% sevoflurane * 1.33 minutes (80 seconds). Sevoflurane was administrated in between two gifts of cardioplegia solution. With this perfusion technique, sevoflurane could not enter the coronary circulation. Exclusion criteria in all three patient groups were pulsatile flow, autologous priming, and ultrafiltration, since these measurements might influence the levels of inflammatory mediators. Heparin was reversed using protamine sulphate. Aspirated chest suction blood was collected in an autotransfusion reservoir (BT894, Dideco, Mirandola, Italy) before heparinization and after administration of protamine, and returned to the patient only after having been washed, using an Electa Concept (Dideco, Mirandola, Italy).

Sample collection

A baseline sample was taken the day before surgery from the brachial vein. Arterial and myocardial venous blood samples were collected simultaneously over the reperfused heart resulting in accurate, and specific measurements of locally ongoing processes in the early (0, 15, 30, 45, and 60 minutes after reperfusion) and late reperfusion phase (2, 4, 8, 16, and 24 hours after reperfusion), as described recently.[17, 18]

Inflammatory mediators

Multiplex analysis was used to evaluate arterial and myocardial venous cytokine patterns. Based on a previous study[18], 12 cytokines were measured with a sensitive custom made multiplex assay according to the manufacturers' instructions (X-plex, Biorad, Veenendaal, The Netherlands): IL-1 receptor antagonist (IL-1ra), IL-6, IL-8, IL-9, IL-10, IL-16, IL-18, granulocyte colony-stimulating factor (G-CSF), interferon-inducible protein-10 (IP-10) also know as C-X-C motif chemokine 10 (CXCL10), monocyte chemoattractant protein-1 (MCP-1), regulated upon activation, normal T cell expressed and secreted (RANTES) also know as chemokine ligand 5 (CCL5) and vascular endothelial growth factor (VEGF).

Intramyocardial p65-nuclear factor-kappa B activity

The nuclear factor (NF)-κB family member p65 controls the transcription of various genes central to inflammation and immunity. Unfettered activation of NF-kB is associated with various inflammatory diseases.[19] To quantify p65-NF-κB activity in myocardial tissue, paired pre- and post-reperfusion myocardial needle biopsies of the left ventricle were analyzed.

Myocardial p65-NF-κB activity was determined as described previously, using a TransAM transcription factor assay kit (DNA binding ELISA) (no. 40097, Active Motif Europe, Rixensart, Belgium).[20]

Myocardial cell damage

Three established blood markers of myocardial cell damage were measured at baseline (brachial vein), at 2, 4, 8, 16, and 24 hours after reperfusion (radial artery), and at 3 and 5 days after surgery (brachial vein). The markers of interest were high sensitive cardiac troponin T (hs cTnT; fifth generation, cat no 05092744190), creatine kinase activity (CK; cat no 12132672216) and creatine kinase-MB subfraction activity (CK-MB; cat no 12132893216). All reagents were purchased from Roche Diagnostics (Mannheim, Germany) and all analyses were done in EDTA plasma on Roche automated clinical chemistry analyzers. CK reference values at 37 °C are < 167 U/L for women and < 190 U/L for males; CK-MB reference values at 37 °C are < 24 U/L. For hs cTnT the 99th percentile, which is per definition the upper reference limit of a normal population, is 0.014 µg/L.

Statistical analysis

Comparisons between continuous variables were made by means of the Mann-Whitney *U*-test. Categorical variables were analyzed with a Chi-square test. The area under the curve (AUC) was calculated for the arterial and venous curves of the plasma measurements for the early and late reperfusion phases. A delta AUC was calculated (venous minus arterial) and the null-hypothesis (delta AUC is 0) was tested by a paired *t*-test. Arterial AUCs were also compared separately using an unpaired *t*-test. AUCs of the three markers of myocardial cell damage were calculated, and compared with a Kruskal-Wallis test. All statistical tests were performed using SPSS statistical analysis software version 20.0 (SPSS Inc, Chicago, IL, USA). A *P* value < 0.05 was considered statistically significant.

RESULTS

Patient characteristics

Baseline patient characteristics and perioperative data are summarized in Tables 1 and 2, respectively. None of the patients died in hospital or within 30 days after surgery.

Table 1. Baseline patient characteristics.

	Intramyocardial Sevoflurane (n = 11)	Control group (n = 10)	Reference group (n = 5)
Age (years)	61 (53-74)	59 (50-76)	69 (65-76)
Gender (no. of male)	7	5	5
Body Mass Index (kg/m^2)	25.46 (23.88-29.59)	25.92 (21.36-30.11)	27.08 (26.58-27.47)
Systolic blood pressure (mmHg)	140 (115-155)	136 (120-141)	140 (140-140)
New York Heart Association class	2 (1-2)	2 (2-2)	2 (2-2)
Logistic EuroSCORE (%)	2.40 (1.51-5.39)	2.08 (1.51-6.28)	4.61 (2.25-5.14)
Creatinine (µmol/L)	87 (74-101)	91 (78-109)	114 (84-118)
C-reactive protein (mg/L)	< 3 (< 3-7.00)	< 3 (< 3-4.50)	< 3 (< 3-< 3)
Hemoglobin (mmol/L)	9.2 (8.6-9.5)	8.7 (8.5-9.2)	9.5 (9.1-9.9)
Diabetes Mellitus (no. of patients)	1 (IDDM)	1 (IDDM)	1 (NIDDM)
COPD (no. of patients)	2	0	0
CAD (no. of patients)	4	2	3
Medication (no. of patients)			
ACE- or Ang II inhibitors	7	4	5
Diuretics	6	5	1
Beta-blockers	6	4	3
Statins	5	3	4
Acetylsalicyl acid	3	1	3
Coumarin derivates	5	5	1

Values are expressed as medians and interquartile ranges, or number of patients.
Abbreviations: ACE, Angiotensin Converting Enzyme; Ang II, Angiotensin-II; CAD, Coronary Artery Disease; COPD, Chronic Obstructive Pulmonary Disease; (N)IDDM, (Non)Insulin-Dependent Diabetes Mellitus.

Table 2. Perioperative data.

	Intramyocardial Sevoflurane (n = 11)	Control group (n = 10)	Reference group (n = 5)
Aortic cross-clamp time (min)	144 (80-172)	130 (104-147)	151 (140-153)
CPB time (min)	178 (123-203)	172 (131-197)	201 (186-217)
Duration of surgery (min)	303 (232-360)	294 (237-328)	335 (296-337)
Performed procedures (n)			
Only MVP (+ ablation)	5 incl. 1 MVR (0)	7 (3)	0
MVP + TVP (+ ablation)	3 (3)	1 (1)	3 (1)
MVP + CABG (+ ablation)	1 (1)	2 (0)	1
MVP + TVP + CABG	1	0	1
MVP + Morrow procedure	1	0	0
Ventilation time (hours)	7 (6-10)	8 (6-11)	8 (4-12)
CRP (mg/L)			
Day after surgery	63 (47-99)	107 (79-144)	58 (58-79)
Two days after surgery	164 (109-210)	231 (132-271)	147 (141-238)
Creatinine (µmol/L)			
Upon arrival ICU	89 (76-105)	82 (65-95)	120 (92-126)
Day one after surgery	84 (58-111)	77 (63-98)	108 (88-148)
Day two after surgery	73 (55-81)	76 (61-98)	98 (72-151)
Total ICU stay (hours)	21 (19-24)	22 (21-24)	22 (20-26)
Total stay in hospital (days)	9 (7-12)	12 (10-16)	9 (8-13)

Values are expressed as medians and interquartile ranges.
Abbreviations: CABG, Coronary Artery Bypass Grafting; CPB, Cardiopulmonary Bypass; ICU, Intensive Care Unit; MVP, Mitral Valve Annuloplasty; MVR, Mitral Valve Replacement; TVP, Tricuspid Valve Annuloplasty.

Inflammatory responses

Sevoflurane and the surgery-induced inflammatory response

Baseline inflammatory parameters were similar in the sevoflurane and control group (Supplementary data, Table 1). Surprisingly, selective myocardial sevoflurane delivery via the cardioplegia solution (that is, prior to reestablishment of coronary blood flow) attenuated the systemic surgery-related inflammatory response as indicated by lower systemic (arterial) IL-6 ($P = 0.03$), IL-8 ($P = 0.009$), IL-16 ($P = 0.007$), IL-18 ($P < 0.001$), and CXCL10 ($P = 0.001$) levels during the first 60 minutes of reperfusion. This attenuation was even observed prior to the moment of reperfusion (Figure 1).

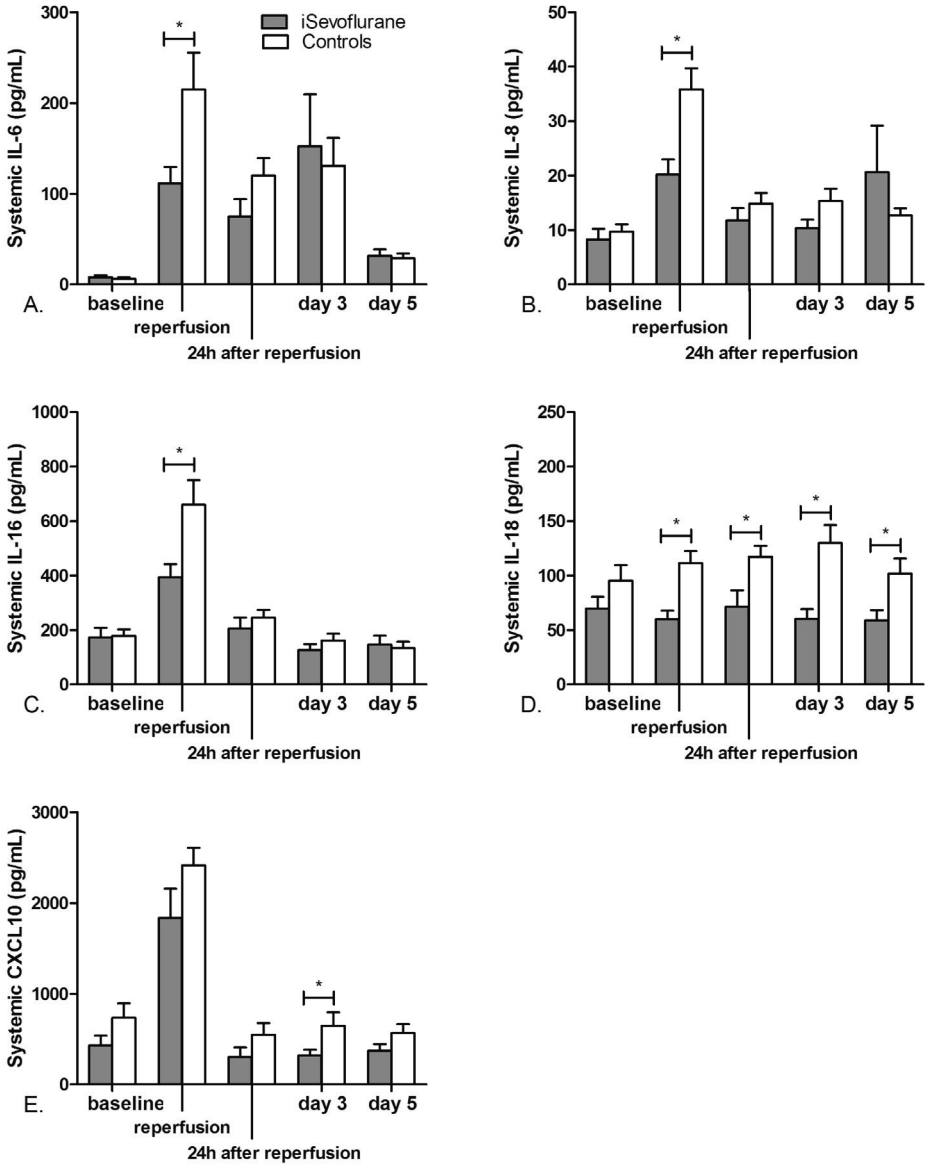


Figure 1. Attenuation of systemic levels of several cytokines by intramyocardial delivery of sevoflurane.

Levels of interleukin (IL)-6, IL-8, IL-16, IL-18, and C-X-C motif chemokines 10 (CXCL10) were already attenuated the moment of reperfusion after intramyocardial delivery of sevoflurane (iSevoflurane). * indicates a *P* value < 0.05. Graph error bars represent the mean ± SEM.

These early systemic effects may be explained by sevoflurane entering the systemic circulation via aspiration of sevoflurane-enriched blood by the cardiotomy sucker. We therefore included a third group, a reference group, in which sevoflurane (0.5%) was intermittently administrated systemically in a scheme that mimicked myocardial delivery. Results of this systemic administration of sevoflurane also resulted in attenuation of the inflammatory response (Figure 2). However, lower systemic levels of IL-6 and IL-8 (both P = 0.001) and the delayed (that is, 2-24 hours after reperfusion) systemic attenuation of CCL5 (P = 0.004) and IL-18 (P < 0.001) by intramyocardial delivery of sevoflurane suggest that intramyocardial delivery is more effective than systemic delivery.

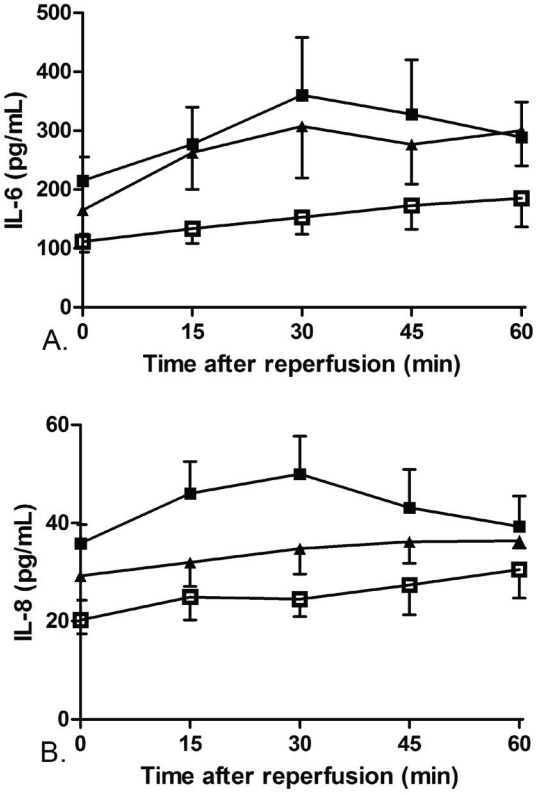


Figure 2. Interleukin-6 and interleukin-8 levels the first hour after reperfusion.
Concentrations of interleukin (IL)-6 and IL-8 were lower in patients who received sevoflurane intramyocardially (□) compared with the reference group (▲) (both P = 0.001). Controls (■) showed the highest levels of both markers. Graph error bars represent the mean ± SEM.

Attenuation of inflammatory cytokines and chemokines was followed by reduced postoperative CRP levels 24 hours after surgery after intramyocardial and systemic delivery of sevoflurane ($P = 0.01$ and $P = 0.04$, respectively). CRP levels were not significantly different at 48 hours, although a trend towards lower levels in the sevoflurane groups was observed ($P = 0.21$ intramyocardial sevoflurane versus controls; $P = 0.45$ systemic sevoflurane versus controls; Figure 3).

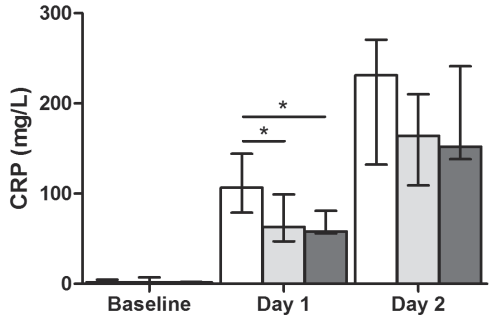


Figure 3. C-reactive protein levels before and after cardiac surgery.
C-reactive protein (CRP) levels were attenuated significantly the first day after surgery in patients treated with sevoflurane (light: intramyocardial sevoflurane; dark: reference group) compared with controls (blanco). Medians and interquartile ranges are shown.

Sevoflurane and the myocardial reperfusion-related inflammatory response

Arteriovenous concentration differences were assessed to selectively evaluate the myocardial reperfusion-related inflammatory response. Neither myocardial delivery nor systemic sevoflurane delivery influenced the immediate myocardial inflammatory response following reperfusion. In other words, during the first hour of reperfusion none of the inflammatory mediators were released by the myocardium. Cardiac selective sevoflurane delivery attenuated IL-6 and MCP-1 release 2-24 hours after reperfusion ($P = 0.004$, $P = 0.03$, respectively).

Since IL-6 and MCP-1 are under transcriptional control of NF-κB, myocardial NF-κB activity was measured in biopsies of the left ventricle to evaluate the effects of sevoflurane on protein level. Time points between aortic cross-clamping and collection of the pre-reperfusion biopsy and reperfusion versus collection of the post-reperfusion biopsy were similar in the intramyocardial group (times of biopsies: 6.71 ± 10.23 min and 29.25 ± 10.26 min, respectively), reference group (5.00 ± 3.35 min and 29.83 ± 9.37 min, respectively), and controls (9.33 ± 10.63 min and 28.63 ± 7.76 min, respectively). Baseline values of NF-κB activity were similar in the three patient groups ($P = 0.64$). In addition, similar NF-κB activity was observed in pre- versus post-reperfusion biopsies in the intramyocardial group ($P = 0.92$), reference group ($P = 1.00$), and control group ($P = 0.25$).

Sevoflurane and clinical parameters

Sevoflurane treatment did not influence plasma creatinine values, ventilation time, or intensive care unit (ICU) length of stay (Table 2). Cardioprotective effects were evaluated by measuring plasma hs cTnT, CK and CK-MB (Figure 4). None of the myocardial cell damage markers were influenced by sevoflurane treatment (P = 0.87, 0.38, and 0.17, respectively).

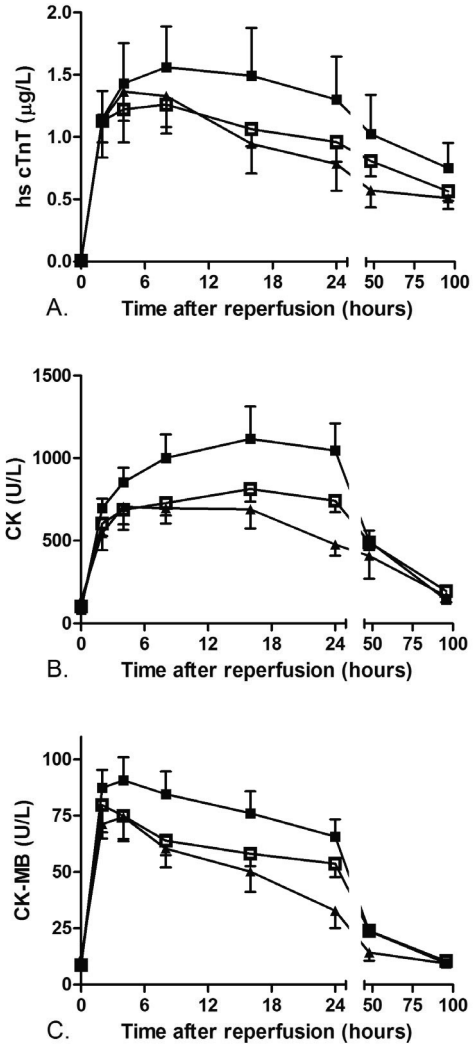


Figure 4. Three biomarkers of postoperative myocardial cell damage.
Similar myocardial cell damage was observed in patients who received sevoflurane intramyocardially (□), the reference group (▲), and control patients (■): (A) high sensitive cardiac troponin T (hs cTnT; P = 0.87), (B) creatine kinase activity (CK; P = 0.38), and (C) creatine kinase-MB subfraction activity (CK-MB, P = 0.17). Graph error bars represent the mean ± SEM.

DISCUSSION

Our results show that intramyocardial and systemic sevoflurane delivery effectively suppress surgery-related inflammatory responses. Sevoflurane did not influence the immediate myocardial inflammatory response following myocardial reperfusion, yet it did reduce the late myocardial inflammatory response. This suggests that the effects of sevoflurane are at the level of gene transcription and/or via an effect on cellular influx. However, effects exerted by sevoflurane are insufficient to result in a clinical benefit.

The observed comprehensive attenuation of systemic inflammation is in line with an earlier study of Nader et al. in patients undergoing coronary artery bypass grafting.[15, 16] Their results with respect to cardioprotective effects were ambiguous with lower troponin I levels measured by a conventional assay but similar CK-MB concentrations. Findings of our evaluation with regard to myocardial cell damage, measured by state-of-the-art assays, were highly consistent with a clear increase in myocardial cell damage upon surgery but absence of sevoflurane-mediated cardioprotection.

The observed early systemic attenuating effects of selective myocardial delivery of sevoflurane on inflammatory markers during on-pump cardiac surgery may appear counterintuitive but is most likely explained by contamination of the circulating blood via the cardiotomy sucker. Therefore, a reference group was included in whom sevoflurane was intermittently administered to the systemic circulation. Observed effects suggest that sevoflurane has a strong anti-inflammatory potential that is already apparent after repeated brief exposure to low levels (as caused by spill-over from the cardioplegia solution).

Clear anti-inflammatory effects but absence of clinical benefits question whether an inflammatory response is beneficial and necessary for tissue repair, or that it is detrimental and aggravates tissue injury. It has been shown that IL-6, IL-8 and MCP-1 showed higher peaks and persisted longer in patients with a complicated course after cardiac surgery in comparison with patients with an uneventful recovery.[21] In addition to the detrimental role of inflammatory mediators, some also appear to have protective effects depending on the presence of responsive cells, the timing, and combination of released cytokines. For example, both receptors of tumor necrosis factor (TNF) have disparate and opposing effects in mice with heart failure. TNFR1 exacerbates and TNFR2 ameliorates NF-κB activation and inflammation.[22] In renal I/R injury, it has been shown that the role of IL-6 is not straightforward either. A renal release of IL-6 was observed upon reperfusion in human kidney transplantation, but neutralization of IL-6 in mice undergoing renal I/R injury resulted in aggravation of tissue injury.[23]

The slow weaning of the systemic effects and the delayed suppression of the post-reperfusion myocardial inflammatory response suggest that the effects of sevoflurane are linked to gene transcription or to an effect on cellular infiltration, an observation that follows

earlier preclinical findings.[5, 24] Reduced systemic inflammation was followed by reduced CRP levels at 24 hours, showing that the anti-inflammatory effect is relevant. CRP levels were not statistically different anymore at 48 hours after surgery, presumably because of weaning of the sevoflurane effects.

The absence of beneficial effects on myocardial cell damage markers do not indicate a protective effect of sevoflurane on myocardial ischemia/reperfusion injury. This observation follows findings of other large clinical trials who failed to show a clinical benefit of sevoflurane.[13, 25] Results of these previous clinical studies may very well be related to the extent and degree of myocardial ischemia between the different studies, the differences in cardioplegic strategy, the possible interference of other drugs, the presence of co-existing diseases, the timing and duration of the administration of volatile anesthetics, and gaps in our understanding of inflammation and its regulation.[26, 27]

Limitations

The sample size of our proof-of-concept study was sufficient to identify differences in the release of inflammatory mediators between the patient groups, although patient numbers were too small to find a clear clinical benefit in relation to mortality and morbidity. However, other studies have repeatedly and consistently fail to show a clinical benefit. A greater number of patients were on statins and aspirin in the experimental group, although this did not influence inflammation at baseline. Furthermore, it is possible that changes in transcription take longer than the on average 30 minutes after reperfusion when collecting these biopsies, explaining similar p65-NF-κB activity in myocardial homogenates.[28]

Conclusion

This clinical proof-of-concept study shows that intramyocardial delivery of sevoflurane is superior to its systemic delivery with respect to attenuation of the systemic inflammatory response. However, these findings were not followed by beneficial clinical outcomes, suggesting that the association between systemic inflammation and postoperative outcome may be more complex than commonly thought.

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Supplementary data, Table 1. Similar baseline values of inflammatory plasma mediators.

	Intramyocardial sevoflurane (n = 11)	Control group (n = 10)	*P* value
IL-1ra	50.10 (18.90-91.24)	68.66 (46.70-131.49)	0.25
IL-6	4.57 (3.07-13.72)	5.42 (2.28-9.51)	0.92
IL-8	6.61 (2.80-13.50)	8.23 (6.11-12.94)	0.40
IL-9	0.45 (0.10-5.14)	1.11 (0.10-8.02)	0.80
IL-10	4.76 (1.64-5.63)	1.85 (1.27-4.41)	0.26
IL-16	141.09 (60.38-285.80)	158.25 (143.53-175.76)	0.44
IL-18	82.74 (35.66-95.05)	85.03 (69.05-126.73)	0.32
G-CSF	30.73 (11.32-51.29)	20.97 (19.40-56.30)	1.00
CXCL10	357.42 (200.08-567.96)	577.18 (428.80-940.24)	0.07
MCP-1	20.21 (12.97-43.68)	21.47 (18.11-38.66)	0.73
CCL5	227.64 (56.26-379.55)	200.41 (98.88-389.87)	0.57
VEGF	3.42 (0.69-14.48)	5.90 (0.76-16.29)	0.70

Values are expressed as medians and interquartile ranges (all pg/mL).
Abbreviations: CCL 5, Chemokine Ligand 5; CXCL10, C-X-C motif Chemokine 10; G-CSF, Granulocyte-Colony Stimulating Factor; IL, Interleukin; IL-1ra, IL-1 receptor antagonist; MCP-1, Monocyte Chemoattractant Protein-1; VEGF, Vascular Endothelial Growth Factor.

CHAPTER TEN

Inflamm Res 2013;62:53-59

Interleukin-9 release from human kidney grafts and its potential protective role in renal ischemia/reperfusion injury

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** Both authors contributed equally to this work*

ABSTRACT

Background: The pathophysiology of ischemia/reperfusion (I/R) injury is dominated by an inflammatory response. In the identification of new therapeutic agents, the role of individual cytokines may be essential. Interleukin (IL)-9 is a pleiotropic cytokine recently identified to be involved in various immune responses. In this study, the role of IL-9 in renal I/R injury was assessed.

Methods: We performed repeated direct measurements of arteriovenous IL-9 concentration differences over the reperfused graft in human kidney transplantation.

Results: Substantial renal IL-9 release was observed from deceased donor kidneys (P = 0.006). In contrast, living donor kidneys, which have a more favourable clinical outcome, did not release IL-9 during early reperfusion (P = 0.78). Tissue expression of IL-9 did not change upon reperfusion in both living and deceased human donor kidneys. To assess the role of IL-9 in I/R injury, an experimental study comprising IL-9 inhibition in mice undergoing renal I/R was performed. Although there was no difference in kidney function, structural damage was significantly aggravated in anti-IL-9 treated mice.

Conclusions: Deceased donor grafts show a substantial IL-9 release upon reperfusion in clinical kidney transplantation. However, inhibition of IL-9 aggravated kidney damage, suggesting a regulating or minor role of IL-9 in clinical I/R injury.

INTRODUCTION

Ischemia/reperfusion (I/R) injury is an inevitable consequence of organ transplantation and a major determinant of patient and graft survival. I/R injury induces delayed graft function, which complicates around 20%[1, 2] of deceased donor kidney transplantations and has a major influence on graft function and survival.[3] The pathophysiology of I/R injury is complex and incompletely understood, although both preclinical[4-6] and clinical studies[7-9] have shown that inflammation is an important mediator of I/R injury. The exact functions of the various cytokines involved in regulating the complex inflammatory events after I/R injury are not fully unraveled yet.[10] Insight in the role of individual cytokines may be essential in the identification of new therapeutic agents.

Interleukin (IL)-9 is a pleiotropic cytokine recently discovered to be involved in various immune responses. Studies on the etiology of asthma and allergies have demonstrated an evident pro-inflammatory role of IL-9.[11, 12] IL-9 is produced by T helper cells[13], regulatory T cells[14], and mast cells[15] and is able to modulate their production of various other cytokines.[12] In a preceding study, we described for the first time a renal release of IL-9 after kidney transplantation.[10]

The aim of the present study was to explore the potential pathophysiological role of IL-9 in renal I/R injury. IL-9 release during reperfusion of both living and deceased human donor kidney grafts was assessed, and the effects of IL-9 inhibition on renal I/R injury were evaluated in an animal experiment.

METHODS

Patient population

Twenty-four patients undergoing renal allograft transplantation were included; of these, eight patients received a kidney from a living donor and sixteen patients received a kidney from a deceased donor (nine brain-dead donors and seven cardiac-dead donors). Kidney transplantations were performed according to the local standardized protocol.[16] In living donors open nephrectomy was performed and Custodiol® histidine–tryptophan–ketoglutarate solution (Tramedico, Weesp, The Netherlands) was used for cold perfusion and storage of the kidney. Brain-dead and cardiac-dead donor kidneys were perfused and stored with either University of Wisconsin solution or Custodiol® histidine–tryptophan–ketoglutarate solution. The immunosuppressive regimen was based on induction therapy with basiliximab on day 0 and 4; and tacrolimus or cyclosporine A in addition with mycophenolate mofetil and steroids. For technical reasons (renal vein sampling) only

patients receiving a left kidney were included. Patient and graft characteristics and ischemia times are summarized in the Supplementary data, Table 1. The postoperative course was uneventful in all patients. One-year patient and graft survival was 100%. The study protocol was approved by the local ethics committee, and informed consent was obtained from each patient.

Interleukin-9 plasma measurements in arteriovenous samples

Arterial and renal venous blood samples were obtained during human kidney transplantation as described previously.[16] In short, blood aliquots were sampled at 30 seconds, 3, 10, and 30 minutes after reperfusion via a catheter placed in the renal vein. Paired arterial blood samples were obtained at 0, 3, 10, and 30 minutes after reperfusion. The same method was used to obtain a control arterial and venous blood sample during donor nephrectomy prior to the induction of renal ischemia. All samples were collected in tubes containing EDTA (BD Vacutainer, Plymouth, UK) and centrifuged twice (1550 *g*, 20 min, 4°C) to deplete it of leukocytes and thrombocytes. Plasma was aliquotted and stored at –70°C until analysis. IL-9 was measured in a custom-made multiplex assay in accordance with the manufacturer's instructions (X-plex, Biorad, Veenendaal, The Netherlands).

Immunohistochemical evaluation of interleukin-9 source in human kidney biopsies

A renal cortical biopsy was obtained before transplantation, after cold storage of a deceased donor graft. Kidney tissue was fixed in formalin for 24 hours and then embedded in paraffin for light microscopy. Longitudinal sections of 4 µm were prepared. After EDTA-retrieval, IL-9 deposition was assessed using a human IL-9 mAb in a 1:100 dilution (cat. no. 507602, Biolegend, Cambridge, UK). Staining was visualized using Nova RED (Vector Labs, Peterborough, UK). Sections were counterstained with hematoxylin (Merck, Darmstadt, Germany).

Interleukin-9 expression in human kidney biopsies

A renal cortical biopsy was obtained before transplantation after cold storage, and a post-reperfusion biopsy was collected 45 minutes after reperfusion. Biopsies were immediately snap frozen in liquid nitrogen and stored at –70°C. Total RNA was extracted from renal tissues using RNAzol (Campro Scientific, Veenendaal, The Netherlands) and glass beads.[17] The integrity of each RNA sample was examined by Agilent Lab-on-a-chip technology using the RNA 6000 Nano LabChip kit and a bioanalyzer 2100 (Agilent Technologies, Amstelveen, The Netherlands). RNA preparations were considered suitable for array hybridization only

if samples showed intact 18S and 28S rRNA bands, and displayed no chromosomal peaks or RNA degradation products (RNA Integrity Number > 8.0).[18] Subsequent microarray analysis was performed using Illumina whole-genome gene expression BeadChips (Illumina BeadArray®, San Diego, CA, USA) according to instructions of the manufacturer at Service XS (Leiden, The Netherlands). IL-9 expression data were obtained from this array.

Animals and surgical procedures

Male BALB/c Jico mice (Charles River, L'Arbresle, France) of 8 weeks old (20-30 g) were divided into the following four groups: (1) I/R pre-treated with anti-IL-9 antibody (Ab) (n = 12); (2) I/R pre-treated with control Ab (n = 12); (3) sham-operated, pre-treated with anti-IL-9 Ab (n = 4), and (4) sham-operated pre-treated with control Ab (n = 4). Monoclonal anti-IL-9 Ab (no. 504802, clone D9302C12, BioLegend, San Diego, CA, USA) or isotype control IgG (no. 400916, clone HTK888, BioLegend, San Diego, CA, USA) was administered at 1 and 2 days before surgery. At 2 days before surgery, 2 ug/g body weight anti-IL-9 Ab or its equivalent of isotype control Ab was administered by i.p. injection, followed by 4 ug/g body weight the day before surgery. This dosage is based on previous research in which a significant effect of anti-IL-9 treatment was observed after administration of a similar dose of the antibody.[19] Mice were anesthetized with isoflurane. Bilateral kidney ischemia was induced by clamping the renal artery and vein for 35 minutes, followed by reperfusion. In the sham group, identical surgical procedures were used, except that clips were not applied.[16] Kidney function was measured by plasma urea concentration (Reflotron®, Roche diagnostics, Almere, The Netherlands) the day before administration of antibodies (day -3), day of surgery (day 0), and the two consecutive days after surgery (day 1 and 2). Two days after surgery, mice were killed and kidneys retrieved. Mice were maintained on standard diet and given water ad libitum throughout the whole experimental period. The study was approved by the veterinary authorities of our institute.

Histological evaluation of murine kidney tissue

The mouse kidneys were fixed in formalin for 24 hours and then embedded in paraffin for light microscopy. Longitudinal sections of 4 µm were prepared and stained with periodic acid-Schiff (PAS). The degree of tissue injury was scored by two blinded observers. Kidney injury was scored semi-quantitatively using the amount of proximal tubule necrosis (score 0–3), band necrosis of the cortex and the medulla (score 0–3), and protein casts in the tubules (score 0–3).

Data collection and statistical analysis

Clinical donor data were retrieved from the Eurotransplant database. Delayed graft function was defined as the need for dialysis within the first week after transplantation. Statistical analysis was performed using SPSS 16.0 statistical analysis software (SPSS Inc, Chicago, IL, USA). Patient characteristics were compared by paired *t*-test and are expressed as mean ± standard deviation (SD). Area under the curve (AUC) was calculated for the arterial and venous curve of the human plasma measurements for the total reperfusion period of 30 minutes. The delta AUC was calculated (venous minus arterial) and the null-hypothesis (delta AUC is 0) was tested by a Wilcoxon signed rank test. Urea concentrations were compared using a Mann Whitney *U*-test, Wilcoxon signed rank test or Kruskal-Wallis test where appropriate. Histological score was tested by the Mann Whitney *U*-test. *P* value < 0.05 was considered significant.

RESULTS

Interleukin-9 is released from deceased donor kidneys during reperfusion

Living donor kidneys released no IL-9 during the first thirty minutes after reperfusion ($P = 0.78$, n = 8, Figure 1A). In contrast, deceased donor kidneys, which generally show more dysfunction after transplantation, released a vast amount of IL-9 during the first thirty minutes after reperfusion ($P = 0.006$, n = 16, Figure 1B). More specifically, only brain-dead donor kidneys showed a significant release of IL-9 ($P = 0.04$, n = 9). However, cardiac-dead donors showed the same trend ($P = 0.07$, n = 7), and therefore deceased donor data are clustered. IL-9 release was specific for I/R, as control measurements over the non-ischemic kidney in the donor did not show a transrenal difference in IL-9 levels ($P = 0.89$, data not shown). This indicates that IL-9 release is not a consequence of manipulation of the kidney during surgery or a reaction to anesthetics.

Source of human interleukin-9 release is mainly tubular epithelial cells

The source of IL-9 released from deceased donor kidneys could be local resident cells or circulating cells. An immunohistochemical staining of IL-9 was performed on renal biopsy tissue from a deceased donor graft, which showed that local stores of IL-9 are present in the kidney. IL-9 signals were present predominantly in renal tubular epithelial cells and also in macrophages and dendritic cells (Figure 2).

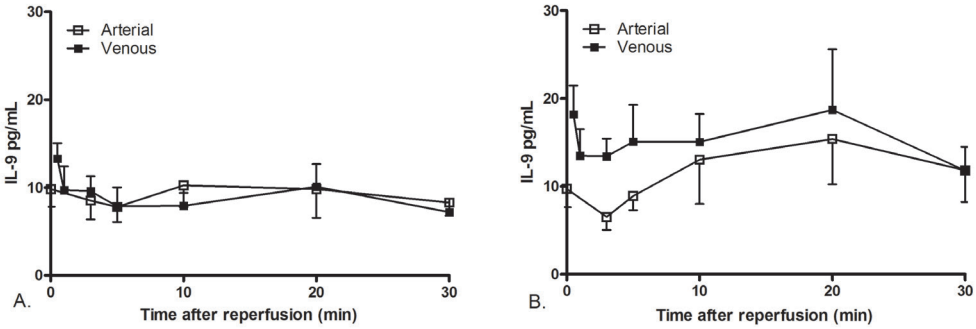


Figure 1. Arterial and renal venous plasma concentrations of interleukin-9 during the first 30 minutes of reperfusion.
(A) No significant release of interleukin-9 (IL-9) from living donor grafts was observed (P = 0.78, n = 8); (B) IL-9 was released significantly from deceased donor grafts (P = 0.006, n = 16).

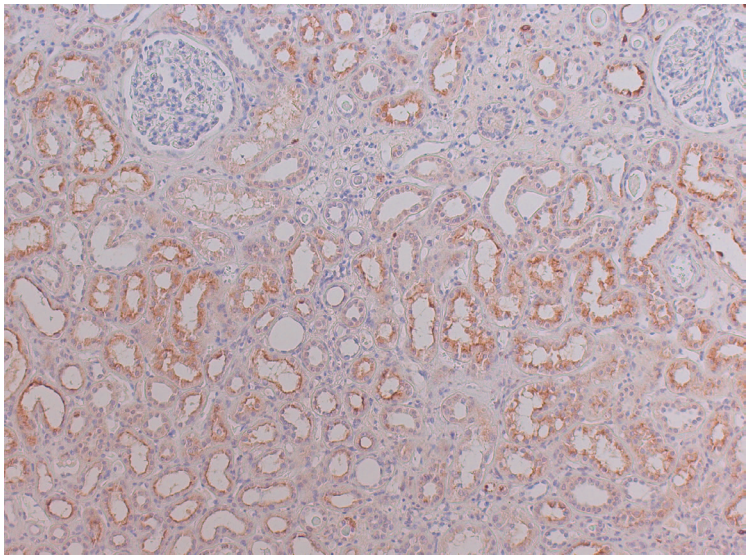


Figure 2. Typical example of interleukin-9 staining in deceased donor kidney biopsy collected before transplantation.
Interleukin-9 signal was positive in renal tubular epithelial cells, macrophages, and dendritic cells. Original magnification x200.

No change in human renal interleukin-9 expression after reperfusion

No differences in IL-9 RNA expression were observed between pre- and post-reperfusion biopsies in living (P = 0.89, Figure 3A) as well as deceased donor kidneys (P = 0.59, Figure 3B). In addition, similar baseline expression of IL-9 in living and deceased donor kidneys was observed (P = 0.57).

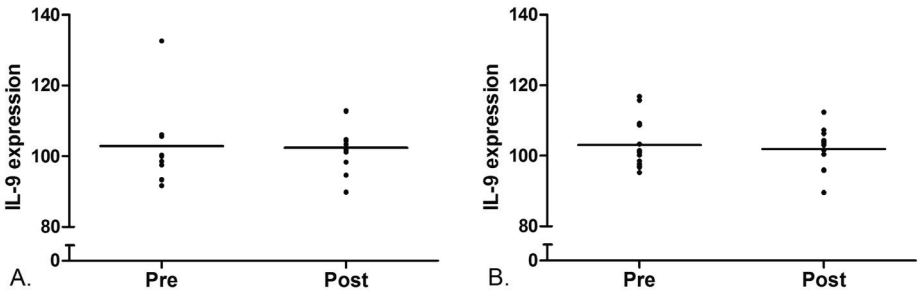


Figure 3. Interleukin-9 expression in human kidney biopsies.
Interleukin-9 (IL-9) expression in human pre- and post-transplantation biopsies of (A) living donor kidneys; and (B) deceased donor kidneys. There was no difference in IL-9 RNA expression between pre- and post-reperfusion biopsies in living and deceased donor kidneys (P = 0.89 and P = 0.59, respectively) nor in baseline IL-9 expression between both donor types (P = 0.57).

Anti-interleukin-9 treatment does not prevent functional and structural kidney ischemia/reperfusion injury

Given the dominance of IL-9 release from deceased donor kidneys, we hypothesized that IL-9 is critical in the initiation of I/R injury. Therefore, mice were treated with anti-IL-9 Ab or isotype control Ab before renal I/R. All mice survived the end of the experiment. Baseline urea concentrations were identical in all animals (Figure 4). In both anti-IL-9 Ab and isotype control Ab treated mice that underwent renal I/R, urea concentration was significantly raised at 1 and 2 days after reperfusion as compared with baseline (all, P = 0.002). As expected, urea of sham-operated animals, both anti-IL-9 Ab and isotype control Ab pre-treated, did not change after surgery as compared with baseline (all, P = NS). Urea concentration was similar in anti-IL-9 treated and control Ab treated mice at day 1 (P = 0.91) and 2 (P = 0.53) after reperfusion (Figure 4).

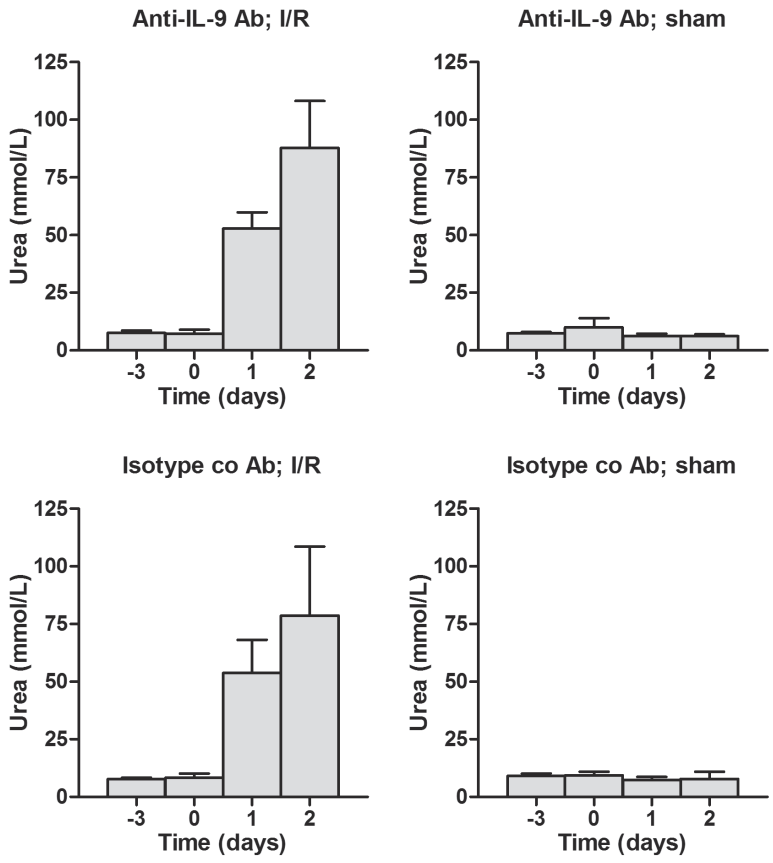


Figure 4. Plasma urea levels after murine renal ischemia/reperfusion.
Plasma urea levels measured at baseline (day -3), at the day of surgery (day 0), and two consecutive days after surgery (day 1 and 2). Plasma urea levels were similar in anti-IL-9 Ab treated mice compared with isotype control Ab treated mice after I/R at day -3 ($P = 0.77$), day 0 ($P = 0.69$), day 1 ($P = 0.91$), and day 2 ($P = 0.53$). The median and interquartile range are plotted.

Structural kidney damage was moderate to severe in all kidneys that underwent I/R, whereas kidneys of sham-operated mice showed normal histology. Kidney injury was scored on several characteristics and then quantified. There was significantly more structural damage in kidneys of anti-IL-9 Ab treated mice than in the control Ab treated group after I/R. In detail, anti-IL-9 Ab treated mice showed more proximal tubule necrosis ($P = 0.02$), band necrosis of the cortex and medulla ($P = 0.01$) and protein casts in the tubules ($P = 0.005$) (Figure 5).

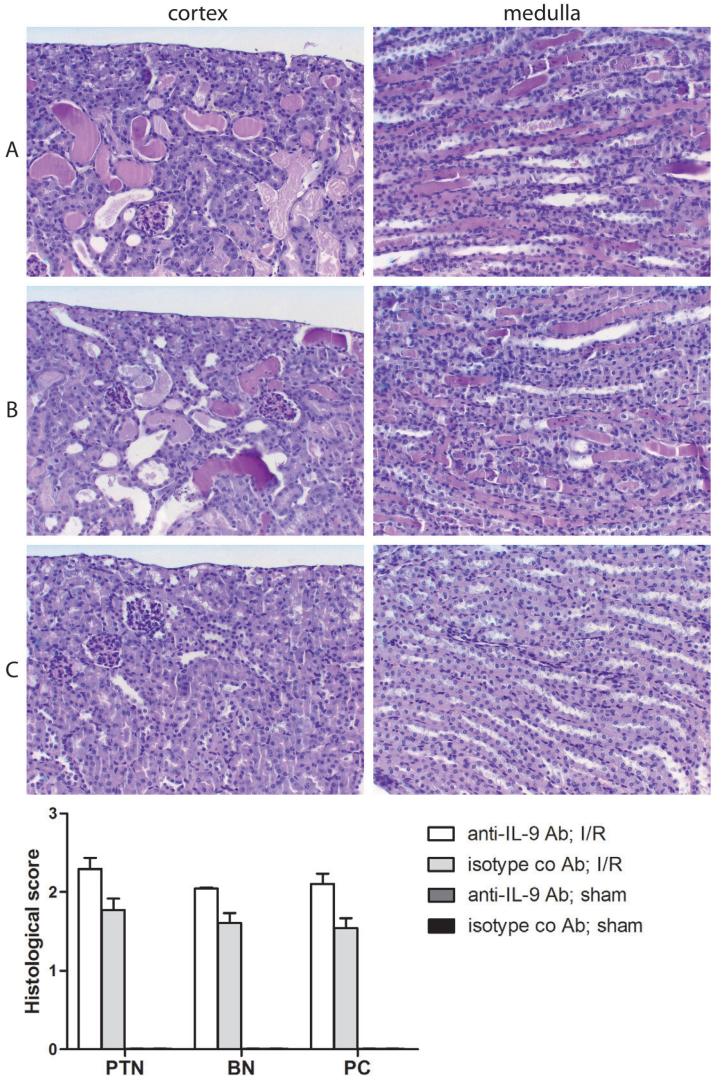


Figure 5. PAS-staining of murine kidney biopsies.
Representative PAS-stained kidney sections harvested 2 days after surgery of (A) mice that received anti-IL-9 Ab before renal I/R; (B) mice that received control Ab before kidney I/R; and (C) sham-operated mice that received anti-IL-9 or control Ab treatment before surgery (no difference). Original magnification x200. Mice receiving anti-IL-9 Ab before I/R had evident protein casts and tubule necrosis, more than isotype controle Ab treated mice. Sham-operated mice receiving either anti-IL-9 Ab or control Ab had no PAS-positive deposits and no structural kidney damage. (D) Semi-quantitative histological damage score for the severity of kidney damage is shown. Damage was scored (0-3) on presence of proximal tubule necrosis (PTN), band necrosis (BN) and protein casts (PC). Kidneys of all mice that underwent kidney I/R showed moderate to severe damage. There were significant differences between anti-IL-9 Ab-treated and isotype control Ab-treated groups (PTN, $P = 0.02$; BN, $P = 0.01$; PC, $P = 0.005$). Kidneys of sham-operated mice scored 0 for all measures. Median and interquartile ranges are plotted.

DISCUSSION

In this study it is shown that IL-9 is released exclusively from human deceased donor grafts directly after reperfusion. Since deceased donor grafts are clinically more affected by I/R injury, we hypothesized that IL-9 is a mediator of renal I/R injury. In a subsequent mouse experiment, inhibition of IL-9 did not influence kidney function after I/R and even aggravated structural kidney injury.

IL-9 was released instantly after reperfusion from deceased donor grafts and its release persisted during the next thirty minutes. The substantial and immediate character of this IL-9 release suggests a pre-stored pool, since no change in IL-9 expression in kidney tissue upon reperfusion was observed. Pre-transplantation infiltrated cells are probably not involved, since baseline biopsies showed no difference between human living and deceased donor IL-9 expression, although there was a vast difference in cellular infiltrate before transplantation.[10] Immunohistochemical staining of IL-9 showed its presence in tubular epithelium and resident macrophages and dendritic cells. These cells are likely responsible for secretion of their pre-stored IL-9.

IL-9 is considered a pro-inflammatory cytokine, although as yet little is known about its exact functions. Preclinical studies have shown that IL-9 promotes mast cell growth and plays a crucial role in the nephroprotective effects of regulatory T cells.[12, 20] In addition, a potential role in the stimulation of erythropoiesis has been suggested[21, 22], as well as an antiapoptotic effect.[23] The observation in our study that IL-9 was released exclusively from deceased donor kidneys, in which tissue damage is most severe, argues for an active role of IL-9 in initiating I/R injury. Therefore, the effect of IL-9 inhibition on kidney I/R injury was assessed in a mouse experiment. Remarkably, results showed aggravated structural kidney damage after I/R in anti-IL-9 Ab treated animals. When extrapolating these findings, it can be suggested that IL-9 plays a regulating role in clinical renal I/R injury or can be an initial trigger for salvaging or limiting injury.

Although no previous data are available regarding the role of IL-9 in I/R injury, the few studies on allograft rejection after transplantation are conflicting. An association between IL-9 and acute, eosinophil-driven rejection has been suggested by Poulin et al. in mismatched heart allografts.[24] However, no involvement of IL-9 was observed in rejection on a longer term in both mouse islets and human kidney allografts.[25]

Limitations

Our arteriovenous sampling time was limited to thirty minutes after reperfusion, the time needed to complete the operative procedure. Deceased donor kidney grafts showed an extensive release of IL-9 within the studied timeframe; therefore it is highly unlikely that living donor kidney grafts will start releasing IL-9 after the studied period. Finally, one might argue that a mouse model of renal I/R injury can only approximate the human situation. Nevertheless, the effects of IL-9 inhibition could be accurately assessed in our animal experiment and a negative effect of anti-IL-9 treatment was indeed observed.

Conclusion

This study shows a substantial and exclusive release of IL-9 from deceased donor grafts in human kidney transplantation, while living donor kidneys, which generally have a more favourable clinical outcome, did not release IL-9 during early reperfusion. The source of released IL-9 was its pre-stored pool in renal tubular cells. Experimental inhibition of IL-9 in mice aggravated kidney damage after I/R injury. All together, these results suggest a regulating or minor role of the released IL-9 on I/R injury in human kidney transplantation.

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Supplementary data, Table 1. Transplantation and outcome characteristics in living donor and deceased donor kidney transplantation.

	LD n = 8	DD n = 16	*P* value
Recipient age (years) Mean ± SD	41.1 ± 10.5	54.6 ± 12.2	0.02
Recipient gender (% male)	38	56	0.39
Donor age (years) Mean ± SD	43.9 ± 10.6	53.5 ± 16.1	0.14
Donor gender (% male)	75	44	0.15
Preservation fluids	HTK (n = 8)	UW (n = 11) HTK (n = 5)	0.001
CIT (min.) Mean ± SD	179.1 ± 18.6	1117.7 ± 299.1	< 0.001
WIT (min.) Mean ± SD	34.0 ± 6.3	33.5 ± 6.1	0.85

Abbreviations: CIT, Cold Ischemia Time; DD, Deceased Donor; HTK, Histidine–Tryptophan–Ketoglutarate; LD, Living Donor; UW, University of Wisconsin; WIT, Warm Ischemia Time.

CHAPTER ELEVEN

Summary and General Discussion

End-stage heart failure is a challenging syndrome with growing incidence and prevalence. There is even a steady increase in the number of heart failure patients that reach an advanced state of the disease due to improved survival after acute myocardial infarction and improvements in the treatment of heart failure itself. In a substantial proportion of these patients, medical therapy for the treatment of heart failure is insufficient despite advances in medical treatment. Surgical procedures may provide an opportunity for additional treatment. Nowadays, the gold-standard therapy in end-stage heart failure is cardiac transplantation. However, there is a persistent shortage of organs available for transplantation. Therefore, surgical treatments, aimed to improve the cardiac function and clinical status of the patient, are therefore of great clinical interest.[1] Although surgical techniques for end-stage heart failure are improving, perioperative morbidity and mortality remain high as outlined in a review by George et al.[2]

In this thesis it has been shown that the observed postoperative complications are only partially explained by the preoperative high-risk state of these heart failure patients or the low cardiac output state. We suggest that postoperative complications are also a reflection of the preexisting autonomic dysregulation in this patient group. We hypothesized that the heart induces processes, such as an inflammatory response, which account for postoperative difficulties with major consequences for the myocardium but also for other organs. Such an effect could result in secondary systemic injury and a vicious circle of circulatory compromise due to the preexisting pump failure, leading to increased morbidity and mortality .

Although generally assumed, the impact of preexisting heart failure on these postoperative complications has never been substantiated. Hence, the impact was evaluated in this thesis in **Chapter 2**. As confirmed, patients with heart failure experience more frequently complications related to their pump failure, resulting in the use of high dosages of inotropic support and various mechanical assist devices. However, complications not related to pump failure per se, such as prolonged mechanical ventilation, sepsis, and vasoplegia, were also more often observed in patients with heart failure. Overall, organ dysfunction, such as circulatory, renal and pulmonary failure, was more common in heart failure patients after cardiac surgery compared with patients without heart failure. This adverse postoperative course in these patients might reflect a systemic dysregulation related to the syndrome of heart failure and can not be directly attributed to the preexisting pump failure. Such an effect could result from a different response to ischemia/reperfusion (I/R) after heart failure surgery. This is however difficult to differentiate from the cardiac response to injury as a result of the surgical procedure and use of cardiopulmonary bypass. As such, arteriovenous measurements were used to discriminate between myocardial and systemic release of various mediators.

In the consecutive chapters of this thesis, perioperative pathophysiological mechanisms were studied in depth in patients with and without heart failure. The aim of this thesis was to systematically evaluate the pathophysiology of human myocardial I/R injury, using cardiac valve surgery as a planned model of clinical I/R. This model provides the opportunity to collect blood samples upon the release of the aortic cross-clamp, the moment of reperfusion, from the radial artery and coronary sinus. This approach allows to measure the release of I/R markers exclusively from the heart.

Oxidative and nitrosative damage after I/R injury were studied in depth since preclinical studies implicate reactive oxygen and nitrogen species (RONS) as key initiators of I/R injury, as outlined in **Chapter 3**.[3-5] Indeed, redox *stress* does occur during reperfusion as shown by Nrf2 activation, the master regulator of the antioxidant stress response. However, the generated RONS apparently do not induce tissue *damage* in human I/R since none of the damage markers – malondialdehyde, 15(*S*)-8-*iso*-prostaglandin F2α, nitrite, and nitrate – were released upon myocardial reperfusion. Organ specificity was excluded by including measurements over the kidney. Findings for all conditions were highly consistent and release of markers of oxidative damage was not observed. These observations suggest an efficient endogenous anti-oxidant system, providing an explanation for the limited efficacy of antioxidant therapy in human I/R.[6]

In **Chapter 4** the activation of the complement cascade after cardiac valve surgery was evaluated since clinical studies showed minute and inconsistent effects of anti-complement therapy in contrast to preclinical studies.[7-9] Our results showed that endomyocardial C3d and C5b-9 depositions were already present in early ischemic biopsies and most abundant in patients with heart failure compared with patients without heart failure. These depositions might be related to the syndrome of heart failure. Remarkably, no myocardial release of C5a or sC5b-9 was observed upon reperfusion in patients with and without heart failure. These results suggest that the contribution of complement activation to human I/R may be less than commonly thought and may explain why anti-complement therapy in humans showed minute and inconsistent effects so far.

A possible role of platelet and endothelial cell activation was studied in **Chapters 5 and 6**. Results do not support preclinical evidence that platelets are involved in human myocardial I/R injury. The activation of platelets was impaired mainly as a result of cardiopulmonary bypass-related hemodilution. Moreover, activated platelets remained inside the cardiopulmonary bypass circuit. Endothelial cell activation markers were analyzed in patients with and without post-cardiac surgery vasoplegia. We observed that patients who develop this vasoplegia have higher preoperative von Willebrand Factor propeptide and soluble P-selectin levels, reflecting a systemic low-grade endothelial cell activation, even before surgery.

In **Chapter 7** the inflammatory response was evaluated in patients with and without heart failure after cardioplegic arrest and reperfusion. Failing hearts showed a different and more pronounced myocardial inflammatory response upon reperfusion than patients without heart failure, which may be associated with the systemic complications as outlined in Chapter 2. This response can be explained by degranulation of preoperatively infiltrated inflammatory cells in myocardial tissue of failing hearts. Directly upon reperfusion, various cytokines and chemokines were released by the myocardium. However, it remains questioned whether this response is beneficial and necessary for tissue repair, or that it is detrimental and aggravates tissue injury. Currently, anti-inflammatory drugs, such as corticosteroids, have not lead to improved clinical outcomes.[10-12]

The role of galectin-3, a marker of myocardial inflammation and fibrosis, has been described in relation to left ventricular reverse remodeling after mitral valve repair in patients with non-ischemic and ischemic heart failure in **Chapter 8**. Results show that low preoperative levels of this marker are associated with left ventricular reverse remodeling six months after surgery. Therefore, measurement of baseline galectin-3 may be useful for the identification of patients who will respond to additional (surgical) therapy with the aim to achieve beneficial left ventricular reverse remodeling after heart failure surgery.

Translation of findings of the above mentioned pathophysiological mechanisms to practical applications is essential to enhance human health. Therefore, the intramyocardial delivery of sevoflurane was studied in detail in **Chapter 9**. Previous studies have shown that systemic administration of sevoflurane has led to differential clinical benefits.[13-16] Our study shows that intramyocardial delivery of sevoflurane during mitral valve surgery attenuates the systemic pro-inflammatory response upon myocardial reperfusion. However, administration of sevoflurane to low-intermediate risk patients undergoing relatively complex valve surgery does not seem to result in less myocardial cell damage. We can not exclude a cardioprotective effect of sevoflurane in more extreme conditions. Again, it remains questioned whether attenuation of the pro-inflammatory responses is beneficial and necessary for tissue repair, or that it is detrimental and may aggravate tissue injury.

The final chapter of this thesis, **Chapter 10**, describes the role of interleukin (IL)-9 in renal I/R injury. *Bedside* results showed that deceased donor grafts release a substantial amount of IL-9 upon reperfusion in kidney transplantation. The source of this released IL-9 originated from pre-stored pools in renal tubular cells. However, *bench* results showed that inhibition of IL-9 aggravated kidney damage. IL-9 may therefore play a regulating role in clinical renal I/R injury or can be an initial trigger for salvaging or limiting injury. A murine renal I/R model was chosen since tissue damage due to I/R was more homogenous in this model compared with a myocardial model. In addition, the read-out of renal tissue damage was easily evaluated with creatinine levels.

Conclusion

The main challenge of this thesis was to evaluate whether the reperfused heart induced systemic effects postoperatively, contributing to morbidity and mortality after cardiac surgery. We identified preexisting processes in patients with heart failure, such as complement activation and influx of inflammatory cells in the myocardium. These changes in their turn altered the post-reperfusion response of the myocardium after cardiac surgery. Moreover, preexisting low-grade endothelial cell activation predicted the development of post-cardiac surgery vasoplegia. No evidence was found for oxidative damage after reperfusion in contrast to the current theory. As expected, targeting the inflammatory response with sevoflurane resulted in attenuation of pro-inflammatory markers, although no cardioprotective effects were observed. A stepwise approach in future research via preclinical to clinical studies, and vice versa, is necessary to ensure the integration of advancements in pathophysiological science with clinical trials.

Future perspectives

Instead of fundamentally different processes following myocardial reperfusion, preexisting mechanisms may contribute to a great extent to different post-reperfusion responses in patients with heart failure. Morbidity and mortality after cardiac surgery are therefore more likely related to the preexisting systemic dysregulation associated with the syndrome of heart failure. Still, many questions are unanswered and new questions arise every day. Despite continuing advances in unravelling the underlying pathophysiological mechanisms of clinical myocardial I/R in patients with heart failure, therapeutic modalities did not show favourable effects or still have to reach human clinical trials as outlined in the introduction of this thesis.

Although beyond the scope of this thesis, the old saying ‘prevention is better than cure’ is applicable to almost all patients with heart failure. Primary prevention would still be the most simple and safe solution. However, due to improvements in secondary and tertiary prevention after myocardial infarction and diagnosis of heart failure itself, there is a steady growth of heart failure patients that reach advanced stages of heart failure. As outlined in this thesis, preexisting processes are present in patients with end-stage heart failure. Cardiac surgery in these heart failure patients can easily lead to dysregulation of their tightly regulated homeostasis. Therefore, preoperative interventions with the aim to optimize their preexisting processes, such as neurohumoral responses, inflammatory pathways, and responsiveness of the vascular beds, might lead to improved outcome after cardiac surgery, as outlined below.

When heart failure is diagnosed and surgical indications are present, it might be beneficial to perform cardiac surgery in an early stage of the disease. Neurohumoral responses – aimed at maintaining arterial and venous pressure, and increased blood volume – are less prominent in early stages. These neurohumoral mechanisms are compensatory to a certain extent, but can also aggravate the syndrome of heart failure by increasing ventricular afterload and preload. This ultimately leads to pulmonary or systemic congestion, and edema. However, timing of surgery has never been linked to the neurohumoral status of patients. After cardiac surgery, a study of Schenk et al. has shown an improvement in neuroendocrine activity a year after left ventricular reconstruction in patients with congestive heart failure.[17]

Interestingly, as outlined in Chapter 7, patients with preexisting heart failure do have a different and more pronounced myocardial inflammatory response upon reperfusion compared with patients without heart failure. In our study, only circulating inflammatory markers and tissue inflammatory cells were evaluated; pathway analysis of pre- and post reperfusion myocardial tissue in patients with preexisting heart failure would reveal additional information. Of note, the application of a common inflammatory pathway may be inappropriate, because of the various etiologies underlying heart failure. Unravelling the complexities of these myocardial inflammatory pathways would be crucial for future studies focussed on anti-inflammatory therapies.[18,19] Furthermore, experimental studies in myocardial infarction models have shown that inflammatory cell subsets could promote either pro-inflammatory and matrix-degrading effects or pro-angiogenetic and reparative effects.[20-22] Consequently, future anti-inflammatory strategies should therefore reduce the deleterious effects of the inflammatory response while maintaining its favourable functions to reduce the risk of heart failure after myocardial infarction.[19]

Another major clinical issue, most likely related to pronounced inflammatory responses, is the (sometimes therapy-resistant) post-cardiac surgery vasoplegia. This syndrome is most frequently observed in patients with preexisting heart failure and is characterized by severe refractory hypotension, metabolic acidosis, and extremely low peripheral vascular resistance. Of note, this is in contrast to the outpatient clinic in which end-stage heart failure patients benefit from medical treatment that lowers the peripheral resistance. In heart failure patients, the (responsiveness of the) vascular system seems to be permanently altered. More precisely, patients suffering from heart failure seem to display an altered vasoresponsiveness to exogenous (adrenergic) stimuli, which might be explained by chronic endogenous adrenergic stimulation and downregulation or desensibilisation of vascular adrenergic receptors.[23,24] Indeed, chronic heart failure patients usually need more support with vasopressor agents after cardiac surgery as compared with patients without heart failure.[25] The maintenance of an adequate blood pressure is often problematic during the early postoperative phase, despite pharmacological adrenergic stimulation. This response can be explained by a limitation to increase the cardiac output resulting in not fulfilling the

needs of the completely (nonresponsive) vasoplegic vascular system. Prolonged hypotension and the accompanying hypoperfusion subsequently lead to end-organ dysfunction that constitutes the main category of complications in this patient population.[26] Understanding of the vascular responsiveness after cardiac surgery in detail might yield therapeutic options or preventive strategies leading to safer surgical interventions and improvement in outcome in this expanding group of patients with heart failure with no reasonable treatment alternatives.

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Samenvatting en Algemene Discussie

Hartfalen is een aandoening waarbij de pompfunctie van het hart verminderd is. Het hart is niet meer in staat voldoende bloed uit te pompen om aan de behoeften van weefsels te voldoen. Er zijn 2 vormen van hartfalen. De eerste vorm is systolisch hartfalen waarbij de hartspier niet krachtig genoeg samentrekt en er dus minder bloed rond wordt gepompt dan normaal. De tweede vorm is diastolisch hartfalen waarbij de hartspier onvoldoende ontspant, zodat het hart zich minder goed kan vullen met bloed en er derhalve ook minder bloed beschikbaar is om rond te pompen. Hartfalen kan verschillende oorzaken hebben, zoals een hartinfarct, langdurig hoge bloeddruk, een hartklepaandoening, ritmestoornissen of een hartspierziekte. Hartfalen ontstaat meestal geleidelijk. Het hart probeert initieel te compenseren voor de verminderde pompfunctie door bijvoorbeeld harder te werken. Op een gegeven moment is het hart verzwakt en treden er klachten op zoals vermoeidheid, kortademigheid bij geringe inspanning, en opgezette benen en enkels. Als het hart eenmaal pompkracht heeft verloren, is het moeilijk dit volledig te herstellen. De behandeling van hartfalen is initieel gericht op het achterhalen en behandelen van de onderliggende oorzaak. Indien de oorzaak niet kan worden behandeld, is de levensverwachting beperkt. Een behandeling met geneesmiddelen is vaak noodzakelijk om het hart te ontlasten en de pompkracht te vergroten met als doel de klachten te verminderen. De geleidelijke verslechtering van het hartfalen kan leiden tot het punt dat deze onbehandelbaar wordt: eindstadium van hartfalen.

Het eindstadium van hartfalen is een uitdagend syndroom met een toenemend aantal (nieuwe) patiënten per jaar. Er is sprake van een gestage groei van het aantal patiënten met hartfalen die uiteindelijk een gevorderd stadium van de ziekte bereikt. Dit is het gevolg van een verbeterde overlevingskans na een acuut hartinfarct en door verbeteringen in de behandeling van hartfalen zelf. Ondanks vooruitgang in de medicamenteuze behandeling is er een toenemende groep patiënten met hartfalen waarbij medicamenteuze ondersteuning alleen onvoldoende is. Chirurgische procedures in combinatie met medicamenteuze therapie bieden een mogelijke behandeling voor deze patiëntgroep. Een harttransplantatie is op dit moment de gouden standaard voor de behandeling van patiënten met eindstadium hartfalen. Een chronisch tekort aan donororganen maakt dat hartchirurgische behandelingen gericht op het behoud van het eigen hart van grote klinische waarde en interesse zijn.[1] Alhoewel chirurgische technieken voor patiënten met hartfalen zijn verbeterd, blijven sterfte en complicaties rondom deze hartoperaties hoog, zoals beschreven in een review door George et al.[2]

In dit proefschrift is beschreven dat complicaties na hartfalen chirurgie maar deels verklaard kunnen worden door het preoperatief hoge risico van deze patiënten als gevolg van het syndroom van hartfalen of de lage cardiac output (de hoeveelheid bloed die het hart in

één minuut kan rondpompen). Wij suggereren dat complicaties na hartfalen chirurgie een reflectie zijn van de al vóór de operatie bestaande dysregulatie in deze patiëntgroep. Wij veronderstellen dat het hart bepaalde processen induceert, zoals een inflammatoire respons (een reactie op een schadelijke prikkel leidend tot onder andere celbeschadiging, vaatverwijding en vochtophoping). De geïnduceerde processen zijn verantwoordelijk voor postoperatieve moeilijkheden met grote consequenties voor het hart, maar ook voor andere organen. Zo'n effect kan resulteren in secundaire systemische schade en een vicieuze cirkel van beperkte circulatie als gevolg van het pre-existente pompfalen bij patiënten met hartfalen, resulterend in verhoogde sterfte en complicaties.

Alhoewel de impact van pre-existent hartfalen op deze postoperatieve complicaties algemeen aanvaard is, is de impact ervan nooit geobjectiveerd. Derhalve werd de impact geëvalueerd in **Hoofdstuk 2**. De resultaten bevestigen dat patiënten met hartfalen vaker complicaties ervaren die gerelateerd zijn aan hun pompfalen. Meer specifiek betreft dit de behoefte aan hogere dosering inotropica en mechanische ondersteuning om de werking van de hartspier te versterken. Daarentegen werden langdurige mechanische beademing, sepsis en vasoplegie (afgenomen vaattonus) – complicaties die niet perse gerelateerd zijn aan pompfalen – ook vaker geobserveerd. In het algemeen trad orgaandysfunctie, zoals hart, long- en nierfalen, vaker op na een hartoperatie bij patiënten met hartfalen. Dit nadelige postoperatieve beloop in deze patiëntgroep zou een systemische dysregulatie kunnen reflecteren, welke gerelateerd is aan het syndroom van hartfalen en niet direct toegewezen kan worden aan het al bestaande pompfalen. Zo'n effect kan veroorzaakt worden door een verschillende reactie van patiënten met hartfalen op ischemie/reperfusie (I/R) ten opzichte van patiënten zonder hartfalen. I/R is de weefselschade die optreedt als gevolg van het weer opnieuw van bloed voorzien van het hart, nadat dit een periode geen bloed heeft gehad. Dit is bijvoorbeeld het geval als er een hartoperatie wordt uitgevoerd waarbij het hart stil moet worden gelegd en deze functie tijdelijk wordt overgenomen door een hart-longmachine. Deze reactie van het hart op I/R is daarentegen lastig te onderscheiden van de reactie van het hart op de schade als gevolg van de operatie en het gebruik van de hart-longmachine. Derhalve werd in dit proefschrift gebruik gemaakt van metingen voor en na het hart (arterioveneuze metingen) om onderscheid te kunnen maken tussen mediatoren die vrijkwamen uit het hart of vrijkwamen uit het lichaam.

In de opeenvolgende hoofdstukken van dit proefschrift werden pathofysiologische mechanismen bestudeerd bij patiënten met en zonder hartfalen die een hartoperatie ondergingen. Pathofysiologie is een onderdeel van het (bio)medisch onderzoek dat tracht te begrijpen hoe ziekten ontstaan en hoe het lichaam zich daaraan aanpast. Het doel van dit proefschrift was om de pathofysiologie van I/R schade van het hart systematisch te

evalueren in de mens, waarbij klepchirurgie gebruikt werd als een gepland model van I/R. Dit model geeft de mogelijkheid om bloedmonsters te verzamelen uit de arteria radialis (een slagader in de pols) en de sinus coronarius (een grote ader die het bloed uit de hartspier verzamelt en naar de rechter boezem voert) op het moment van reperfusie. Reperfusie na een hartoperatie treedt op zodra de chirurg de aortaklem verwijderd heeft aan het einde van de operatie. Een aortaklem is noodzakelijk voor het stilleggen en openen van het hart. Door bepaalde mediatoren te vergelijken in het slagaderlijk en aderlijk bloed kan de vrijlating van I/R markers specifiek uit het hart gemeten worden.

Allereerst werd schade als gevolg van reactieve zuurstof- en stikstofverbindingen na I/R in detail bestudeerd aangezien preklinische studies laten zien dat deze radicalen belangrijke initiatoren zijn van I/R schade, zoals beschreven in **Hoofdstuk 3**.[3-5] Zoals verwacht, trad er redox *stress* op tijdens reperfusie welke werd aangetoond middels de activatie van Nrf2, de hoofdregulator van de antioxidant stress reactie. Opvallend was dat de gegenereerde reactieve zuurstof- en stikstofverbindingen niet tot *schade* leiden in de mens na I/R. Geen van de schade markers – malondialdehyde, 15(*S*)-8-*iso*-prostaglandine F2α, nitriet en nitraat – werd namelijk door het hart vrijgelaten na myocardiale reperfusie. Orgaanspecificiteit werd uitgesloten door ook metingen over de nier te verrichten. De resultaten waren voor beide condities zeer consistent en er werd geen vrijlating van oxidatieve schade markers geobserveerd. Deze resultaten suggereren een efficiënt endogeen antioxidant systeem in de mens en geven ook een verklaring voor de beperkte effectiviteit van antioxidant therapie bij humane I/R tot op heden.[6]

In **Hoofdstuk 4** wordt de activatie van het complement systeem na hartklepchirurgie geëvalueerd. Dit systeem omvat een groot aantal eiwitten die deel uitmaken van het afweersysteem. Het complement systeem werd bestudeerd aangezien klinische studies zeer kleine en inconsistente effecten van anti-complement therapie tonen in tegenstelling tot preklinische studies.[7-9] Onze bevindingen toonden aan dat endomyocardiale afzettingen van C3d en C5b-9 reeds aanwezig waren in de vroeg ischemische biopten. Bovendien was dit meer uitgesproken bij patiënten met hartfalen in vergelijking tot patiënten zonder hartfalen. Deze afzettingen kunnen mogelijk gerelateerd worden aan het syndroom van hartfalen, aangezien deze al aanwezig waren op het moment dat de operatie nog maar kortdurend bezig was. Opvallend was dat het hart, zowel van patiënten met als zonder hartfalen, geen C5a of sC5b-9 vrij maakte gedurende de reperfusie fase. De bovenstaande resultaten suggereren dat de bijdrage van complement activatie aan I/R schade in de mens misschien wel minder is dan algemeen gedacht. Dit zou ook verklaren waarom anti-complement therapie tot op heden bij de mens minimale en inconsistente effecten heeft laten zien.

Een mogelijke rol van bloedplaatjes en endotheelcel activatie werd bestudeerd in de **Hoofdstukken 5 en 6**. Endotheel is de bedekkende laag cellen die de binnenkant van hart en bloedvaten bedekt. Onze resultaten ondersteunen het preklinisch bewijs voor

betrokkenheid van bloedplaatjes bij I/R schade van het menselijke hart niet. De activatie van bloedplaatjes was beperkt met name als gevolg van hart-longmachine gerelateerde verdunning van het bloed. Bovendien bleven geactiveerde plaatjes achter in het circuit van de hart-longmachine. Endotheelcel activatie mediatoren werden geanalyseerd in patiënten met en zonder vasoplegie na hartchirurgie. In onze studie werd aangetoond dat patiënten die vasoplegie ontwikkelden al voor de operatie hogere waarden van P-selectine en van Willebrand Factor propeptide hadden. Dit duidt op een systemische laaggradige endotheelcel activatie die al aanwezig is voor de hartoperatie.

In **Hoofdstuk 7** werd de inflammatoire reactie bij patiënten met en zonder hartfalen bestudeerd, na het stilleggen van het hart en de daaropvolgende reperfusie. Falende harten tonen een andere en meer uitgesproken inflammatoire respons na reperfusie dan harten van patiënten zonder hartfalen. Dit kan geassocieerd worden met de systemische complicaties zoals beschreven in Hoofdstuk 2. Deze reactie kan verklaard worden door degranulatie van preoperatief geïnfiltreerde inflammatoire cellen in het hartweefsel van falende harten. Direct na reperfusie werden diverse cytokines en chemokines – eiwitten die immuunreacties reguleren – door het hart vrijgelaten. Desondanks blijft het de vraag of deze reactie gunstig is en of deze noodzakelijk is voor herstel van het weefsel, of dat deze respons ongunstig is en weefselschade verergerd. Tot op heden hebben anti-inflammatoire medicamenten, zoals corticosteroiden, niet geleid tot verbeterde klinische uitkomsten.[10-12]

De rol van galectine-3, een marker van inflammatie en fibrose van het hart, werd beschreven in **Hoofdstuk 8** in relatie tot linker kamer reverse remodeling (afname van linker kamer hypertrofie en -dilatatie) na herstel van de mitralisklep bij patiënten met niet-ischemisch en ischemisch hartfalen. Resultaten tonen aan dat lage preoperatieve waarden van deze marker geassocieerd zijn met linker kamer reverse remodeling 6 maanden na de operatie. Derhalve kan het meten van galectine-3 voor de operatie bruikbaar zijn om patiënten te identificeren die zullen reageren op de additionele (chirurgische) therapie met als doel reverse remodeling te bereiken.

Translatie van de resultaten van de bovengenoemde pathofysiologische mechanismen naar praktische toepassing is essentieel om de gezondheid van patiënten met hartfalen te bevorderen. Derhalve werd toediening van sevoflurane, een anti-inflammatoir anestheticum, rechtstreeks in het hart (intramyocardiaal) in detail bestudeerd in **Hoofdstuk 9**. Eerdere klinische studies hebben contrasterende resultaten laten zien na systemische toediening van sevoflurane.[13-16] Dit kan wijzen op het feit dat er sprake is van een dosis-respons relatie, wat vraagtekens oproept over de concentratie van sevoflurane in het hart na systemische toediening. Onze studie toont aan dat na intramyocardiale toediening van sevoflurane tijdens mitraalklepchirurgie de systemische pro-inflammatoire reactie na reperfusie van het hart verminderd is. Toediening van sevoflurane aan laag tot intermediair

risico patiënten, die relatief complexe klepchirurgie ondergaan, lijkt niet te resulteren in minder hartschade. We kunnen niet uitsluiten dat in meer extreme condities sevoflurane wel deze cardioprotectieve effecten heeft. Ook in deze studie blijft het de vraag of de vermindering van de pro-inflammatoire respons gunstig en noodzakelijk is voor herstel van het weefsel, of dat deze respons ongunstig is en weefselschade verergert.

In het laatste hoofdstuk van dit proefschrift, **Hoofdstuk 10**, werd de rol van interleukine (IL)-9 bij nier I/R schade beschreven. *Klinische* resultaten tonen aan dat nieren van overleden donoren bij een niertransplantatie een substantiële hoeveelheid IL-9 vrijlaten op het moment van reperfusie. Dit IL-9 bleek opgeslagen te liggen in niertubuli. *Preklinische* resultaten tonen daarentegen aan dat remming van IL-9 nierschade verergert. IL-9 kan daarom een regulerende rol spelen bij klinische nier I/R schade, maar kan ook een initiële trigger zijn voor beperking van schade. Er werd gebruik gemaakt van een muismodel van nier I/R aangezien weefselschade als gevolg van I/R meer homogeen was in dit model in vergelijking met een hartmodel. Bovendien was de uitleesparameter van nierschade makkelijk te evalueren middels creatinine waarden.

Conclusie

Dé uitdaging van dit proefschrift was te evalueren of het hart na reperfusie effecten elders in het lichaam induceert, die bijdragen aan sterfte en complicaties na hartchirurgie. Pre-existente processen, zoals afzettingen van complement en influx van inflammatoire cellen in het hart, werden geïdentificeerd bij patiënten met hartfalen. Deze veranderingen op hun beurt veranderden de reactie van het hart na reperfusie. Bovendien voorspelde een pre-existente laaggradige endotheelcel activatie het ontstaan van vasoplegie na hartchirurgie. Er werd geen bewijs gevonden voor oxidatieve schade na reperfusie in tegenstelling tot de huidige theorie. Tot slot resulteerde de beïnvloeding van de inflammatoire respons middels sevoflurane inderdaad in een vermindering van pro-inflammatoire markers, maar werden er geen cardioprotectieve effecten geobserveerd. Een stapsgewijze benadering voor verder onderzoek van preklinisch naar klinisch onderzoek en vice versa is essentieel om de vooruitgangen in de pathofysiologische wetenschap te kunnen integreren in klinische studies.

Toekomstperspectieven

Pre-existente mechanismen lijken een grotere bijdrage te leveren aan de verschillende respons op reperfusie van het hart bij patiënten met hartfalen dan fundamenteel verschillende processen tussen mensen met en zonder hartfalen. Sterfte en complicaties na hartchirurgie zijn derhalve waarschijnlijk gerelateerd aan de pre-existente systemische

dysregulatie geassocieerd met het syndroom van hartfalen. Diverse vragen zijn nog altijd onbeantwoord en nieuwe vragen ontstaan dagelijks. Ondanks continue vooruitgang wat betreft het ontrafelen van de onderliggende pathofysiologische mechanismen van I/R van het hart bij patiënten met hartfalen, tonen behandelingen tot op heden geen gunstige effecten of moeten nog bestudeerd worden in klinische studies zoals beschreven in de introductie van dit proefschrift.

Hoewel het buiten het bestek van dit proefschrift valt, blijft het gezegde 'voorkomen is beter dan genezen' toepasbaar op bijna alle patiënten met hartfalen. Primaire preventie zou nog steeds de meest simpele en veilige oplossing zijn. Door verbeteringen in secundaire en tertiaire preventie na een hartinfarct en de diagnose van hartfalen zelf, is er een gestage groei van patiënten met hartfalen die een gevorderd stadium van de ziekte bereiken. Zoals beschreven in dit proefschrift zijn pre-existente processen aanwezig bij patiënten met eindstadium hartfalen. Hartchirurgie bij deze patiënten kan gemakkelijk leiden tot dysregulatie van hun strak gereguleerde homeostase. Derhalve zouden interventies gericht moeten zijn op de optimalisatie van deze pre-existente processen, wat mogelijk kan leiden tot verbeterde uitkomsten na hartchirurgie. Te denken valt hierbij aan neurohumorale reacties, inflammatoire netwerken en reactiviteit van het vasculaire systeem (bloedvaten), welke hieronder nader worden toegelicht.

Als hartfalen gediagnosticeerd is en chirurgische indicaties gesteld zijn, dan kan het gunstig zijn om hartchirurgie in een vroeg stadium van de ziekte te verrichten. Neurohumorale reacties – gericht op het behoud van arteriële / veneuze druk en toename van het bloedvolume – zijn minder prominent aanwezig in vroege stadia van de ziekte. Deze neurohumorale mechanismen zijn tot op zekere hoogte compensatoir, maar kunnen ook het syndroom van hartfalen verergeren door toename in voor- en nabelasting van de hartkamers. Dit leidt uiteindelijk tot verhoogde drukken in het longvaatbed en oedeem. Vermeldenswaardig is dat het moment van chirurgie nog nooit gerelateerd is aan de neurohumorale status van patiënten. In een studie van Schenk et al. is wel aangetoond dat er een verbetering in neuroendocriene activiteit werd gezien een jaar na reconstructie van de linker kamer in patiënten met hartfalen.[17]

Zoals beschreven in Hoofdstuk 7 hebben patiënten met pre-existent hartfalen een verschillende en meer uitgesproken inflammatoire respons van het hart na reperfusie dan patiënten zonder hartfalen. In onze studie werden alleen circulerende inflammatoire mediatoren en inflammatoire cellen in het hartweefsel bestudeerd. Analyse naar inflammatoire netwerken in hartweefsel van voor en na reperfusie bij patiënten met pre-existent hartfalen zou extra informatie op kunnen leveren. Gezien de diverse oorzaken van hartfalen is het waarschijnlijk niet juist te verwachten dat er één algemeen inflammatoir netwerk is. Het ontrafelen van de complexiteit van deze netwerken is cruciaal voor verdere studies gericht op anti-inflammatoire therapieën voor patiënten met hartfalen.[18,19]

Bovendien hebben preklinische studies laten zien dat diverse subtypen van inflammatoire cellen zowel pro-inflammatoire als matrix-degraderende effecten kunnen bewerkstelligen of juist pro-angiogenetische en herstellende effecten hebben in hartinfarct modellen.[20-22] Dit heeft tot gevolg dat toekomstige anti-inflammatoire strategieën de verwoestende effecten van de inflammatoire respons zouden moeten reduceren, maar daarnaast zouden ze ook de gunstige functies moeten behouden die het risico op hartfalen na een hartinfarct verminderen.[19]

Een andere belangrijke observatie in de dagelijkse praktijk is (therapieresistente) vasoplegie, wat meest waarschijnlijk gerelateerd is aan de prominente inflammatoire reacties postoperatief. Dit syndroom wordt het meest gezien bij patiënten met pre-existent hartfalen en wordt gekenmerkt door lage bloeddruk, metabole acidose en een extreem lage perifere vaatweerstand. Opvallend is dat dit tegengesteld is aan de dagelijkse klinische praktijk waarin patiënten met eindstadium hartfalen profiteren van medicamenteuze behandeling die de perifere vaatweerstand juist verlaagd. Bij patiënten met hartfalen is de reactiviteit van het vasculaire systeem soms permanent veranderd. Patiënten met hartfalen tonen een andere vasoreactiviteit op exogene (adrenerge) stimuli, die verklaard kunnen worden als gevolg van chronische endogene adrenerge stimulatie en het naar beneden bijstellen of het verminderd gevoelig worden van vasculaire adrenerge receptoren.[23,24] Anders uitgelegd is er sprake van een chronisch verhoogde stimulatie van sympathische zenuwvezels door het lichaam zelf, met verhoogde productie van adrenaline en noradrenaline tot gevolg. Dit leidt weer tot minder of het minder gevoelig zijn van die receptoren in de bloedvaten. Patiënten met chronisch hartfalen hebben over het algemeen meer ondersteuning met inotropica en vasopressiva nodig na hartchirurgie ter ondersteuning van de hemodynamiek dan patiënten zonder hartfalen.[25] Ondanks medicamenteuze adrenerge stimulatie is het behoud van een adequate bloeddruk vaak problematisch tijdens de vroege postoperatieve fase. Deze respons kan verklaard worden doordat de cardiac output onvoldoende verhoogd kan worden om aan de behoeften van het vasculaire systeem tegemoet te komen tijdens vasoplegie. Langdurige lage bloeddruk en de bijbehorende verminderde doorbloeding leiden tot eind-orgaan dysfunctie, een van de belangrijkste complicaties in deze patiëntenpopulatie na hartchirurgie.[26] Gedetailleerd begrip van de vasculaire reactiviteit na hartchirurgie zou tot therapeutische opties of preventieve strategieën kunnen leiden. Deze zouden op hun beurt weer tot veiligere chirurgische interventies en verbeteringen in uitkomsten kunnen leiden voor deze patiënten met hartfalen zonder geschikte alternatieven voor behandeling.

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ABBREVIATIONS

6MWT	six-Minute Walk Test
15(*S*)-8-*iso*-PGF2α	15(*S*)-8-*iso*-prostaglandin F2α
Ab	Antibody
ACE	Angiotensin Converting Enzyme
ADP	Adenosine Diphosphate
AF	Atrial Fibrillation
Ang-2	Angiopoietin-2
ANOVA	One-way between-groups Analysis of Variance
AoX	Aortic cross-clamping
APACHE	Acute Physiology and Chronic Health Evaluation
ARB	Angiotensin Receptor Blocker
ARDS	Acute Respiratory Distress Syndrome
ARE	Antioxidant Responsive Element
AT-I/II	Angiotensin I/II
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
BDD	Brain-Dead Donor
Beta-TG	Beta-Thromboglobulin
BM	Basement Membrane
BN	Band Necrosis
CABG	Coronary Artery Bypass Grafting
CAD	Coronary Artery Disease
CCL	Chemokine Ligand
CD	Cluster of Differentiation
CDD	Cardiac-Dead Donor
CIT	Cold Ischemia Time
CK	Creatine Kinase
CO	Cardiac Output
COPD	Chronic Obstructive Pulmonary Disease
COX	Cyclooxygenase
CPB	Cardiopulmonary Bypass
CRP	C-Reactive Protein
C-RP	Collagen-Related Peptide
CRT	Cardiac Resynchronization Therapy
cTnT	cardiac Troponin T
CXCL	C-X-C Chemokine Ligand

DD	Deceased Donor
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EF	Ejection Fraction
eGFR	estimated Glomerular Filtration Rate
ELISA	Enzyme-Linked Immunosorbent Assay
EROA	Effective Regurgitation Orifice Area
FACS	Fluorescence-Activated Cell Sorting
FMR	Functional Mitral Regurgitation
G-CSF	Granulocyte Colony-Stimulating Factor
GAPDH	Glyceraldehyde Phosphate Dehydrogenase
GC-MS	Gas Chromatography-Mass Spectrometry
GC-MS/MS	Gas Chromatography-Tandem Mass Spectrometry
GSH	Glutathione
GSTA2	Glutathione S-transferase A2
HF	Heart Failure
HMOX-1	Heme Oxygenase-1
HLA	Human Leucocyte Antigen
HTK	Histidine-Tryptophan-Ketoglutarate
I/R	Ischemia/Reperfusion
ICD	Implantable Cardioverter Defibrillator
ICU	Intensive Care Unit
IHF	Ischemic Heart Failure
IL	Interleukin
IP-10	Interferon-inducible Protein-10
LAVI	Left Atrial Volume Index
LD	Living Donor
LV	Left Ventricle/Ventricular
LVEDP	Left Ventricular End-Diastolic Pressure
LVEDV	Left Ventricular End-Diastolic Volume
LVEF	Left Ventricular Ejection Fraction
LVESV	Left Ventricular End-Systolic Volume
MCP-1	Monocyte Chemoattractant Protein-1
MDA	Malondialdehyde
MFI	Median Fluorescence Intensity
MI	Myocardial Infarction
MMP	Matrix Metallopeptidase
MPO	Myeloperoxidase

MVP	Mitral Valve Annuloplasty
MODS	Multiple Organ Dysfunction Syndrome
mPTP	mitochondrial Permeability Transition Pore
MR	Mitral Regurgitation
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF-κB	Nuclear Factor-κB
NHF	Non-Heart Failure
NIHF	Non-Ischemic Heart Failure
NQO-1	NAD(P)H Quinone Oxidoreductase
Nrf2	Nuclear factor (erythroid-derived 2)-related factor 2
NT-proBNP	N-Terminal pro-Brain Natriuretic Peptide
NYHA	New York Heart Association
OPG	Osteoprotegerin
PAS	Periodic Acid-Schiff
Pcr	Phosphocreatine
PCWP	Pulmonary Capillary Wedge Pressure
PDGF	Platelet-Derived Growth Factor
PGE_2	Prostaglandin E_2
PTCA	Percutaneous Transluminal Coronary Angioplasty
QoL	Quality of Life
RAAS	Renin-Angiotensin-Aldosterone System
RANTES	Regulated upon Activation, Normal T-cell Expressed and Secreted
RMA	Restrictive Mitral Annuloplasty
ROC	Receiver Operating Characteristic
RONS	Reactive Oxygen and Nitrogen Species
R Vol	Regurgitant Volume
SD	Standard Deviation
SEM	Standard Error of the Mean
SIRS	Systemic Inflammatory Response Syndrome
SNS	Sympathetic Nervous System
SPAP	Systolic Pulmonary Artery Pressure
SOD	Superoxide Dismutase
TBARS	Thiobarbituric Acid-Reactive Substances
TNF	Tumor Necrosis Factor
TRAP	Thrombin Receptor-Activating Peptide
TVP	Tricuspid Valve Annuloplasty
VEGF	Vascular Endothelial Growth Factor
VT	Ventricular Tachycardia

vWF	von Willebrand Factor
vWFpp	von Willebrand Factor propeptide
WIT	Warm Ischemia Time
XO	Xanthine Oxidase

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CURRICULUM VITAE

Kirsten Aimee Kortekaas werd geboren op 26 september 1985 te Leiderdorp. In 2003 behaalde zij haar gymnasium diploma aan het Stedelijk Gymnasium te Leiden. In datzelfde jaar begon zij aan haar studie Biomedische Wetenschappen aan de Universiteit Utrecht na uitloting voor de studie Geneeskunde. Na de felbegeerde inloting in 2004 startte zij met de studie Geneeskunde aan de Universiteit Leiden, de stad van haar keuze. Tijdens haar studie verrichtte zij wetenschappelijk onderzoek op de afdeling Algemene Interne Geneeskunde in het Leids Universitair Medisch Centrum naar het gebruik van klinische beslisregels in de diagnostiek van longembolieën onder leiding van dr. M.V. Huisman. Bovendien nam zij in 2007 deel aan het Honours Programme van de Universiteit Leiden middels de Honours Class "Health, Science and Technology". Zij behaalde haar doctoraalexamen in 2008 na het afronden van haar afstudeerproject getiteld 'Myocardial Ischemia/Reperfusion Injury' op de afdeling Thoraxchirurgie in het Leids Universitair Medisch Centrum gesuperviseerd door prof. dr. R.J.M. Klautz en dr. J.H.N. Lindeman. Aansluitend werd gestart met een promotietraject op dezelfde afdeling, waaruit dit proefschrift tot stand is gekomen. In mei 2011 startte zij met haar coschappen en behaalde haar artsexamen in april 2013. In de toekomst ambieert Kirsten een klinische en wetenschappelijke carrière binnen de cardiologie. Derhalve start zij per oktober 2013 als arts-assistent in het Onze Lieve Vrouwe Gasthuis te Amsterdam onder supervisie van dr. G.A. Somsen.

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**Both authors contributed equally to this work*

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