UNIVERSITÄT BERN

Graduate School for Cellular and Biomedical Sciences

University of Bern

Role of the Plasma Cascade Systems in Endothelial Cell Activation Induced by Peripheral Ischemia / Reperfusion Injury

PhD Thesis submitted by

Shengye Zhang

from **China**

for the degree of

Doctor of Medicine and Philosophy (MD, PhD)

Supervisor

Prof. Dr. Robert Rieben

Cardiovascular Research
Department of Clinical Research
Medical Faculty of the University of Bern

Co-advisor

PD Dr. Jan A. Plock
Department of Plastic and Hand Surgery
University Hospital Zurich

Accepted by the Faculty of Medicine, the Faculty of Science and the Vetsuisse Faculty of the University of Bern at the request of the Graduate School for Cellular and Biomedical Sciences

Bern, Dean of the Faculty of Medicine

Bern, Dean of the Faculty of Science

Bern, Dean of the Vetsuisse Faculty Bern

Preface

Peripheral ischemia/reperfusion (I/R) injury may occur in a variety of clinical settings including peripheral vascular diseases, limb transplantation, trauma, sepsis, and tourniquet application in limb surgery. A complex inflammatory response can be evoked due to activation of the plasma cascade systems and endothelial cell (EC) activation, leading to potentially life-threatening disorders. As a result, it is of fundamental importance to understand the mechanisms and find therapeutic approaches to attenuate the extent of I/R injury.

The aim of my MD-PhD was to investigate the underlying mechanisms of I/R injury in terms of activation of the plasma cascade systems and EC in a hind limb I/R injury model in rats including the skeletal muscle and bone. Furthermore, in order to reduce the number of animals used for in vivo studies, in accordance with 3R principles, EC activation was also carried out in a microfluidic system under flow conditions in the context of xenotransplantation. Complement inhibitors were tested both in vivo and in vitro.

In this thesis, 5 papers/manuscripts have been included. All of them are original articles. I am the first author for paper I -IV and I contributed to paper V as a co-author. Two papers have been published. The other three manuscripts are in submission. Three papers/manuscripts concern I/R injury, one involves xenotransplantation, and one focuses on an ischemic skin flap model. The thesis ends with an overall discussion and outlook, acknowledgements, and curriculum vitae.

At the end of this preface, I would like to express my appreciation for my supervisor Prof. Dr. Robert Rieben, who accepted me as a MD-PhD student to work in his lab and guided me throughout the thesis with full support. I would also like to thank all of my colleagues, friends, and other lab members for their contribution to this thesis. Finally, I would like to give a big thank you to my parents, my sisters and my brother for their consistent encouragement, love, and support.

TABLE OF CONTENTS

Abstract

Peripheral ischemia/reperfusion (I/R) injury is a critical pathology of clinical entities such as peripheral vascular diseases, limb transplantation, trauma, and sepsis, as well as tourniquet use in surgical procedures on the extremities. It fuels inflammatory responses to local tissue as well as distant organs, resulting in the systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) under severe conditions. To date, the mechanisms are not clearly understood and promising therapeutic interventions are still absent.

In this thesis, a rat hind limb I/R injury model was used to investigate the inflammatory mechanisms of peripheral I/R injury induced by activation of the plasma cascade systems (i.e. complement, coagulation, fibrinolysis, and kinin), as well as endothelial cells (EC). Also the effect of C1 inhibitor on EC activation was explored in our model. In addition, an in vitro microfluidic system was developed to grow and investigate EC under physiological flow conditions. One application of this system was to test whether it can be used to reproduce complement activation, as well as EC activation in a xenotransplantation model. Three different complement inhibitors were tested in this model. All of our studies were in accordance with the 3R principles (Reduction, Replacement, Refinement of animal experiments) and the ARRIVE (Animal Research: Reporting of in vivo experiments) guidelines.

The main objective of this thesis was to investigate key mechanisms of I/R injury and shed light on potential therapeutic targets for clinical patients suffering from reperfusion injury. Our data showed that the complement, coagulation and fibrinolytic systems were activated in our rat hind limb I/R injury model by analyzing the skeletal muscle and bone tissue, as well as endothelial activation. Pre-treatment with C1 inhibitor (C1 INH) significantly attenuated reperfusion injury of rat hind limbs by preventing EC activation. Moreover, a simple in vitro model is able to reproduce key findings of complement- and EC-activation in a xenotransplantation model. Complement inhibitors C1 INH, APT070, and low molecular weight dextran sulfate (DXS) effectively reduced complement and EC activation in this in vitro xenotransplantation model.

In summary, activation of the plasma cascade systems and EC are involved in the pathogenesis of peripheral I/R injury as well as xenotransplantation. C1 INH is a promising multifunctional complement inhibitor, which not only attenuates activation of the plasma cascades but also preserves endothelial function.

Introduction

Ischemia is caused by a restriction or deprivation of blood supply to a tissue or organ for a limited period of time. The consequences of ischemia have long been associated with clinical outcomes in many diseases [1-3]. Therefore, the basic and prime salvage - timely restoration of oxygenated blood to the ischemic tissue or organ - is of critical importance for preserving viability and function. However, reperfusion, paradoxically, can exacerbate tissue injury in excess of that produced by ischemia alone. On this basis tissue damage caused by reperfusion to the previously ischemic bed is defined as ischemia/reperfusion injury (I/R injury). I/R injury has been involved in the pathological processes of many clinical diseases, such as myocardial infarction, cardiopulmonary bypass, coronary angioplasty, tourniquet application in a surgical setting, organ transplantation, trauma, sepsis, stroke, as well as peripheral vascular diseases such as thrombotic occlusion and embolism formation. It may initiate both local and systemic inflammation, such as systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS), which are potential life-threatening disorders and have high morbidity and mortality worldwide [4, 5]. Therefore, it is of major importance to understand the underlying mechanisms and define potential therapeutic approaches in reperfusion injury.

Essentially, reperfusion injury has tissue or organ specificity. For example, brain is particularly vulnerable to ischemia, which causes cells to undergo permanent damage within a few minutes after blood supply disruption due to the high energy requirement [6]. Myocardium can tolerate up to 15 min of severe ischemia before developing myocardial infarction with manifestations of microvascular and endothelial injury, myocardial stunning, arrhythmias, and myocyte death [7]. Lung I/R injury is more complicated due to its dual blood supply systems and available oxygen from alveolar ventilation. It is characterized by increases in microvascular permeability, vascular resistance, edema, and blood pressure, as well as impaired oxygenation [8]. In the most severe conditions, acute respiratory distress syndrome ensues [9]. As for reperfusion injury in liver, this mainly results from liver transplantation and major hepatic resection. Liver I/R injury has often been associated with microvascular failure [10, 11], free radical oxygen species [12, 13], as well as inflammatory reactions mediated by Kupffer cells, neutrophils, complement, and cytokines [14, 15]. Distant organ damages caused by liver I/R injury is also a detrimental consequence [16]. Similar to hepatic I/R injury, reperfusion injury to the kidney often occurs in the setting of kidney transplantation [17]. This may lead to kidney dysfunction and failure [18], which is characterized by a decreased glomerular filtration rate and subsequent loss of the ability to excrete wastes, concentrate urine, and conserve electrolytes [19].

In contrast to those organs, skeletal muscle tissue can survive up to 60-90min of ischemia without irreversible damages. However, a longer time of ischemia can lead to microvascular disruption, muscle edema, compartment syndrome, no-reflow, disseminated intravascular coagulation, or SIRS and MODS upon reperfusion [20-22].

During ischemia, nutrient depletion and hypoxia may lead to release of damage associated molecule patterns (DAMP) and neo-epitope expression particularly on the affected endothelium [23, 24], which may be recognized by pre-formed antibodies circulating in the blood. This activates the complement system via all three pathways leading to formation of the anaphylatoxins C3a and C5a as well as the membrane attack complex C5b-9. After re-establishment of blood supply, metabolites [25-27] produced in ischemia are flushed into the circulating system, which may trigger a profound inflammatory response. The extent of I/R injury depends on the duration of ischemia and /or reperfusion [28-30], and also the severity of ischemia [31, 32].

Not only the complement system plays an important role in reperfusion injury, but also the coagulation and the kallikrein/kinin cascades contribute to the pathogenesis of reperfusion injury in heart and kidney [33-35]. Also endothelial cell (EC) activation is also known to be involved in I/R injury [36]. Moreover, plasma cascades and EC activation are interactive and inter-dependent. However, to date, the defined mechanisms of plasma cascade and EC activation are still not clearly understand and promising therapeutic strategies are missing. Therefore, the following overview will focus the role of the plasma cascades and endothelial cell activation in I/R injury. In addition, the potential of effective therapeutic approaches will also be included.

1. Role of reactive oxygen species in ischemia/reperfusion injury

Reactive oxygen species (ROS) are free radicals containing oxygen with at least one unpaired electron, such as superoxide radical ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$), and hydrogen peroxide (H_2O_2). ROS are generated continuously in mitochondria as by-products of cellular metabolism and are balanced by endogenous scavenging mechanisms, maintaining homeostasis in aerobic organisms. Oxidative and antioxidant enzymes are of importance to ROS formation. The former mainly includes endothelial xanthine oxidase and nicotinamide adenine dinucleotide phosphate (NADP) oxidase, while superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase are predominant in the latter. Overproduced ROS may cause progressive oxidative stress and contribute to reperfusion injury.

Ischemia is associated with gene reprogramming. For example, prolylhydroxylase (PHD) activity is inhibited because oxygen is needed as a cofactor. This hydroxylates specific proline residues in the oxygen-dependent degradation domain of hypoxia-inducible transcription factor-1α (HIF-1 α), leading to upregulation of HIF-1 α [37]. More over, the production of adenosine triphosphate (ATP) is decreased due to the lack of oxygen in ischemia, leading to an accumulation of hypoxanthine. During this process, xanthine dehydrogenase is converted into xanthine oxidase [38]. When oxygen is restored in reperfusion, xanthine oxidase can oxidize hypoxanthine to xanthine and other forms of ROS, which triggers the onset of reperfusion injury [39, 40]. NADPH oxidase has also been shown to be critical for ROS production in pulmonary and myocardial studies [41, 42]. Free radical scavengers like SOD and/or CAT have been demonstrated to effectively prevent reperfusion injury to heart and skeletal muscle [43, 44].

Overproduced ROS may lead to progressive oxidative stress that causes damage to protein, lipids, DNA, and RNA. Much of the damage is caused by hydroxyl radicals and lipids are the major targets. Malondialdehyde (MDA) and 4-hydroxynonenal (HNE) are the main products of lipid peroxidation, which decreases the membrane fluidity and disrupt membrane-bound proteins. They are produced during ischemia and increased after reperfusion, triggering the onset of reperfusion injury in rat liver and myocardium [45, 46]. Oxidative stress may even be involved in distant organ failure in the tourniquet shock model in mice [47].

However, this may not be universal since we did not find significantly elevated concentrations of MDA in plasma, nor did we find significantly reduced SOD activities in our rat hind limb I/R injury model, with the exception of the 2 h reperfusion value in the 40% FiO2 group [48]. These results are consistent with a recently published clinical study [49], as well as earlier data from rat hind limb I/R injury [50], which questions the role of oxidative stress in I/R injury. The controversial results might be due to differences in

animal species and/or experimental techniques.

Nevertheless, reactive nitrogen species (RNS) are also important components of free radicals, including nitric oxide (NO) and peroxynitrite ($ONOO^-$). Low concentrations of NO produced by endothelial nitric oxide synthase (eNOS) has physiological functions [51] like inducing vasodilation, preventing neutrophil/platelet adhesion to endothelium, inhibiting smooth muscle cell proliferation and migration, and maintaining endothelial cell barrier integrity. However, higher concentrations of NO produced from inducible NOS (iNOS) and neuronal NOS (nNOS) are detrimental to ischemic tissue [52]. $ONOO^-$ is reported to oxidize membrane lipids and permeate lipid bilayers [53], inactivate SOD [54], increase NO-induced blood–brain barrier permeability, and trigger apoptosis [55] (Figure 1).

It has been demonstrated that RNS play a critical role in the pathophysiology development of I/R injury in other organs such as lung, liver, and heart [56-58]. An inhibitor of iNOS reduced the generation of RNS in the skeletal muscle I/R injury in extracellular SOD knockout mice [59]. The inhibitor of iNOS reduced the generation of RNS in the skeletal muscle I/R injury in extracellular SOD knockout mice [59]. Treatment with peroxynitrite scavengers like uric acid and phenolic compound showed the neuroprotective effects in ischemic stroke [60, 61].

However, scavengers did not work consistently in reperfusion injury. It was reported that scavengers against ROS did not attenuate the extent of reperfusion injury in skeletal muscle [62]. Also Hawkes et al demonstrated that scavengers of SOD and allopurinol did not have beneficial effects on the length, blood flow, or histological changes in pedicle skip flap in pigs [63]. In clinical studies, anti-oxidant interventions, quite often, did not show benefits as evidenced in human coronary artery bypass surgery [64] and in acute ischemic stroke even with an increased endpoint of death[65]. Consequently, it still remains controversial as to the exact role of ROS and RNS, as well as the effectiveness of therapeutic interventions in the context of reperfusion injury.

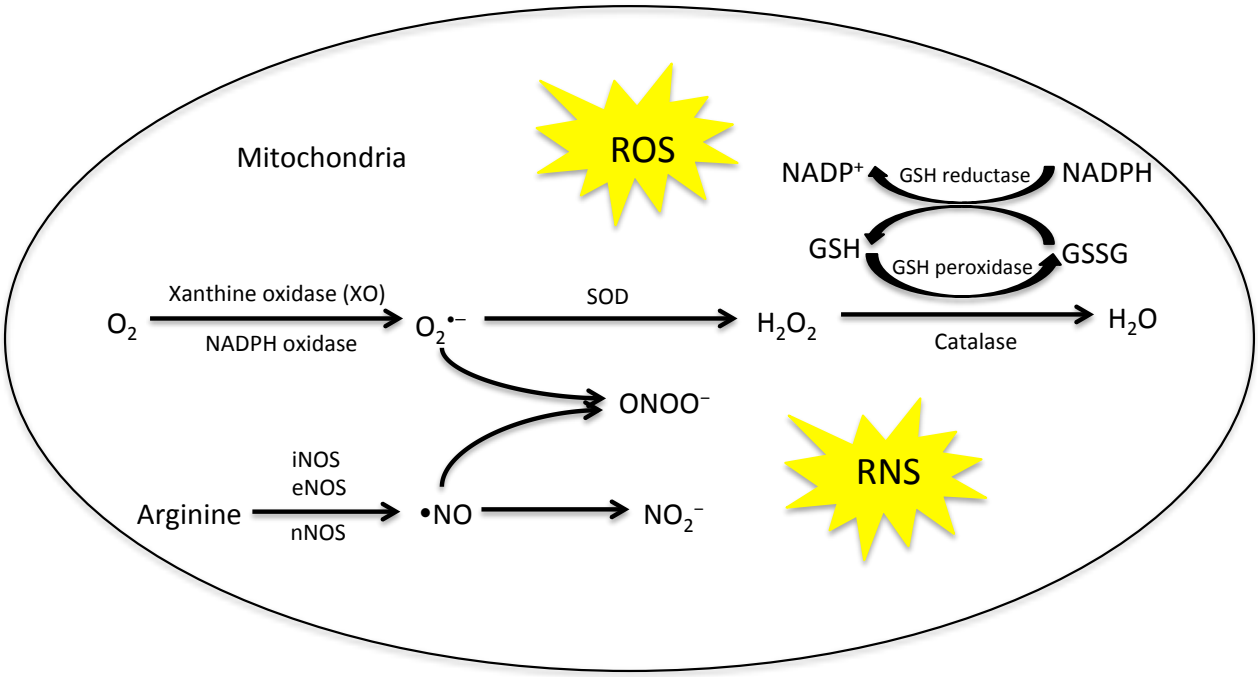


Figure 1: Schematic presentation for reactive oxygen and nitrogen species. GSH: glutathione; GSSG: glutathione disulfide: iNOS: inducible nitric oxidase; eNOS: endothelial nitric oxidase; nNOS: neuronal nitric oxidase. $NADP^+$: nicotinamide adenine dinucleotide phosphate; NADPH: the reduced form of $NADP^+$. Modified from Wulf Dröge. Free radicals in the physiological control of cell function. Physiological Reviews. 2002.

2. The plasma cascade systems

2.1 The complement system

The complement system has long been viewed as the first line of defense against pathogens in innate immunity. More than 30 components and regulators comprise the complement system. They are synthesized and secreted by hepatocytes, as well as other cells stimulated by cytokines and hormones. Activation of complement leads to opsonization and lysis of pathogens via C3b and pore formation by membrane attack complex (MAC) as well as the release of potent inflammatory molecules anaphylatoxins C3a and C5a. Beyond the elimination of microbes, complement can also participate in a sterile inflammation in I/R injury [66]. Under normal conditions, the complement system is tightly regulated by fluid-phase and membrane-bound proteins. These include C1 esterase inhibitor (C1 INH), C4 binding protein (C4BP), factor H (fH), factor I (fI), S-protein, and clusterin in plasma. Membrane cofactor protein (MCP/CD46), decay-accelerating factor (DAF/CD55), complement receptor 1 (CR1), and CD59 are in a membrane-bound format. C1 INH inactivates C1r, C1s, MASP-1, and MASP-2. C4BP, MCP, and CR1 act as cofactors for factor-I-mediated cleavage of C3b and C4b. DAF and fH destabilize C3 and C5 convertases. CD59, S-protein, and clusterin inhibit C9 polymerization to prevent C5b-9 generation [67] (Table 1).

Regulator	Function	Location
C1 inhibitor (C1 INH)	Inactivates C1r and C1s, MASP-1 and MASP-2	Plasma
MCP/CD46	Cofactor for factor I-mediated cleavage of C3b and C4b	Membrane-bound
DAF/CD55	Destabilizes C3/C5 convertase of the CP and AP (accelerating activity)	Membrane-bound
CR1	Decay accelerating activity as well as cofactor activity for factor I-mediated cleavage of C3b and C4b	Membrane-bound
C4 binding protein (C4BP)	Binds to C4b; decay accelerating and cofactor activity	Plasma
Factor H	Binds to C3b; has accelerating activity of the AP C3 and C5 convertase and cofactor activity	Plasma
Thrombomodulin	Increases fH cofactor activity, activates TAFI-mediated C3a and C5a inactivation	Membrane-bound
Factor I	Degrades C3b and C4b aided by cofactors	Plasma
CD59	Blocks the C9 association with C5b-7 to prevent C5b-9 formation on host cells	Membrane-bound
S-protein	Binds to C5b-7 and inhibits C9 polymerization	Plasma
Clusterin (SP-40, 40)	Prevents membrane binding of C5b-7 and inhibits MAC formation membrane as well as sC5b-9	Plasma

Table 1: Complement regulatory proteins in human beings and their functions. MCP: membrane cofactor protein; DAF: Decay accelerating factor; CR1: complement receptor 1; TAFI: thrombin activatable fibrinolysis inhibitor; CP: classical pathway; AP: Alternative pathway. Adapted from [67].

Typically, there are three pathways for the activation of the complement system (Figure 2), namely the classical pathway (CP), lectin pathway (LP), and alternative pathway (AP). All of the three pathways are initiated by different molecules and converge at C3. The classical pathway is initiated by the binding of C1q to the Fc portion of the antigen-antibody immune complex as well as acute phase C-reactive protein (CRP) [68]. This binding causes a conformational alteration, leading to the sequential activation of C1r and

C1s. Activated C1s cleaves C4 into C4a and C4b, also C4b-bound C2 into C2a and C2b, resulting in a generation of the C3 convertase C4b2a.

Similarly to CP, LP is initiated by the binding of mannose–binding lectin (MBL) and/or ficolins to antibody-antigen complexes and carbohydrate patterns in the microorganisms. These recognition molecules are in complex with MBL-associated serine proteases (MASP-1, MASP-2 and MASP-3), which cleave C4 and C2 and generate C3 convertase C4b2a.

Distinct from CP and LP, AP is induced by hydrolysis of a thioester bond within C3, either spontaneously at a low rate or accelerated by contact of C3 with various surfaces (tick over) [69]. This leads to a conformational change in the structure of C3, resulting in the formation of C3b(H_2O) and exposure of new binding sites. Factor B (fB) binds to C3b(H_2O) and is cleaved by factor D (fD), leading to the formation of C3 convertase C3bBb. Newly formed C3bBb is further stabilized by properdin.

C3 convertases including C4b2a and C3bBb cleave C3 into more C3b and C3a. More C3b binds to C3 convertase, leading to the formation of C5 convertases C4b2a3b and C3bBb3b, respectively. These C5 convertases cleave C5 into C5b and C5a. C5b assembles with C6, C7, C8, and C9 to form MAC inserted into the membrane and soluble sC5b-9 in the circulation. During the process, anaphylatoxins of C3a and C5a are produced and function as chemoattractants for macrophage and neutrophils, stimulating release of histamine, regulating vasodilation, contracting smooth muscle cells, and triggering an oxidative burst [70, 71].

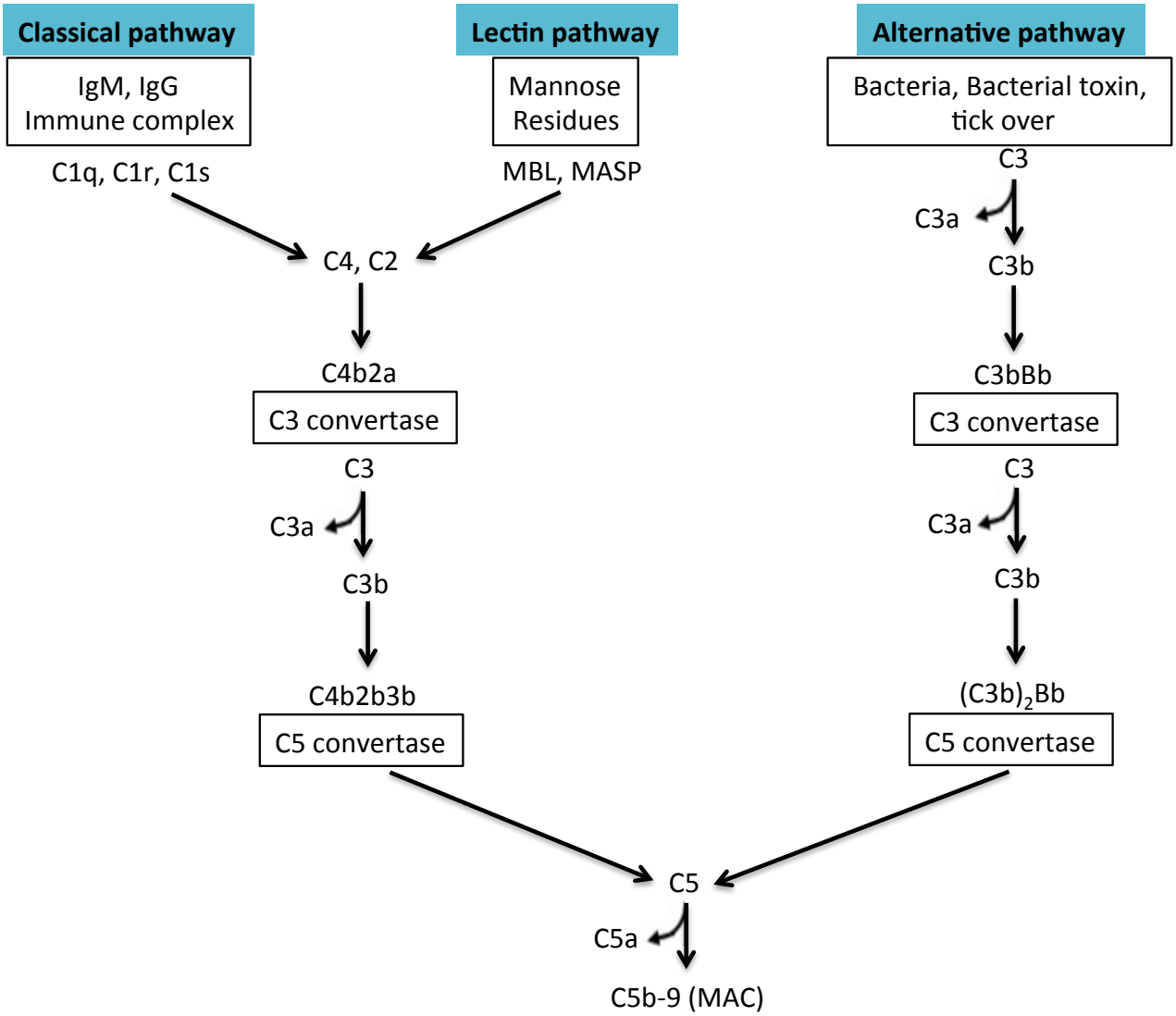


Figure 2: Schematic overview of the complement cascade illustrating the three activation pathways (Classical, lectin, and alternative) and the membrane attack complex. MBL: mannose binding lectin; MASP: mannose binding lectin associated serine protease; MAC: membrane attack. Adapted from [67].

2.1.1 Role of complement in ischemia/reperfusion injury

In 1971, Hill and Ward first reported the involvement of complement in myocardial infarction in rats in which C3 fragments had chemotactic activity for neutrophils [72]. Since then, myocardial infarction has been used as a good model to investigate the role of complement in I/R injury [73, 74]. Also reperfusion injury to other organs like brain, lung, liver, kidney, and limbs were extensively studied and it was shown that he complement system plays an important role in these organs too [75-79].

The functional importance of an individual complement component in the pathophysiology of I/R injury has usually been investigated in animals lacking that component or its receptors. Vakeva et al demonstrated that deposition of C1 started after 2-4h of myocardial ischemia, even in the absence of reperfusion [80]. However, it was reported that C1q was not involved in gastrointestinal I/R injury [81], and C1q deficiency of mice did not prevent tissue injury in a transient focal cerebral ischemia model [82]. MBL-deficient mice were protected from necrotic tissue injury, but not from edema or distant lung injury in the skeletal muscle I/R injury model, whereas the opposite effects were shown in C1q-deficient mice, which were protected from edema formation and remote lung injury, but not from necrotic tissue injury [83]. Clinically, stroke patients with MBL deficiency experienced smaller infarct size and had a favorable outcome while receiving conservative treatment [84]. Also, the MBL null allele preserved epithelial cell integrity following intestine I/R injury in a case report [85]. This is in line with the finding C4 deficiency prevented tissue injury from skeletal muscle and intestinal I/R injury in mice [86, 87].

Factor B deficiency in mice was shown to protect kidneys from ischemic acute renal failure [88], sustain the subacute stage of infarct development in stroke [89], reduce inflammation in renal I/R injury [90], but not in intestinal I/R injury [91]. Factor H knock out mice did not have increased infarct volume and severe brain damage in a stroke model, indicating that factor H may not be involved in transient focal ischemia [92]. C3-deficient mice were protected from transient focal cerebral ischemia [82] and skeletal muscle I/R v [86].

Kyriakides et al [93] demonstrated that C5b-9 was critical in the development of skeletal muscle I/R injury by using C5-deficient mice. C5-deficient mice were also proven to be effective in reducing reperfusion injury in intestine [94]. It was reported that C3-, C5-, and C6-deficient mice were protected from renal I/R injury, while it was not the case for C4-deficient mice, underlining the importance of complement activation downstream of C3 [78]. However, mice deficient in C5 were not protected from tissue damage arising from transient focal cerebral ischemia [82].

In investigating the function of complement regulatory proteins, CD55 deficiency was reported to increase renal I/R injury in mice, and double deficiency of CD55/CD59 greatly exacerbated the extent of reperfusion injury [95]. In the meantime, CD59(-/-) alone was not detrimental to renal I/R injury [95]. Recently, Miwa et al demonstrated that deletion of C5aR, C3, fB, fP, C3aR, or C5aR attenuated renal I/R injury in DAF/CD59 double knockout mice, whereas C4, Ig, or MBL did not show efficiency [96]. This revealed that MAC and anaphylatoxins contributed to renal tissue injury via AP [96]. Xu et al showed that C5aR knockout mice were protected against intestinal I/R injury as well as neutrophil infiltration in the remote lung [97]. Peng et al also demonstrated a predominant role of C5aR by using C3aR, C5aR, or C3aR/C5aR deficient mice in renal I/R injury [98]. In contrast, C3aR deficiency was shown to have an opposing role in worsening intestinal I/R injury by constraining neutrophil mobilization in mice[99].

2.1.2 Complement-targeted therapeutics in ischemia/reperfusion injury

Based on the prominence of complement activation during I/R injury, it may be promising to use complement-specific therapeutics. Specific complement inhibitors, pooled plasma proteins, and monoclonal antibodies are often administrated to investigate these effects.

C1 INH, a member of the serpin (serine protease inhibitor) superfamily, was originally used for treatment of hereditary angioedema (HAE) which is due to the deficiency of functional C1 INH [100]. Later on, C1 INH was also applied to different organ I/R injury. For instance, C1 INH was shown to have beneficial effects in heart, liver, brain and skeletal muscle I/R injury [101-104]. It could also prevent organ transplantation-mediated reperfusion injury [105, 106]. In a clinical trial, administration of C1 INH showed its beneficial effects on attenuating renal dysfunction in patients with severe sepsis and septic shock [107]. Acting also at the very beginning of the complement cascade, a specific C1s inhibitor exerted cardioprotective effects in a rabbit model of myocardial I/R injury [108]. Moreover, administration of endogenous MBL/ficolin-associated protein-1 (MAP-1) was demonstrated to ameliorate myocardial I/R v through displacing MASP-1, MASP-2, and MASP-3 [109].

Soluble complement receptor 1 (sCR1) is a modified form of complement receptor 1, which lacks the transmembrane and cytoplasmic domains. It has the potential to inhibit I/R injury -mediated tissue damage due to its decay-accelerating activity for C3 and C5 convertases and co-factor activity for C3b and C4b degradation. Its effective benefits were manifested in myocardial infarction, liver and skeletal I/R injury [110-112], as well as lung transplantation [113]. Recombinant sCR15-18 domain of human sCR1 exerted cardioprotective and neuroprotective effects in myocardial and cerebral I/R injury [114, 115]. APT070 (Mirococept), a recombinant derivative of sCR1-3 domain of sCR1, was shown to be protective against myocardial, intestinal, and skeletal I/R injury [112, 116, 117]. However, APT070 was not able to attenuate reperfusion injury in our recently published lower extremity I/R injury study [118]. TP10, another soluble derivative of sCR1, was reported to inhibit inflammatory reactions in lung and renal transplantation [119, 120]. In a larger multicenter clinical trial including 564 high-risk patients undergoing cardiopulmonary bypass (CPB), TP10 effectively inhibited complement activation during the process, however, it did not reduce the incidence of primary endpoints (death, myocardial infarction, intra-aortic balloon pump support, and prolonged intubation) [121].

Inhibition of C5 with monoclonal antibodies was demonstrated to effectively reduce I/R injury in heart, brain, intestine, and kidney [75, 122-124]. Its protective effects were also seen in heart transplantation [125]. Pexelizumab (Alexion Pharmaceuticals, Inc.), one anti-C5 monoclonal antibody, is worthy of

discussion and review due to its extensive clinical trials. For example, Pexelizumab was demonstrated to significantly reduce the mortality and morbidity following acute myocardial infarction (AMI) or coronary artery bypass graft (CABG) in a prospective, randomized, double-blinded, placebo-controlled trial [126]. In a another clinical trial involving 914 patients undergoing coronary artery bypass grafting requI/R injuryng CPB, Pexelizumab reduced the risk of death or MI on post-operative day 4 and day 30 [127]. However, no protective effects were shown on primary endpoints (composite of death, new Q-wave, or non-Q-wave, myocardial infarction, left ventricular dysfunction, or new central nervous system deficits) [127]. Later on, it was found that Pexelizumab did not show its clinical benefits in overall analysis for major adverse events, death, MI, stroke, and heart failure in seven trials including 15196 patients with ST elevation MI and CABG [128]. However, it can reduce the risk of death in patients undergoing CPB grafting [128]. In 2011, a clinical phase II study: the COMMA trial and phase III study: the PRIMOCABG trial were performed in AMI and CABG patients. They accordingly commented that a mediational model should be used to investigate the effectiveness of Pexelizumab, which was affected by the baseline risk factors in patients [129]. Also Pexelizumab may be harmful to patients with ST-segment elevation myocardial infarction undergoing primary percutaneous interventions [130]. Considering the recent unconvincing clinical results of Pexelizumab recently, its continued use should be seriously questioned. However, the concept of anti-inflammation accompanying CPB may deserve further investigation [131]. In 2013, Eculizumab (SolI/R injurys, Alexion Pharmaceuticals, Inc.), another anti-C5 monoclonal antibody, showed its efficiency and safety in kidney transplantation [132, 133]. In 2015, Eculizumab was raised as a promising intervention in renal transplantation [134].

Cobra venom factor (CVF) [135] is isolated from cobra venom and is an analog of C3 in structure and function. It activates complement and thus depletes the activity of complement in serum. CVF was shown to alleviate myocardial necrosis after coronary artery occlusion [136], diminish remote lung injury following intestinal I/R injury in rats [137], and reduce cerebral infarct volume in adult rats [138]. Humanized CVF (HC3-1496), a non-immunogenic compatible CVF-like molecule, displayed its effective benefits in myocardial infarction [139]. Similarly, a C3a receptor antagonist was shown to reduce the extent of focal cerebral ischemia [82, 140]. An inhibitor of the AP, CR2-fH, was shown to be associated with better outcomes in ischemic stroke by preserving neurological function, decreasing infarct volume, and reducing inflammation [89].

2.2 The coagulation system

Blood clotting is normally activated to avoid the loss of blood volume during bleeding but can also be activated in the presence of inflammation like I/R injury [141-143]. Classically, there are two pathways for coagulation activation, the extrinsic pathway and the intrinsic pathway. They can be initiated by different molecules and converge at Factor X (FX). Most coagulation factors are serine proteases apart from the glycoproteins of factor V and factor VIII (FVIII), and the transglutaminase of Factor XIII (FXIII).

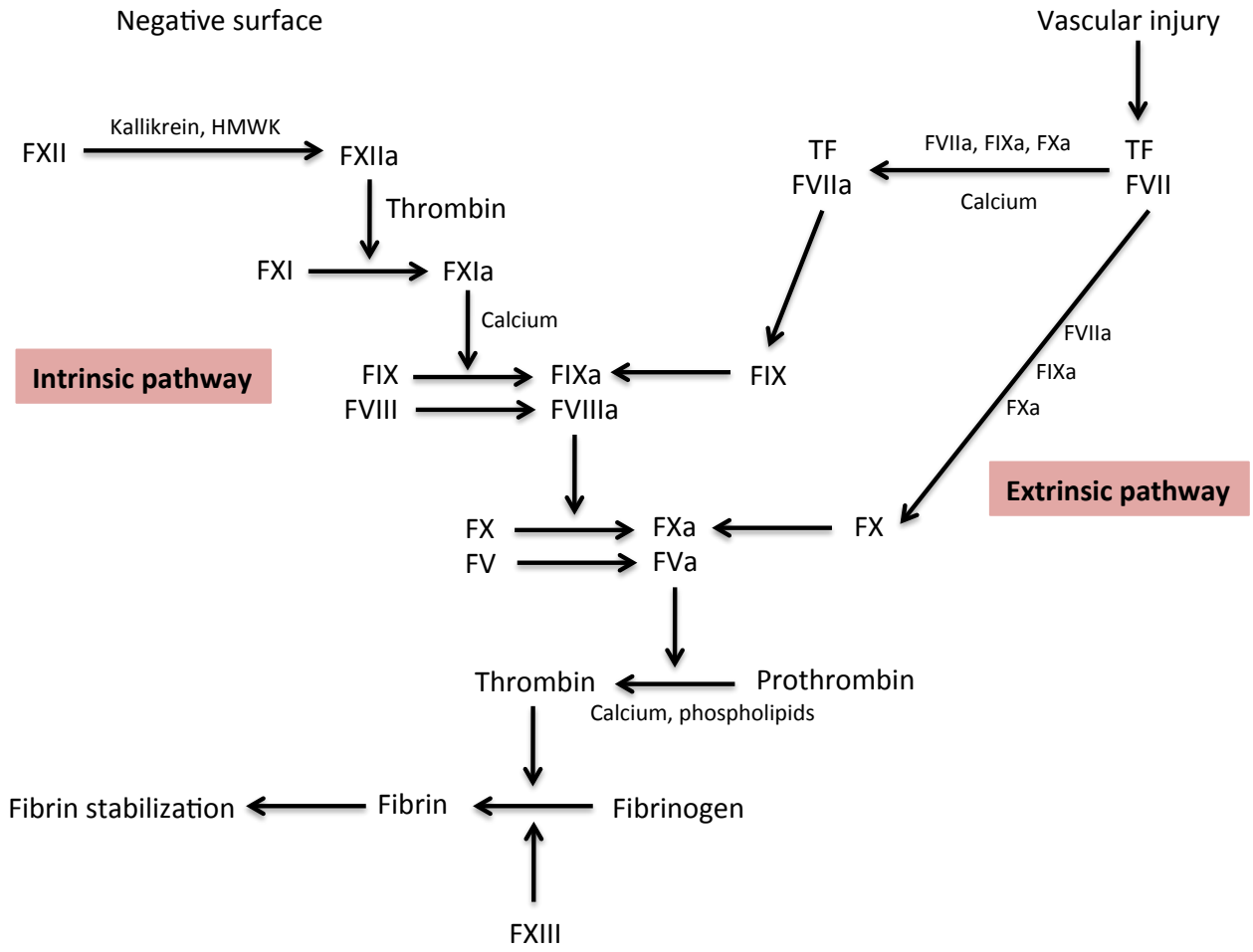


Figure 3: Schematic representation of the coagulation cascade. Adapted from [143].

The extrinsic pathway is initiated by tissue factor (TF), which is located in sub-endothelial cells in physiological conditions but can be exposed after vascular injury [144, 145], e.g. trauma, reperfusion

injury. After exposure, TF is activated and binds to circulating activated factor VIIa (FVIIa), leading to the formation of a TF-FVIIa complex [146]. Afterwards, this complex activates FX to FXa.

The intrinsic pathway is also called the contact activation pathway, which is activated by factor XIIa (FXIIa) due to auto-activation of FXII and triggered by contact with the activating surface [147, 148]. Activated FXIIa catalyzes conversion of factor XI (FXI) to FXIa, which then converts FXI to FIXa. FIXa catalyzes the proteolytic conversation of FX to FXa.

Following FXa formation, factor V (FV) is converted to FVa, leading to a FXa-FVa complex. In the presence of cofactors such as FVIIIa, calcium, and platelet phospholipids, FXa-FVa complex cleaves prothrombin to thrombin, which induces the conversion of fibrinogen to fibrin, ultimately leading to cross-linked fibrin clot formation with the aid of FXIII.

Under quiescent conditions, the body maintains homeostasis by regulation of the fibrinolytic system. This fibrinolysis mainly involves tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). These can cleave plasminogen into plasmin, which degrades fibrin into soluble fragments. Inhibitors of the fibrinolysis system include plasminogen activator inhibitor 1 and 2 (PAI-1 and PAI-2), as well as thrombin activatable fibrinolysis inhibitor (TAFI), which inhibit the activity of plasminogen and tPA. Endogenous anti-coagulants also contribute to the homeostasis, such as anti-thrombin III (ATIII), tissue factor pathway inhibitor (TFPI), protein C, protein S, and thrombomodulin. High levels of PAI-1 can bind more tPA to form a tPA/PAI-1 complex, in which bound tPA does not have any pro-fibrinolytic function [149]. A few studies [149-151] have shown that the tPA/PAI-1 complex may be a risk marker for thrombus formation. D-dimer, one of the fibrin degradation products containing two D fragments, is usually used as a marker to indicate a high thromboembolic state in clinical patients [152, 153].

2.2.1 Role of coagulation in ischemia/reperfusion injury

Accumulating studies have demonstrated that the coagulation cascades participate and play a prominent role in the pathogenesis of I/R injury. For example, in 1976, it was found that plasma levels of FVIII, FIX, and FXI were significantly increased following myocardial infarction, whereas values of FXII in plasma were significantly reduced in patients with sustained MI [154]. Also Vaziri et al et al reported that plasma levels of FXIIa and high molecular weight kininogen (HMWK) were decreased in patients with AMI and unstable angina, while FXI, FIX, fibrinogen, and D-dimer were elevated, implicating the activation of the intrinsic pathway [155]. The coagulation system is not only involved in myocardial ischemia or MI, but also in reperfusion injury in other organs. In 1998, activated TF was shown to contribute to the breakdown of microvascular circulation during hepatic I/R injury [156]. It was postulated that TF contributed to I/R injury via protease activated receptor 1 (PAR-1) signaling [157]. Production of fibrin contributed to microvascular occlusion and tissue damage in cerebral I/R injury [158]. Serum levels of PAI-1 started to increase at 6h of reperfusion, peaked at 24h of reperfusion, and then recovered gradually in renal I/R injury [159].

Mice deficient in coagulation factors are not as common as complement-deficient mice. TF deficiency protected mice from renal failure, mortality and tubular inflammation in a renal I/R injury model, and similar benefits were shown in PAR-1-deficient mice [157]. PAI-1 knockout mice had less fibrin deposition, mild histological changes, and a lower plasma level of cytokines and chemokines in lung I/R injury [160]. Ajay et al reported that fibrinogen deficiency protected against tubular injury, kidney dysfunction, necrosis, apoptosis, and inflammation in mouse kidney I/R injury [161]. PAR-2-deficient mice were protected from myocardial infarction and heart remodeling following cardiac I/R injury [162]. FXII-deficient mice that were subjected to transient cerebral ischemia had a reduction in infarct volume and fibrin deposition, which was also shown to be true in FXI-deficient mice [163]. Pham et al demonstrated that the recovery of cortical blood flow was better in FXII-deficient mice than wild type-mice after ischemic stroke [164].

2.2.2 Coagulation-targeted therapeutics for ischemia/reperfusion injury

Similar to the concept of complement-targeted therapeutics, also synthetic anticoagulant proteins and specific antibodies have been used to inhibit coagulation-related effects in I/R injury. Administration of anti-TF monoclonal antibody (mAb) led to a smaller infarct size by reducing thrombin-induced inflammation in a rabbit myocardial I/R injury model [165]. It was also described that anti-sense oligodeoxynucleotides for rat TF ameliorated hepatic I/R injury [166]. Anti-human TF mAb showed promising results in the treatment of distant lung injury induced by intestinal I/R injury in human TF knock-in transgenic mice, but not in wild type mice [167]. Finally, in a baboon transient focal cerebral ischemia model, intravenous treatment with anti-TF mAb TF9-6B4 significantly increased reflow in each class of microvessels [168].

Kleinschnitz et al demonstrated that mice treated with the FXII inhibitor PCK were protected from ischemic injury in stroke [163]. Inhibition of FXIIa with rHA-infestin-4 was shown to reduce ischemic damage and pathological coagulation after silent brain ischemia in mice [169]. However, the FXII inhibitor COU254 did not show any effects in acute ischemic stroke (AIS) [170]. Mice that received an antibody against FXII-mediated activation of FXI displayed a significant reduction in cerebral infarction, fibrin deposition, and neurological deficits after AIS [171]. There are fewer I/R injury studies concentrating on the use of TFPI. Yoshimura et al noted that TFPI decreased the activity of procoagulation and inflammation in liver I/R injury in rats [172].

It was reported that recombinant human, active site-blocked FVIIa effectively inhibited TF procoagulant activity and reduced the infarct size and no-reflow area in myocardial I/R injury in rabbits [173]. Again, active site inhibited FVIIa attenuated the extent of myocardial I/R injury in mice through the NF-κB pathway [174]. The selective FXa inhibitor DX-9065a reduced the levels of TF and monocyte chemoattractant protein-1 (MCP-1) in liver I/R injury [175]. In a randomized, double-blinded, placebo-controlled phase 2 study, the FXa inhibitor Apixaban significantly reduced the coagulation activity markers D-dimer and prothrombin fragment 1.2 (F1.2) in a dose-dependent manner in patients with acute coronary syndrome (ACS) [176]. The ATLAS ACS-2-TIMI 51 trial investigated the effects of Rivaroxaban, another inhibitor of FXa, and found it had a potential role in reducing the rates of cardiovascular death, MI, and stroke at the primary end point [177, 178]. However, use of other FXa inhibitors like Darexaban and TAK-442 did not improve outcomes for cardiovascular events such as MI, bleeding, stroke, and thrombolysis in recent large phase II trials of ACS [179, 180]. In addition, defibrinogenating rabbits with ancrod did not

effectively reduce infarct size following myocardial I/R injury, suggesting fibrin deposits do not make a significant contribution to infarct size [165, 181].

Accumulating studies have shown that anti-coagulants can reduce I/R injury. For example, low molecular weight heparin (LMWH) is routinely used in acute myocardial infarction patients for its safety and efficiency without significant increase in major hemorrhagic events [182, 183]. The potential of LMWH was also shown in I/R injury studies [184, 185], supporting its anti-inflammatory role. The beneficial effects of LMWH are also seen in stroke patients [186]. However, from the reviewed and meta-analysis-based papers, the standard use of LMWH in stroke patients still remains controversial [186, 187]. Inhibition of thrombin with hirudin reduced the infarct size in myocardial I/R injury [181]. Another anticoagulant protein, activated protein C (APC), was capable of reducing reperfusion injury to heart, kidney, and skeletal muscle [188-190]. In a murine model of focal ischemia, APC administration showed neuroprotective, anti-inflammatory, and anti-thrombotic effects [191]. Clinically, APC has only been tested in septic patients. However, its use is not recommended in severe sepsis due to its clinical inefficiency [192, 193]. Similary, ATIII was shown to reduce reperfusion injury in various organs [194-196].

Aprotinin, a non-specific serine protease inhibitor, displayed a broad anti-inflammatory action in preclinical I/R injury models [197-199]. However, in a clinical trial involving 4373 patients undergoing revascularization, aprotinin application was associated with high risks of MI, heart failure, stroke, or encephalopathy, which limited its continued clinical use [200]. Nevertheless, it was apparent that the benefits of aprotinin outweighed its risks in CABG patients worldwide [201].

2.3 The kinin system

The kinin system was discovered at the beginning of the nineteenth century. Due to its duality and complexity, it started to attract attention only 60 years ago. The central component of this system is bradykinin, which is mainly generated from two types of precursors, tissue and plasma kallikrein (KK) .

The kinin system is part of the contact activation system, which is initiated by FXII (Figure 4). FXII can be activated by contact with negatively charged surfaces such as glass, kaolin, silica, ellagic acid, dextran sulfate, over sulfated chondroitin sulfate and nanoparticles *in vitro* [202, 203]. Endogenous (patho)physiological activators of FXII have also been reported *in vivo*, such as polyphosphate, collagen, misfolded protein aggregates, lipopolysaccharides, glycosaminoglycans, nucleic acids, and phosphatidylserine [203-205]. On contact with these molecules, FXII is converted into FXIIa, which converts prekallikrein (PK) into KK. This subsequently cleaves HMWK, leading to the release of the vasoactive peptide bradykinin. In turn FXII can be further activated by KK and FXIIa thereby forming a positive feedback loop [203], which explains the massive amplification of protein activation that occurs in this system.

Under normal situations, about 75% of PK is bounded noncovalently to HWMK in a 1:1 ratio to form the PK-HMWK complex, which is stabilized by C1 INH [206]. The rest is free in the circulation.

There are two receptors for bradykinin (BK) in the body, bradykinin receptor 1 and 2 (BR1 and BR2), the binding to which can regulate blood pressure, increase vascular permeability, cause vasodilation, and mediate inflammation [207]. BR2 is constitutively present in many tissues, while BR1 is only expressed in response to proinflammatory stimuli [207, 208]. In vivo, bradykinin has a short half-life of about 17s [209], due to its rapid degradation by angiotensin-converting enzyme, carboxypeptidase N, neutral endopeptidase, and aminopeptidase P [210]. Laine et al reported that BK1-5 is a stable metabolite [211].

Introduction – Kallikrein/kinin

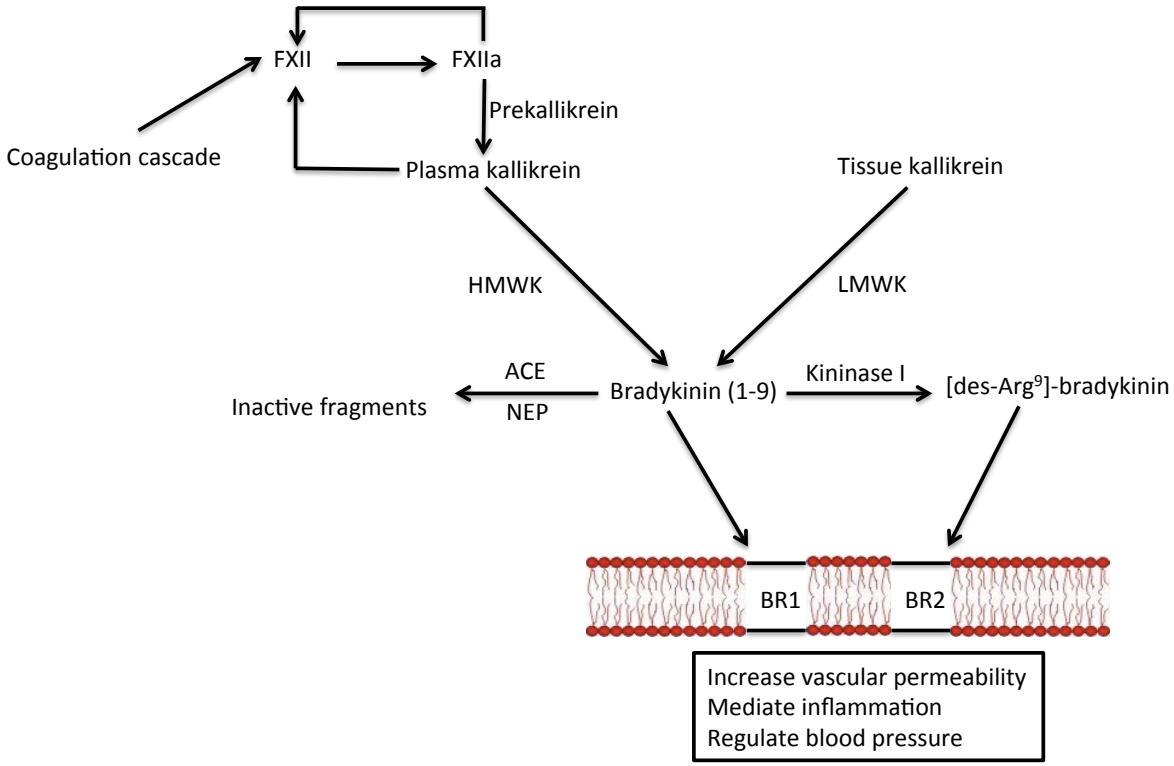


Figure 4: Schematic presentation of the kallikrein/kinin system. HMWK: high molecular weight kininogen; BK: bradykinin; BR: bradykinin receptor; ACE: angiotensin-converting enzyme; NEP: neutral endopeptidase;

2.3.1 Role of the kinin system in ischemia/reperfusion injury

The role of the kallikrein/kinin system in I/R injury has been defined in ischemic stroke and traumatic brain injury and is eloquently described in a review paper [212]. FXII-deficient mice showed a defective formation of thrombi revealed by intravital microscopy and blood flow measurements [213]. It was shown that FXII deficiency suppressed the production of bradykinin in plasma stimulated by contact with an artificial surface [214]. Cheng et al reported that FXII or FXI deficiency significantly inhibited thrombosis formation in the $FeCl_3$-induced carotid artery injury model [215]. FXI deficiency also showed antithrombotic efficiency in a $FeCl_3$-induced vena cava thrombosis model [216]. Depletion of plasma PK or FXII using antisense oligonucleotide (ASO) technology reduced thrombosis formation without affecting homeostasis [217]. However, congenital PK-deficiency does not protect patients from thrombotic events [218, 219]. Pons reported that tissue KK-deficient mice displayed an increased risk of mortality rate and left ventricular hypertrophy and dilation [220]. In contrast, Gob et al reported that plasma KK deficiency played a protective role in ischemic stroke by reducing thrombus formation and inflammation [221]. In a multicenter case-reported study with 1268 stroke patients, plasma levels of tissue kallikrein was found to be low, which was negatively correlated with the first-ever stroke and was an independent predictor for stroke recurrence [222]. Langhauser et al demonstrated HMWK-deficient mice were protected from brain infarction, neurologic deficits, thrombus formation, and inflammation in an ischemic stroke model [223].

Souza et al demonstrated that BR1 deficiency was more efficient in providing anti-inflammatory and anti-lethality effects than the absence of BR2 or BR1/BR2 following intestinal I/R injury in mice [224]. This study also found that BR2 was a major driving force for BR1 activation, and may have a protective role in I/R injury that depends on its vasodilatory function [224]. The beneficial effects of BR1 deficiency were also seen in kidney and myocardium [225, 226]. Nonetheless, Kakoki et al reported Both BR1/BR2-deficient mice had the highest mortality rate and displayed the most severe degree of inflammation, apoptosis, and histological and functional changes during renal I/R injury, which contradicted the above studies [227].

In stroke, BR2 knockout mice showed a reduction in infarct volume and edema formation, as well as better motor function and longer survival time as compared with the wild type mice [228]. However, Austinat et al demonstrated that BR2 deficiency did not provide neuroprotection in a transient middle cerebral artery occlusion (tMCAO) model [229]. Whereas, it was BR1 knockout mice that were protected from brain infarction, neurological deficits, tissue edema, and inflammation in stroke [229].

2.3.2 Kinin system–targeted therapeutics in ischemia/reperfusion injury

In transient focal cerebral ischemia, a BR1 inhibitor dose dependently showed a significant reduction in infarct volume [229]. In contrast, a BR2 inhibitor did not change the outcome of stroke in mice [229]. Pretreatment with the BR1 antagonist R-954 selectively showed anti-inflammatory effects following renal I/R injury, compared with treatment with the BR2 antagonist HOE-140 [35]. Another inhibitor of BR1, BI-113823, was also demonstrated to improve the cardiac function and attenuate the proinflammatory reactions after myocardial infarction [230]. Blockade of BR1 with (des-Arg^{9}, Leu^{8})-bradykinin reduced the infarct size in the Langendorff-perfused myocardial I/R injury model [231]. However, no protection was conferred using the BR1 antagonist B9958 in an experimental model of myocardial infarction in rats [232].

The BR2 antagonists CP-0597 effectively reduced the ischemic hemisphere size and infarct volume in stroke in rats [233]. The protective effects of the BR2 antagonist FR173657 were similarly confirmed in small intestinal I/R injury and pulmonary I/R injury in dogs [234, 235] as well as in a canine lung transplantation model [236]. It was shown that the BR2 antagonist HOE-140 protected mice from inflammatory responses and lethality in an intestinal I/R injury model [224]. However, the efficiency of HOE-140 was not proven in lung I/R injury [237], nor in a rat hind limb I/R injury model [238, 239]. Similarly, the nonselective BR1/BR2 antagonist B9430 did not reduce the mortality after global cerebral ischemia even through it inhibited leukocyte-endothelium interaction[240].

Gene delivery of tissue kallikrein was shown to protect against myocardial infarction and stroke in rats [241, 242]. It was reported that inhibition of plasma kallikrein with DX-88 reduced brain infarct volume, edema formation, and neurological deficits following transient focal brain ischemia [243].

Few clinical studies have focused on bradykinin receptors. In a double-blind, crossover trial, HOE-140 did not show any protective effects in forearm I/R injury with preconditioning [244]. This suggested that endogenous BR2 did not play a major role in I/R injury or remote ischemic preconditioning in man (Clinical trial registration information: NCT00965120 and NCT00965393) [244]. In another randomized clinical study including 115 patients with CPB, HOE-140 decreased the capacity of intraoperative fibrinolysis (higher PAI-1 to tPA molar ratio), suggesting a contribution of endogenous BK to tPA generation during CPB [245]. However, HOE-140 did not reduce plasma levels of D-dimer or postoperative need of blood transfusions [245]. So far, discrepancies relating to the beneficial and detrimental effects of BRs still exist in the field of cardiovascular diseases. Therefore, more experimental studies need to be conducted to address the issues surrounding BR-targeted therapeutics [246].

2.4 Interactions of the plasma cascade systems

Elements of both the complement and coagulation cascades belong to the serine proteases of the chymotrypsin family, sharing structural and functional similarities [247]. They exist as inactive zymogens and are activated by upstream proteases [247]. Considering their common origin [248, 249], an extensive crosstalk between the complement and the coagulation systems has been unmasked. It was for example reported that MASP-1 had thrombin-like activity and promoted blood clotting by cleaving FXIII and fibrinogen [250, 251]. MASP-1 also interacts with the kallikrein/kinin system by cleaving HMWK and releasing BK [252]. Moreover, MASP-1 participants in the alternative pathway by converting pro-factor D to the active form of factor D [253]. However, a recent study reported that MASP-3, instead of MASP-1 and MASP-2, is able to activate pro-factor D [254]. Furthermore, activation of MASP-2 can lead to fibrin clot formation, which bridges the complement and coagulation systems [255, 256].

The anaphylatoxin C3a was also demonstrated to activate platelets to potentiate coagulation [257]. Similarly, platelet activation was induced by MAC by exposure of procoagulant lipids and the secretion of granules from platelet cytoplasm [258, 259]. It was reported that C5, C5a, and the inactive form of MAC increased the expression of TF in leukocytes and ECs which links the complement and coagulation systems [260-262]. Amara et al demonstrated that thrombin, FXIa, FXa, FIXa, and plasmin effectively cleaved C3 and C5, demonstrating the intercommunications between the coagulation and fibrinolysis cascades and the complement system [263]. However, TF, FVII, FVIIa, and activated protein C did not affect the cleavage of C3 and C5 in this study [263].

Nevertheless, the coagulation proteinases potentiate the complement cascades. It was shown that thrombin cleaved C5 into C5a in C3-deficient mice, suggesting a new complement pathway for C5a production that is mediated by thrombin [264]. FXIIa activated C1 to initiate activation of the classical complement pathway [265, 266]. Activated platelet surfaces were shown to bind to C1q, C4BP, and fH through chondroitin sulfate mediation, while binding to C1 INH was C1q-dependent [267]. It was observed that alpha- and beta-chains of fibrinogen and fibrin were able to bind to rMBL-A and rMBL-C to augment the activity of the lectin pathway [268].

Further, crosstalk between the complement, coagulation, and fibrinolysis cascades has been identified. Wojta et al demonstrated that incubation of rhC5a with human mast cells and primary blood basophils led to an increased level of PAI-1 in a dose- and time- dependent manner, which inhibited the enzymatic activity of tPA [269]. Treatment of mast cells with C5a led to higher expression of PAI-1, facilitating

healthy mast cells conversion from a resting profibrinolytic state to an activated prothrombotic phenotype [270]. With the polymerization of C9 to C5b-8 complex, plasminogen was induced to bind to membrane

C9 and enhanced its activity by tPA [271]. Keizer et al showed that the Kunitz-2 domain of TFPI inhibited the activity of MASP-2, suggesting the use of TFPI in I/R injury might be an interesting therapeutic approach.

Intercommunication also exists between the kinin and complement cascades. Wiggins et al showed that incubation of plasma kallikrein with rabbit C5 led to chemotactic C5a production [272]. Plasma kallikrein was able to cleave fB in a similar pattern to fD to activate the alternative pathway [273, 274]. The receptor gC1qR/p33 for C1q played an essential role in mediating the generation of BK and related kinins [275, 276].

As for the interactions between the coagulation and kallikrein/kinin systems, in fact, they share the common initiator FXIIa. This is a group of plasma proteins including coagulation FXII and FXI, KK and high molecular weight kininogen (HMWK). Puri et al demonstrated that thrombin induced platelet aggregation in plasma, which was mainly inhibited by HMWK [277, 278]. BK and its metabolites were confirmed to be selective inhibitors of α-thrombin-induced platelet aggregation [279, 280]. It was shown that mutant APC with minimal anticoagulant activity preserved the anti-inflammatory carboxypeptidase activity of TAFI by decreasing the production of BK in plasma [281] (Figure 5).

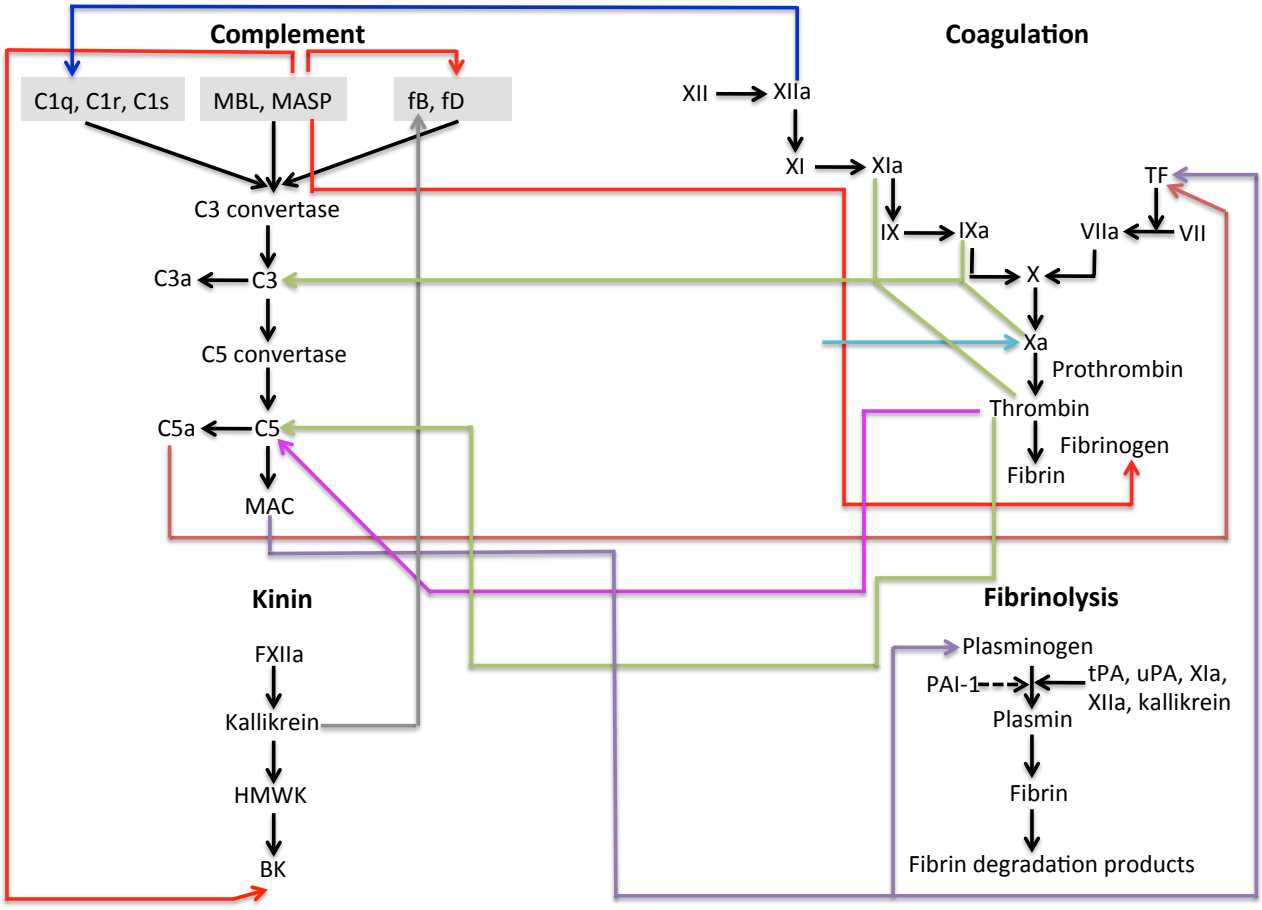


Figure 5: Interactions of the plasma cascade systems. The plasma cascade systems involve complement, coagulation, kallikrein/kinin and fibrinolysis. They are highly interactive and interconnected. fB: factor B; fD: factor D; HMWK: high molecular weight kininogen; BK: bradykinin; tPA: tissue plasminogen activator; uPA; urokinase plasminogen activator;

3. Role of endothelial cell activation in ischemia/reperfusion injury

3.1 Endothelial cell barrier

The endothelium forms a physical barrier that separates blood from tissue. This barrier is a continuous semipermeable membrane rather than simply a passive one. It has long been known to act as a gatekeeper and plays a pivotal role in exchanging fluid and electrolytes, controlling inflammation and coagulation, regulating vascular tone, and maintaining hemostasis in equilibrium. These vital functions depend on the intact structure of the glycocalyx layer.

This endothelial glycocalyx layer, whose "backbone" molecules are proteoglycans and glycoproteins, is decorated by a pyramid of membrane-bound and soluble molecules [282, 283]. The major proteoglycans are syndecan and glypican families. They have long unbranched glycosaminoglycan (GAG) side-chains that covalently link with heparan sulfate (HS), as well as chondroitin sulfate, dermatan sulfate, and keratin sulfate. HS has been shown to bind to many plasma proteins via HS binding sites, such as SOD, thrombomodulin, AT-III, TFPI, C1 INH, and IL-8, which gives ECs a natural phenotype of anti-inflammation and anti-coagulation [284]. Also, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and hepatic growth factor (HGF) are present in the glycocalyx and HS regulates their transport and effector functions [285]. In addition, hyaluronic acid is also linked with GAGs (Figure 6).

Glycoproteins are composed of short branched carbohydrate side-chains comprising endothelial adhesion molecules and components of the coagulation and fibrinolytic systems [282]. Three families of selectins, integrins, and immunoglobulins comprise the cell adhesion molecules. The immunoglobulins mainly include intravascular adhesion molecule 1 and 2 (ICAM-1 and -2), vascular cell adhesion molecule 1 (VCAM-1), and platelet endothelial cell adhesion molecule 1 (PECAM-1 or CD31) [282]. They can be upregulated to mediate inflammatory cell recruitment and cell signaling in vascular injury with the aid of thrombin, interleukin-1 (IL-1), and tumor necrosis factor α (TNF-a) [286, 287]. Glycoprotein Ib-IX-V complex is a good example of a component of the coagulation and fibrinolysis system, which is the platelet receptor for von Willebrand factor (vWF) and P-selectin, involved in maintaining hemostasis [282, 288].

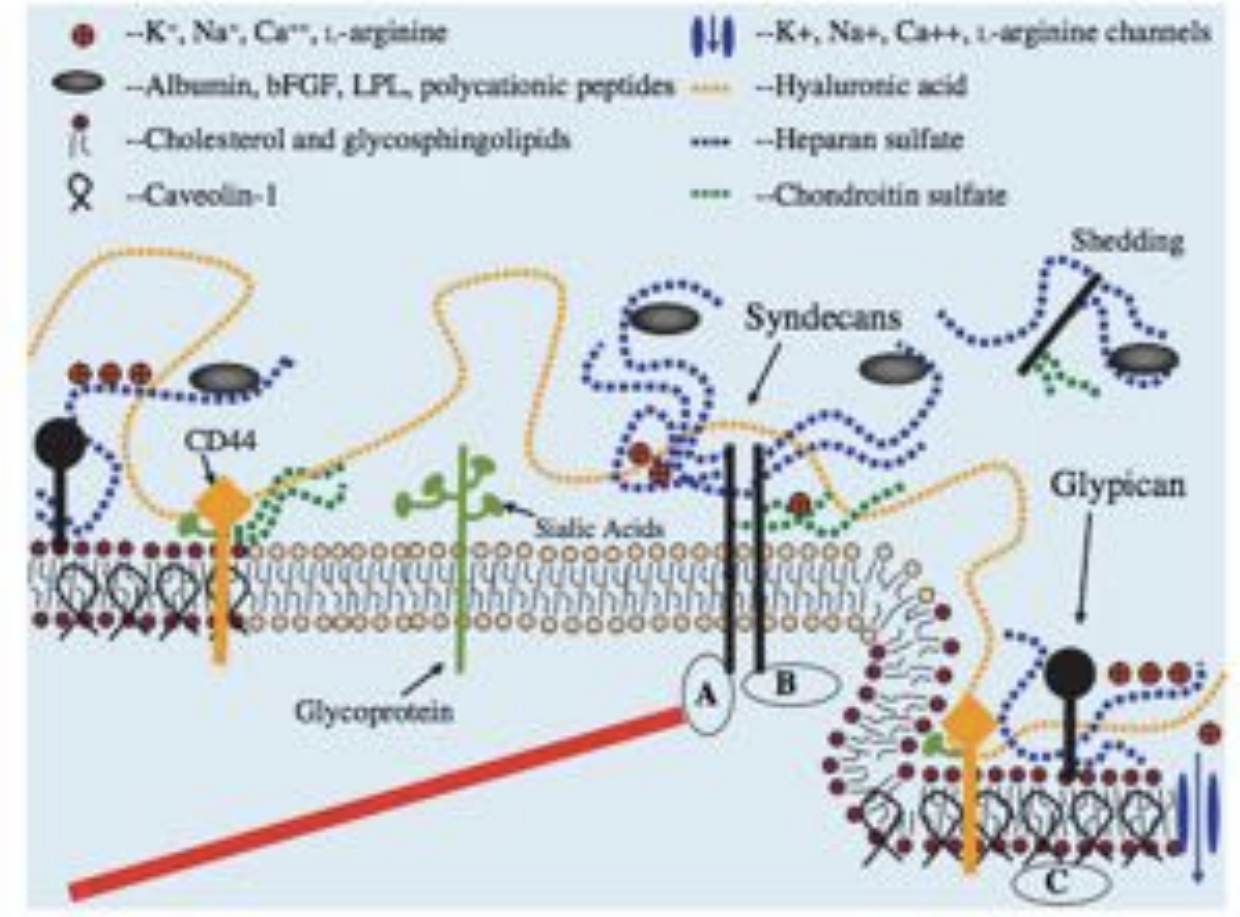


Figure 6: A cartoon representation of proteoglycans and glycoproteins on the surface of endothelial cells. Caveolin-1 associates with regions high in cholesterol and sphingolipids in the membrane (darker circles, left), and forms cave-like structures, caveolae (right). Glypicans, along with their heparan sulfate chains (blue dotted lines) localize in these regions. Transmembrane syndecans are shown to cluster in the outer edge of caveolae. Besides heparan sulfate, syndecans also contain chondroitin sulfate, lower down the core protein (green dotted lines). A glycoprotein with its short oligosaccharide branched chains and their associated SA 'caps' are displayed in the middle part of the figure (green). Hyaluronic acid or hyaluronan is a very long glycosaminoglycans (orange dotted line), which weaves into the glycocalyx and binds with CD44. Transmembrane CD44 can have chondroitin sulfate, heparan sulfate and oligosaccharides attached to it, and localizes in caveolae. Plasma proteins (grey), along with cations and cationic amino acids (red circles) are known to associate with glycosaminoglycans. (A) The cytoplasmic domains of syndecans link it to cytoskeletal elements (red line). (B) Oligomerization of syndecans helps them make direct associations with intracellular signalling effectors. (C) A series of molecules involved with endothelial nitric oxide synthase signalling localize in caveolae. Adapted from [283].

Not only the endothelial cell glycocalyx plays an important role in maintaining the integrity of the barrier, but also the interendothelial cell junctions. They include adherens junctions (AJs), tight junctions (TJs), gap junctions (GJs) [289]. They are often intermingled with the junctional structure and play a fundamental role not only in maintaining tissue integrity but also vascular permeability, intercellular communication, leukocyte extravasation, and angiogenesis [289]. For example, VE–cadherin, is one of the predominant AJs [290], while other cadherins like T-cadherin, and Pcdh12 [291, 292] are located in EC junctions. Claudins are the major transmembrane components of TJs by forming TJ strands [293]. Occludin can increase the TJ barrier function although not necessary for TJ strand formation. The junctional adhesion molecules (JAMs) colocalize with TJs but are not involved in TJ strands [294]. Nectin was found associated with both AJs and TJs, while PECAM, Pcdh12, CD99 and S-endo-1 are still unknown heterophilic ligands. Both TJs and AJs are linked to the actin cytoskeleton, and VE–cadherin may also be associated with vimentin filaments in some vascular beds. Connexins participate in gap junctions [295] (Figure 7).

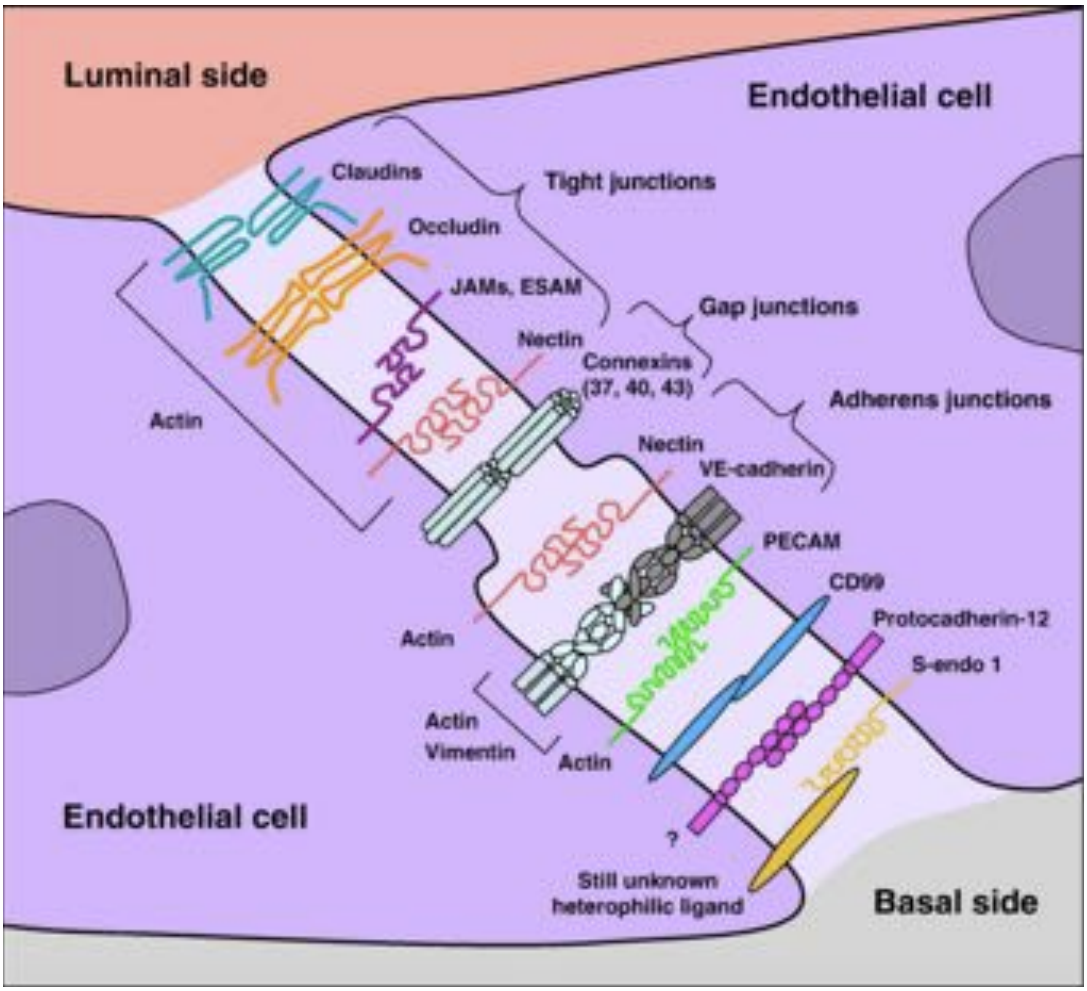


Figure 7: Adhesive proteins within the interendothelial cleft. Claudins, occluding, JAMs and ESAM are located in TJs, VE–cadherin in AJs and connexins in gap junctions. Nectin was detected in both AJs and TJs, while PECAM, Pcdh12, CD99 and S-endo-1 are outside of these structures. TJs and AJs are both linked to the actin cytoskeleton and VE–cadherin may also be associated with vimentin filaments in some vascular beds. Adapted from [289].

3.2 Endothelial cell activation

The EC barrier can be activated by pathogen and non-pathogen induced inflammatory stimuli. The latter is known as the sterile inflammation and occurs in the context of I/R injury. There are two stages of EC activation, endothelial stimulation (an early event) and endothelial activation (a later event), respectively [296]. In the first stage, EC activation is referred to as type I EC activation. It is characterized by the release of von Willebrand factor (vWF) and IL-8, as well as the shedding of heparan sulfate proteoglycan, which does not depend on gene transcription or de novo protein synthesis [297-299]. ECs are transiently retracted from each other to increase intracellular gap formation [300]. In the second stage, EC activation depends on gene transcription and de novo protein synthesis. The expression of adhesion molecules E-selectin, P-selectin, ICAM-1, and VCAM-1 is upregulated and promotes leukocyte-endothelial, leukocyte-platelet, and endothelial-matrix interactions. Other proinflammatory cytokines/chemokines including IL-1α, IL-1β, IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-1 α (MIP-1) are also synthesized and secreted. Meanwhile, procoagulant activity is enhanced with a higher expression of PAI-1 and TF [296, 301]. Reversible EC activation, corresponding to type I and II, does not alter the endothelial integrity. However, irreversible endothelial activation leads to decreased integrity of the endothelial barrier, endothelial dysfunction, and cell death [302] (Figure 8).

Moreover, accumulating studies confirmed endothelial activation in inflammatory environments. Shedding of the glycocalyx in I/R injury was first demonstrated by Czarnowska et al [36]. Later, Rehm et al reported an elevated plasma concentration of syndecan-1 and heparan sulfate in patients after cardiac ischemia on CPB [303]. Endothelial glycocalyx shedding was also documented in trauma patients [304], I/R of isolated guinea pig hearts [305], and vascular inflammation in rats [306]. In our hind limb I/R injury study, we also found the glycocalyx layer was largely shedded compared to our normal controls (own study, paper II of this thesis, unpublished).

Devaraj et al reported that incubation of human aortic endothelial cells with CRP resulted in an elevated secretion and activity of PAI-1 in a time- and dose-dependent manner [307]. This was novel and had implications for the underlying mechanism of the metabolic syndrome and atherothrombosis [307]. TF in the circulating microparticles was demonstrated to be elevated in heart transplantation patients, which was induced by activated ECs during allograft rejection [301]. The elevated level of PAI-1 favors fibrin clot formation. We had consistent PAI-1 expression in reperfused skeletal muscle tissue from the vehicle control group as reported here (own study, paper II and paper III of this thesis, unpublished).

Furthermore, adhesion molecules were shown to be associated with inflammatory EC activation in dilated, congestive and ischemic cardiomyopathy [308, 309]. Expression of adhesion molecules was also evident following the immunological changes of endothelial cell activation in inflammatory bowel disease [310].

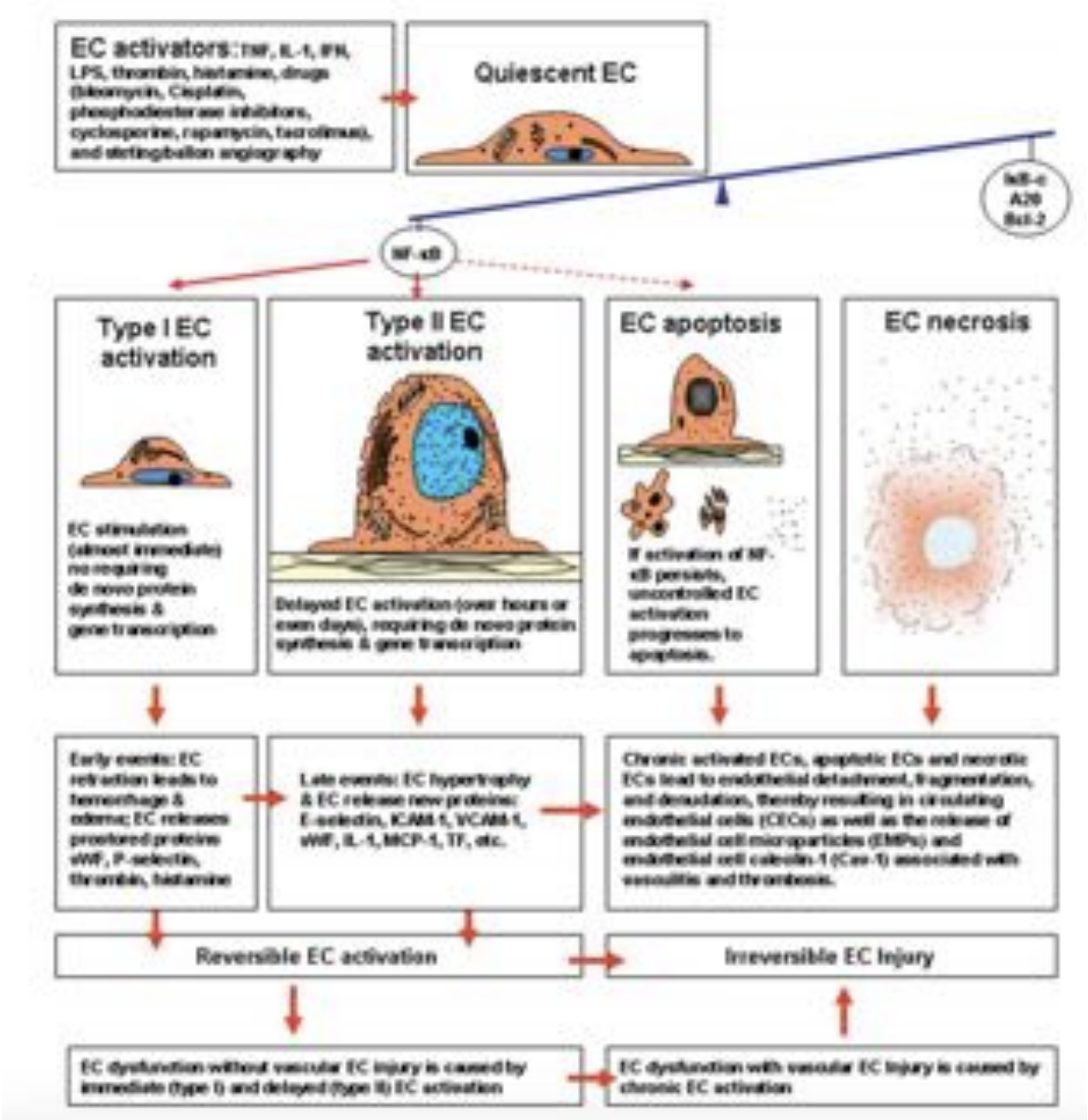


Figure 8: Schematic representation of the relationship of endothelial cell (EC) activation, EC apoptosis, EC dysfunction, and EC injury. Endothelial cell activation and EC apoptosis are strictly controlled through the expression of a set of protective genes (IkB-a, A20, Bcl-2) that down-regulate the expression of the transcription factor NF-kB. Activation of NF-kB triggers EC activation and renders the endothelium more susceptible to apoptosis. Activated ECs enhance expression of specific gene products (i.e., proteins), with resultant new capacities and new functions in inflammation, coagulation, and immunity, but no visible evidence of EC injury is present. The EC activation process, if uncontrolled, can progress to EC apoptosis. Endothelial cell activation is distinct from EC injury; however, the two phenomena are likely to overlap in the activation process. Endothelial cell activation may be associated with vascular EC injury in the development of vasculitis, thrombosis, and other vascular diseases that are mediated by leukocyte adhesion molecules, vasoactive mediators, pro-inflammatory cytokines, chemokines, and procoagulant molecules released by activated ECs. Type I EC activation (immediate event) and Type II EC activation (over hours or days) are reversible when EC activators are withdrawn, whereas chronic EC activation (over months) leads to EC injury with EC detachment from the underlying basement membrane and denudation of the vessel wall, resulting in circulating endothelial cells (CECs) and the release of EC microparticles (EMPs) and EC caveolin-1 (Cav-1) from the plasmalemmal membranes. TNF, tumor necrosis factor; IL-1, interleukin-1; IFN, interferon; LPS, lipopolysaccharide. Adapted from[302].

The interactions may be mediated by enhancing the activity of granulocytes and lymphocytes as well as interactions of lymphocyte-platelet and lymphocyte-endothelium.

Damaged EC also lead to the activation of the complement system. In 1986, Seifert et al demonstrated that ischemic EC injury resulted in complement activation [311]. Also, apoptotic human umbilical vascular endothelial cells (HUVEC) mediated the activation of AP in the complement system [312]. Another study reported that apoptotic ECs, induced by hypoxia/reoxygenation, triggered the activation of the complement system by binding C1 and contributed to the inflammatory responses [313]. It was shown that contact activation of ECs was FXIIa-dependent and zinc-dependent through binding to gC1qR [314, 315]. Based on this, Ghebrehiwet et al proposed that at the site of vasculitis and atherosclerosis, expression of gC1qR on activated ECs may be enhanced and lead to high-affinity of C1q and HMWK binding, as well as possible activation of the coagulation cascades and the generation of BK [315].

Collectively, EC activation occurs in an inflammatory environment and in turn intensifies the extent of inflammation as a consequence of the activation of plasma cascades as well as proinflammatory cytokines and chemokines.

3.3 Role of the plasma cascades on endothelial cell activation

Once the plasma cascades are triggered, they act on ECs and augment damage. Excessive complement deposits on the endothelium increase vascular permeability, potentiate endothelial procoagulant activity, and stimulate the release of cytokines and chemokines. For example, the binding of C1q to its receptors cC1qR and gC1qR on endothelium augmented the expression of proinflammatory adhesion molecules, leading to the activation of ECs mainly through NF-κB signaling [296, 316]. The cooperation of C1q receptors and β-integrins was required to mediate EC adhesion and spreading in a C1q dose dependent manner [317, 318]. Anaphylatoxin C5a increased the expression of P-selectin on ECs [319]. Similarly, C5b-9 coordinated with TNF-α to stimulate the expression of E-selectin and ICAM-1 [320]. Sublytic concentrations of C5b-9 were shown to induce the release of IL-8 and MCP-1 [321]. C5b-9 also stimulated ECs via a higher secretion of vWF and new surface expression of intracellular granule membrane protein GMP-140 [322]. Furthermore, C5b-9 was confirmed to induce TF expression and enhance endothelial procoagulation activity [323].

Procoagulant activity also contributes to EC activation. Cell surface expression of TF was induced by TNF-α and played an important role in increasing the permeability of the endothelial barrier, as well as the down-regulation of VE-cadherin expression [324]. It was revealed that an accumulated expression of TF on ECs induced cell apoptosis via P38 and P53 pathways [325]. Masanobu et al demonstrated that TF was significantly expressed in liver sinus ECs from the rejected grafts in a clinical liver transplantation study [326]. Laposata et al reported that thrombin induced gap formation in the confluent monolayer of HUVECs, while bradykinin, C3a, and C5a did not affect the integrity of ECs [327]. Later on, it was found that the ability of thrombin-induced EC activation was enhanced in the presence of TNF-α through NF-κB signaling [328]. And thrombomodulin ameliorated atherosclerosis and neointima formation through inhibiting thrombin-induced EC activation in a carotid ligation model [329]. It was reported that platelets adhered to endothelium induced by thrombin, leading to enhanced endothelial procoagulant activity [330]. Li et al demonstrated that adhesion of activated platelets to thrombin-induced human saphenous vein ECs (HSVEC) was mediated through glycoprotein GPIIb/IIIa [331]. For the effects of the fibrinolysis system on EC activation, it was reported that incubation of plasminogen and urokinase-like plasminogen activator (uPA) with confluent HUVECs caused a retraction of the monolayer and increased the permeability [332].

The kallikrein/kinin system also acts on EC activation. BK and kinin metabolites were demonstrated to mediate the adherence of monocytes to ECs accompanied with increased expression of ICAM-1 and CD11b, contributing to EC activation [333]. Carl et al demonstrated that BK increased the permeability of EC monolayers via BR1 and BR2 as well as elastase released by activated polymorphonuclear cells [334]. This BK-induced endothelial leakage was confirmed to be transcellular passage instead of paracellular diffusion [335] (Figure 8).

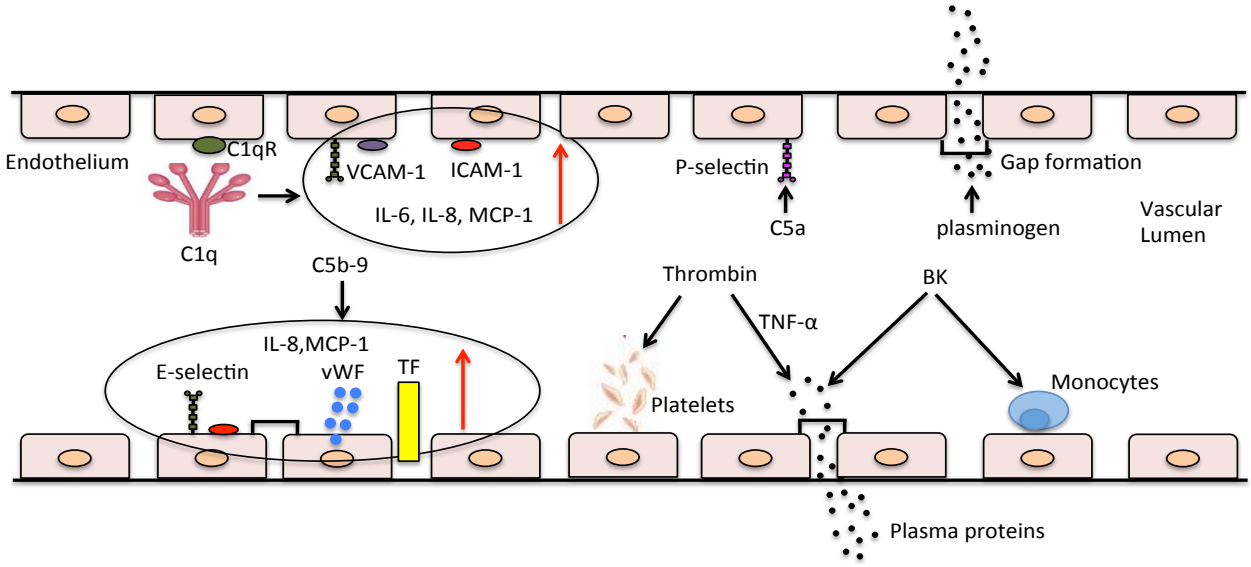


Figure 8: Role of the plasma cascade systems on endothelial cell activation. C1qR: C1q receptors; VCAM-1: vascular cell adhesion molecule 1; ICAM-1: intracellular adhesion molecule 1; MCP-1: monocyte chemoattractant protein-1; vWF: von Willebrand factor; TF: tissue factor; TNF-α: tumor necrosis factor-α; BK: bradykinin. Schematic illustration is not drawn to scale.

3.4 Combined inhibition of plasma cascades and endothelial cell activation

Based on the reviewed data and our work, here we put forward a novel concept to attenuate I/R injury by means of simultaneous inhibition of the plasma cascades and EC activation. C1 INH, low molecular weight dextran sulfate, AT III, as well as combined inhibitory approaches, will be discussed.

C1 INH was first discovered in 1957 [336]. Twenty years later, it was demonstrated that C1 INH inactivated C1r and C1s proteases from the first complement component C1 [337, 338]. Wuillemin et al found that the inhibitory activity of C1 INH on C1s was increased by 6-130 fold in the presence of GAG [284]. Recently, C1 INH has been shown to inhibit MASP-2 50-fold faster than C1s, implying MASP-2 is a major physiological target for CI INH [339]. Nielsen et al first reported that supraphysiologic concentrations of C1 INH were able to inhibit the activity of three complement pathways in a different inhibition pattern [340]. Also C1 INH interacted with C3b to prevent fB from binding to C3b and thus inhibited the activation of AP [341].

In fact, C1 INH not only regulates the complement system, but also the contact system. Jansen et al demonstrate that C1 INH treatment significantly reduced the plasma level of FXIIa and prekallikrein in a primate sepsis model [342]. Previous studies also showed that C1 INH was a major inhibitor of the human plasma KK [343, 344]. The full inhibition of plasma kallikrein by C1 INH explained the activation of plasma KK in HAE patients [345]. The role of C1 INH in coagulation is also evidenced in vivo in that FVIIa, prothrombin fragments F1 + 2, and thrombin-antithrombin complex (TAT) are significantly elevated and recovered to normal in remission [346, 347]. Kusumam et al found that HAE patients treated with nanofiltered C1 INH had a reduced level of FXIIa, kallikrein, plasmin, and bradykinin in plasma [348].

The fibrinolytic system is also affected by C1 INH. The complex of tPA/PAI-1 is used as a marker for investigating the effects of C1 INH on tPA inhibition. Huisman et al demonstrated that a higher plasma level of tPA/C1 INH complex was detected in patients with venous occlusion and peritoneal inflammatory disease, indicating an inhibitory activity of C1 INH on the fibrinolytic cascade [349]. It was also reported that C1 INH in its native form was able to inhibit the activity of plasmin without degradation [350].

In addition, C1 INH provides EC protection from I/R injury. C1 INH prevented the release of HS into the circulation in a dose dependent manner in an in vitro model of xenograft hyperacute rejection [351]. Our previous lower extremity I/R injury study [118] showed that 50IU/kg C1 INH preserved the expression of HS in the reperfused muscle tissue. However, in our current hind limb I/R injury study, we found 100IU/kg

C1 INH protected the endothelial HS layer, but not 50IU/kg C1 INH (own study, paper II of this thesis, unpublished). Cai and Davis reported that C1 INH interfered with leukocyte-EC interaction by binding to P- and E-selectin, which may confer a certain degree of EC protection by C1 INH [352].

Furthermore, C1 INH has anti-inflammatory functions that are independent of serine protease inhibition. Zeerleder et al reported that C1 INH administration protected patients from neutrophil accumulation that was supposed to correlate with severe organ dysfunction in sepsis [353]. Inhibition of leukocyte infiltration by C1 INH is also evidenced in an inflammatory bowel disease model[354].

As C1 INH is a multifunctional protein in a serine protease inhibition-dependent and -independent manner, it may be a promising intervention to attenuate I/R injury. In experimental animal studies, the beneficial effects of C1 INH were shown in reperfusion injury to heart, liver, brain, skeletal muscle, and intestine [355-359], as well as in transplantation-induced I/R injury in lung and heart [106, 360]. In a randomized, placebo-controlled, double-blinded trial, C1 INH was proven to be a safe and efficient treatment for acute attacks in HAE [361]. In a phase 3, randomized, placebo-controlled trial, consistent benefits of C1 INH were shown in HAE patients [362]. The efficiency and safety of nanofiltered C1 INH was also reported in a short term treatment for HAE attacks in an open-label trial (TRIAL REGISTRATION: clinical trials.gov Identifier:NCT00438815) [363]. In addition, C1 INH was identified to effectively inhibit the systemic inflammation in trauma patients in the CAESAR study [364]. The effects of C1 INH were also investigated in antibody-mediated rejection (AMR) in highly human leukocyte antigen (HLA)-sensitized renal transplant recipients. It appeared that C1 INH may be a safe therapy, which reduced I/R injury but further control studies need to be performed, because the levels of C3 and C4 were increased in the C1 INH treated group [365].

Low molecule weight dextran sulfate 5000 (DXS 5000, M_r 5KDa) is a highly sulfated polyglucose and belongs to a member of the GAG family. It is known as a glycosaminoglycan analogue with EC protection and inhibition of the complement and coagulation cascades. Laumonier et al demonstrated that DXS acted as an EC protectant to replace the shedded HS proteoglycan layer on porcine ECs treated with normal human serum (NHS), which mimicked the xenograft rejection model in vitro [366]. Our group also showed that DXS was effective in ameliorating acute vascular rejection in a cardiac xenotransplantation model *in vivo*, which accorded with protection of the xenograft endothelium [367]. In our human-to-pig xenoperfusion model, DXS showed its EC protection on porcine aortic endothelial cells (PAECs) (own study, paper IV of this thesis, unpublished). Wuillemin et al reported that DXS effectively reduced C3 and C4 deposition on cells, and it potentiated the complement inhibitory activity of C1 INH [284]. Our group

also showed that DXS clearly reduced the deposition of C1q, C3b/c, C4b/c, and C9 in a rat aortic clamping model [368]. Also it was confirmed that DXS effectively decreased the deposition of IgM, IgG, C1q, and C3b/c in a tissue in tourniquet-induced skeletal muscle I/R injury model [369].

As for the influence of DXS on the coagulation cascades, Zeerleder et al demonstrated that DXS inhibited the activity of FXa and platelet aggregation, as well as prolonging the prothrombin time (PT), the activated partial thromboplastin time (APTT), and the thrombin time (TT) [370]. It was also shown that DXS decreased fibrin glomerular thrombosis formation score in rats with thrombotic microangiopathy [371]. Our previous study showed that DXS also decreased the level of thrombin-antithrombin (TAT) complex in plasma in a CPB model in pigs [372]. Mauron et al reported that DXS potentiated the inhibitory activity of FXIa by C1 INH to more than 90%, while C1 INH alone inhibited FXIa activation to 50% [373].

DXS is also active on fibrinolytic system. It was reported that DXS activates plasminogen via urokinase-type plasminogen activator (uPA) [374, 375]. Miles et al showed that DXS-induced fibrinolytic activity relied on the presence of FXII, prekallikrein, and HMWK [376]. FXII was activated by DXS in an in intro system including low ionic strength, low concentration of FXII, and an excess of exogenous PK [377]. APTT was prolonged after uPA treatment, but it was shortened with the use of DXS [378]. Therefore it was recommended to combine the use of uPA and DXS in the clinical thromboembolism cases.

AT III is a glycoprotein synthesized by liver and is a multifunctional protein similar to C1 INH. It regulates the coagulation cascades with inhibitory activities on FIXa, FXa, FXIa, FXIIa, thrombin, and FVIIa/TF complex [379-381]. In the kallikrein/kinin system, plasma KK was reported to be inhibited by AT III [382]. The fibrinolytic component plasmin was also shown to be regulated by AT III, which can be enhanced by heparin [383]. Ogston et al demonstrated that AT III suppressed the esterase activity of C1s in a the presence of heparin [384]. The activity of MASP-1 and MASP-2 was also inhibited by AT III in the presence of heparin [250]. Furthermore, AT III was shown to prevent the shedding of the endothelial glycocalyx layer as well as adherence of platelets to vascular beds in an isolated guinea pig heart I/R injury model [385]. The protective effects of AT III were also documented in animal I/R injury studies in renal and liver as well as distant lung damage induced by skeletal muscle I/R injury [194, 196, 386]. In a clinical phase 2 trial, AT III treatment exerted a significant reduction in the 30-day all-cause mortality in severe septic patients [387, 388]. The clinical benefits of AT III were later confirmed on the 90-day quality of life score (QoL), in particular, in social and psychologic functioning [389], in a later phase III clinical trial (ISRCTN22931023). However, ATIII did not reduce overall mortality and increased the risk of bleeding

based on a systemic review with meta-analysis and trial sequential analysis [390]. It was therefore not recommend for use in critically ill patients [390].

A combined inhibition of the plasma cascades and EC protectants might reduce the extent of inflammation in I/R injury. For example, Dickneite et al demonstrated that coinfusion of C1 INH with either the inhibitor of thrombin recombinant hirudin or AT III showed significantly higher survival rates than thrombin inhibitor alone [391]. The combined efficiency of C1 INH and AT III was also reported to decrease intravascular fibrin clot formation and improve cardiovascular functions in mean arterial pressure and cardiac output following endotoxin shock in rabbits [392]. Combined C1 INH and AT III treatment improved mortality in haemorrhagic pancreatitis, while either C1 INH or AT III alone did not show beneficial effects [393].

4. Conclusion

Many studies have elaborated the role of the complement, coagulation, kallikrein/kinin, and EC activation in reperfusion injury. Also many papers have documented the beneficial effects of inhibitors of these systems or EC protectants. Essentially, not only the plasma cascades are interacting, but also the plasma cascades and EC activation. Activation of the plasma cascades acts on EC activation, and EC activation in turn influences the activities of the plasma cascade systems. Therefore, it looks promising to aim at the inhibition of both plasma cascades and EC activation for attenuating I/R injury. This is a new concept for treatment of reperfusion injury and a combined and simultaneous inhibition of the plasma cascades and/or EC activation has not been conducted in the field of I/R injury so far. However, we should be very careful when interpreting our animal experiments in a clinical setting and even more prudent when transferring our therapeutic concepts from animal studies into clinical patients.

5. References

1. S. Haji, R. Planchard, A. Zubair, J. Graff-Radford, C. Rydberg, R. D. Brown, Jr., and K. D. Flemming, "The clinical relevance of cerebral microbleeds in patients with cerebral ischemia and atrial fibrillation," *Journal of Neurology,* 2015.
2. G. Yan, Y. Xuan, Z. Dai, G. Zhang, H. Xu, D. Mikulis, and R. Wu, "Evolution of blood-brain barrier damage associated with changes in brain metabolites following acute ischemia," *Neuroreport,* vol. 26, no. 16, pp. 945-51, 2015.
3. A. Farzaneh-Far, H. R. Phillips, L. K. Shaw, A. Z. Starr, M. Fiuzat, C. M. O'Connor, A. Sastry, L. J. Shaw, and S. Borges-Neto, "Ischemia change in stable coronary artery disease is an independent predictor of death and myocardial infarction," *JACC: Cardiovascular Imaging,* vol. 5, no. 7, pp. 715-24, 2012.
4. H. Yasuhara, and T. Muto, "[Ischemia/reperfusion injury and organ failure]," *Nihon Geka Gakkai Zasshi,* vol. 99, no. 8, pp. 510-7, 1998.
5. M. M. Yassin, D. W. Harkin, A. A. Barros D'Sa, M. I. Halliday, and B. J. Rowlands, "Lower limb ischemia-reperfusion injury triggers a systemic inflammatory response and multiple organ dysfunction," *World Journal of Surgery,* vol. 26, no. 1, pp. 115-21, 2002.
6. J. Pan, A. A. Konstas, B. Bateman, G. A. Ortolano, and J. Pile-Spellman, "Reperfusion injury following cerebral ischemia: pathophysiology, MR imaging, and potential therapies," *Neuroradiology,* vol. 49, no. 2, pp. 93-102, 2007.
7. S. Verma, P. W. Fedak, R. D. Weisel, J. Butany, V. Rao, A. Maitland, R. K. Li, B. Dhillon, and T. M. Yau, "Fundamentals of reperfusion injury for the clinical cardiologist," *Circulation,* vol. 105, no. 20, pp. 2332-6, 2002.
8. W. A. den Hengst, J. F. Gielis, J. Y. Lin, P. E. Van Schil, L. J. De Windt, and A. L. Moens, "Lung ischemia-reperfusion injury: a molecular and clinical view on a complex pathophysiological process," *American Journal of Physiology: Heart and Circulatory Physiology,* vol. 299, no. 5, pp. H1283-99, 2010.
9. C. F. Su, S. J. Kao, and H. I. Chen, "Acute respiratory distress syndrome and lung injury: Pathogenetic mechanism and therapeutic implication," *World J Crit Care Med,* vol. 1, no. 2, pp. 50-60, 2012.
10. B. Vollmar, J. Glasz, R. Leiderer, S. Post, and M. D. Menger, "Hepatic microcirculatory perfusion failure is a determinant of liver dysfunction in warm ischemia-reperfusion," *American Journal of Pathology,* vol. 145, no. 6, pp. 1421-31, 1994.
11. B. Vollmar, M. D. Menger, J. Glasz, R. Leiderer, and K. Messmer, "Impact of leukocyte-endothelial cell interaction in hepatic ischemia-reperfusion injury," *American Journal of Physiology,* vol. 267, no. 5 Pt 1, pp. G786-93, 1994.
12. D. Galaris, A. Barbouti, and P. Korantzopoulos, "Oxidative stress in hepatic ischemia-reperfusion injury: the role of antioxidants and iron chelating compounds," *Current Pharmaceutical Design,* vol. 12, no. 23, pp. 2875-90, 2006.
13. S. Tselepidis, L. Papazoglou, A. Dessiris, I. Vlemas, G. Papageorgiou, A. Stournara, and A. Minas, "Liver injury after ischemia and reperfusion: the role of oxygen free radicals," *Military Medicine,* vol. 169, no. 7, pp. 531-5, 2004.
14. A. G. Sutter, A. P. Palanisamy, J. D. Ellet, M. G. Schmidt, R. G. Schnellmann, and K. D. Chavin, "Intereukin-10 and Kupffer cells protect steatotic mice livers from ischemia-reperfusion injury," *European Cytokine Network,* vol. 25, no. 4, pp. 69-76, 2014.
15. G. M. Diepenhorst, W. de Graaf, H. W. Niessen, A. K. van Vliet, C. E. Hack, and T. M. van Gulik, "Immunoglobulin M, C-reactive protein and complement activation in rat hepatic ischemia-reperfusion injury," *European Surgical Research,* vol. 52, no. 1-2, pp. 50-62, 2014.
16. A. Oguz, M. Kapan, I. Kaplan, U. Alabalik, B. V. Ulger, O. Uslukaya, A. Turkoglu, and Y. Polat, "The effects of sulforaphane on the liver and remote organ damage in hepatic ischemia-reperfusion model formed with pringle maneuver in rats," *International Journal of Surgery (London, England),* vol. 18, pp. 163-8, 2015.
17. M. Kosieradzki, and W. Rowinski, "Ischemia/reperfusion injury in kidney transplantation: mechanisms and prevention," *Transplantation Proceedings,* vol. 40, no. 10, pp. 3279-88, 2008.
18. E. A. Hoste, G. Clermont, A. Kersten, R. Venkataraman, D. C. Angus, D. De Bacquer, and J. A. Kellum, "RIFLE criteria for acute kidney injury are associated with hospital mortality in critically ill patients: a cohort analysis," *Critical Care (London, England),* vol. 10, no. 3, pp. R73, 2006.
19. R. W. Schrier, W. Wang, B. Poole, and A. Mitra, "Acute renal failure: definitions, diagnosis, pathogenesis, and therapy," *Journal of Clinical Investigation,* vol. 114, no. 1, pp. 5-14, 2004.
20. R. Silver, J. de la Garza, M. Rang, and J. Koreska, "Limb swelling after release of a tourniquet," *Clinical Orthopaedics and Related Research,* no. 206, pp. 86-9, 1986.
21. A. Szijarto, Z. Turoczi, J. Szabo, P. Kaliszky, E. Gyurkovics, P. Aranyi, L. Regali, L. Harsanyi, and G. Lotz, "Rapidly progressing fatal reperfusion syndrome caused by acute critical ischemia of the lower limb," *Cardiovascular Pathology,* vol. 22, no. 6, pp. 493-500, 2013.
22. F. W. Blaisdell, "The pathophysiology of skeletal muscle ischemia and the reperfusion syndrome: a review," *Cardiovascular Surgery,* vol. 10, no. 6, pp. 620-30, 2002.

23. R. F. van Golen, M. J. Reiniers, P. B. Olthof, T. M. van Gulik, and M. Heger, "Sterile inflammation in hepatic ischemia/reperfusion injury: present concepts and potential therapeutics," *Journal of Gastroenterology and Hepatology,* vol. 28, no. 3, pp. 394-400, 2013.
24. P. Matzinger, "The danger model: a renewed sense of self," *Science,* vol. 296, no. 5566, pp. 301-5, 2002.
25. A. Chauhan, U. Sharma, N. R. Jagannathan, and Y. K. Gupta, "Rapamycin ameliorates brain metabolites alterations after transient focal ischemia in rats," *European Journal of Pharmacology,* vol. 757, pp. 28-33, 2015.
26. W. L. Lanier, R. E. Hofer, and W. J. Gallagher, "Metabolism of glucose, glycogen, and high-energy phosphates during transient forebrain ischemia in diabetic rats: effect of insulin treatment," *Anesthesiology,* vol. 84, no. 4, pp. 917-25, 1996.
27. G. Wikstrom, G. Ronquist, S. Nilsson, E. Maripu, and A. Waldenstrom, "Continuous monitoring of energy metabolites using microdialysis during myocardial ischaemia in the pig," *European Heart Journal,* vol. 16, no. 3, pp. 339-47, 1995.
28. J. Bardutzky, Q. Shen, N. Henninger, S. Schwab, T. Q. Duong, and M. Fisher, "Characterizing tissue fate after transient cerebral ischemia of varying duration using quantitative diffusion and perfusion imaging," *Stroke,* vol. 38, no. 4, pp. 1336-44, 2007.
29. C. Duehrkop, and R. Rieben, "Refinement of tourniquet-induced peripheral ischemia/reperfusion injury in rats: comparison of 2 h vs 24 h reperfusion," *Laboratory Animals,* vol. 48, no. 2, pp. 143-54, 2014.
30. D. A. Parks, and D. N. Granger, "Contributions of ischemia and reperfusion to mucosal lesion formation," *American Journal of Physiology,* vol. 250, no. 6 Pt 1, pp. G749-53, 1986.
31. M. Furlan, G. Marchal, F. Viader, J. M. Derlon, and J. C. Baron, "Spontaneous neurological recovery after stroke and the fate of the ischemic penumbra," *Annals of Neurology,* vol. 40, no. 2, pp. 216-26, 1996.
32. W. D. Heiss, R. Graf, T. Fujita, K. Ohta, B. Bauer, J. Lottgen, and K. Wienhard, "Early detection of irreversibly damaged ischemic tissue by flumazenil positron emission tomography in cats," *Stroke,* vol. 28, no. 10, pp. 2045-51; discussion 2051-2, 1997.
33. H. Grundt, D. W. Nilsen, O. Hetland, E. Valente, and H. E. Fagertun, "Activated factor 12 (FXIIa) predicts recurrent coronary events after an acute myocardial infarction," *American Heart Journal,* vol. 147, no. 2, pp. 260-6, 2004.
34. M. C. Minnema, R. J. Peters, R. de Winter, Y. P. Lubbers, S. Barzegar, K. A. Bauer, R. D. Rosenberg, C. E. Hack, and H. ten Cate, "Activation of clotting factors XI and IX in patients with acute myocardial infarction," *Arteriosclerosis, Thrombosis, and Vascular Biology,* vol. 20, no. 11, pp. 2489-93, 2000.
35. P. H. Wang, G. Campanholle, M. A. Cenedeze, C. Q. Feitoza, G. M. Goncalves, R. G. Landgraf, S. Jancar, J. B. Pesquero, A. Pacheco-Silva, and N. O. Camara, "Bradykinin [corrected] B1 receptor antagonism is beneficial in renal ischemia-reperfusion injury," *PloS One,* vol. 3, no. 8, pp. e3050, 2008.
36. E. Czarnowska, and E. Karwatowska-Prokopczuk, "Ultrastructural demonstration of endothelial glycocalyx disruption in the reperfused rat heart. Involvement of oxygen free radicals," *Basic Research in Cardiology,* vol. 90, no. 5, pp. 357-64, 1995.
37. G. D'Angelo, E. Duplan, N. Boyer, P. Vigne, and C. Frelin, "Hypoxia up-regulates prolyl hydroxylase activity: a feedback mechanism that limits HIF-1 responses during reoxygenation," *Journal of Biological Chemistry,* vol. 278, no. 40, pp. 38183-7, 2003.
38. A. C. Cave, J. S. Ingwall, J. Friedrich, R. Liao, K. W. Saupe, C. S. Apstein, and F. R. Eberli, "ATP synthesis during low-flow ischemia: influence of increased glycolytic substrate," *Circulation,* vol. 101, no. 17, pp. 2090-6, 2000.
39. G. De Santis, and M. Pinelli, "Microsurgical model of ischemia reperfusion in rat muscle: evidence of free radical formation by spin trapping," *Microsurgery,* vol. 15, no. 9, pp. 655-9, 1994.
40. W. Inauen, M. Suzuki, and D. N. Granger, "Mechanisms of cellular injury: potential sources of oxygen free radicals in ischemia/reperfusion," *Microcirculation, Endothelium, and Lymphatics,* vol. 5, no. 3-5, pp. 143-55, 1989.
41. Z. Yang, A. K. Sharma, M. Marshall, I. L. Kron, and V. E. Laubach, "NADPH oxidase in bone marrow-derived cells mediates pulmonary ischemia-reperfusion injury," *American Journal of Respiratory Cell and Molecular Biology,* vol. 40, no. 3, pp. 375-81, 2009.
42. S. Matsushima, H. Tsutsui, and J. Sadoshima, "Physiological and pathological functions of NADPH oxidases during myocardial ischemia-reperfusion," *Trends in Cardiovascular Medicine,* 2014.
43. D. Hou, W. Y. Liu, F. Z. Fang, J. Mo, S. F. Sun, W. R. Shi, J. M. Ye, Y. F. Guan, and J. Liu, "Prevention of myocardial reperfusion injury with free radical scavengers. An experimental study," *Chinese Medical Journal (Engl.),* vol. 102, no. 10, pp. 768-73, 1989.
44. A. M. Feller, A. C. Roth, R. C. Russell, B. Eagleton, H. Suchy, and N. Debs, "Experimental evaluation of oxygen free radical scavengers in the prevention of reperfusion injury to skeletal muscle," *Annals of Plastic Surgery,* vol. 22, no. 4, pp. 321-31, 1989.
45. M. Fukai, T. Hayashi, R. Yokota, T. Shimamura, T. Suzuki, M. Taniguchi, M. Matsushita, H. Furukawa, and S. Todo, "Lipid peroxidation during ischemia depends on ischemia time in warm ischemia and reperfusion of rat liver," *Free Radical Biology and Medicine,* vol. 38, no. 10, pp. 1372-81, 2005.

46. I. E. Blasig, T. Grune, K. Schonheit, E. Rohde, M. Jakstadt, R. F. Haseloff, and W. G. Siems, "4-Hydroxynonenal, a novel indicator of lipid peroxidation for reperfusion injury of the myocardium," *American Journal of Physiology,* vol. 269, no. 1 Pt 2, pp. H14-22, 1995.
47. R. Nishikata, N. Kato, and K. Hiraiwa, "Oxidative stress may be involved in distant organ failure in tourniquet shock model mice," *Legal Medicine (Tokyo, Japan),* vol. 16, no. 2, pp. 70-5, 2014.
48. S. Zhang, C. Duehrkop, J. A. Plock, and R. Rieben, "Inhalation anesthesia of rats: influence of the fraction of inspired oxygen on limb ischemia/reperfusion injury," *Laboratory Animals,* 2015.
49. D. K. de Vries, K. A. Kortekaas, D. Tsikas, L. G. Wijermars, C. J. van Noorden, M. T. Suchy, C. M. Cobbaert, R. J. Klautz, A. F. Schaapherder, and J. H. Lindeman, "Oxidative damage in clinical ischemia/reperfusion injury: a reappraisal," *Antioxid Redox Signal,* vol. 19, no. 6, pp. 535-45, 2013.
50. M. Da Silveira, and W. B. Yoshida, "Trimetazidine and N-acetylcysteine in attenuating hind-limb ischemia and reperfusion injuries: experimental study in rats," *International Angiology,* vol. 28, no. 5, pp. 412-7, 2009.
51. M. Rosselli, P. J. Keller, and R. K. Dubey, "Role of nitric oxide in the biology, physiology and pathophysiology of reproduction," *Human Reproduction Update,* vol. 4, no. 1, pp. 3-24, 1998.
52. X. M. Chen, H. S. Chen, M. J. Xu, and J. G. Shen, "Targeting reactive nitrogen species: a promising therapeutic strategy for cerebral ischemia-reperfusion injury," *Acta Pharmacologica Sinica,* vol. 34, no. 1, pp. 67-77, 2013.
53. S. S. Marla, J. Lee, and J. T. Groves, "Peroxynitrite rapidly permeates phospholipid membranes," *Proceedings of the National Academy of Sciences of the United States of America,* vol. 94, no. 26, pp. 14243-8, 1997.
54. J. S. Beckman, H. Ischiropoulos, L. Zhu, M. van der Woerd, C. Smith, J. Chen, J. Harrison, J. C. Martin, and M. Tsai, "Kinetics of superoxide dismutase- and iron-catalyzed nitration of phenolics by peroxynitrite," *Archives of Biochemistry and Biophysics,* vol. 298, no. 2, pp. 438-45, 1992.
55. K. Jian Liu, and G. A. Rosenberg, "Matrix metalloproteinases and free radicals in cerebral ischemia," *Free Radical Biology and Medicine,* vol. 39, no. 1, pp. 71-80, 2005.
56. I. N. Hines, S. Kawachi, H. Harada, K. P. Pavlick, J. M. Hoffman, S. Bharwani, R. E. Wolf, and M. B. Grisham, "Role of nitric oxide in liver ischemia and reperfusion injury," *Molecular and Cellular Biochemistry,* vol. 234-235, no. 1-2, pp. 229-37, 2002.
57. B. V. Naidu, C. Fraga, A. L. Salzman, C. Szabo, E. D. Verrier, and M. S. Mulligan, "Critical role of reactive nitrogen species in lung ischemia-reperfusion injury," *Journal of Heart and Lung Transplantation,* vol. 22, no. 7, pp. 784-93, 2003.
58. X. L. Wang, H. R. Liu, L. Tao, F. Liang, L. Yan, R. R. Zhao, B. L. Lopez, T. A. Christopher, and X. L. Ma, "Role of iNOS-derived reactive nitrogen species and resultant nitrative stress in leukocytes-induced cardiomyocyte apoptosis after myocardial ischemia/reperfusion," *Apoptosis,* vol. 12, no. 7, pp. 1209-17, 2007.
59. J. W. Park, W. N. Qi, J. Q. Liu, J. R. Urbaniak, R. J. Folz, and L. E. Chen, "Inhibition of iNOS attenuates skeletal muscle reperfusion injury in extracellular superoxide dismutase knockout mice," *Microsurgery,* vol. 25, no. 8, pp. 606-13, 2005.
60. J. A. Shin, H. Lee, Y. K. Lim, Y. Koh, J. H. Choi, and E. M. Park, "Therapeutic effects of resveratrol during acute periods following experimental ischemic stroke," *Journal of Neuroimmunology,* vol. 227, no. 1-2, pp. 93-100, 2010.
61. Y. Zhou, S. H. Fang, Y. L. Ye, L. S. Chu, W. P. Zhang, M. L. Wang, and E. Q. Wei, "Caffeic acid ameliorates early and delayed brain injuries after focal cerebral ischemia in rats," *Acta Pharmacologica Sinica,* vol. 27, no. 9, pp. 1103-10, 2006.
62. K. B. Faust, V. Chiantella, J. Vinten-Johansen, and J. H. Meredith, "Oxygen-derived free radical scavengers and skeletal muscle ischemic/reperfusion injury," *American Surgeon,* vol. 54, no. 12, pp. 709-19, 1988.
63. J. S. Hawkes, C. M. Young, and L. G. Cleland, "Ischaemia reperfusion injury in pedicle skin flaps in the pig: lack of protective effect of SOD and allopurinol," *British Journal of Plastic Surgery,* vol. 42, no. 6, pp. 668-74, 1989.
64. I. El-Hamamsy, L. M. Stevens, M. Carrier, M. Pellerin, D. Bouchard, P. Demers, R. Cartier, P. Page, and L. P. Perrault, "Effect of intravenous N-acetylcysteine on outcomes after coronary artery bypass surgery: a randomized, double-blind, placebo-controlled clinical trial," *Journal of Thoracic and Cardiovascular Surgery,* vol. 133, no. 1, pp. 7-12, 2007.
65. P. M. Bath, R. Iddenden, F. J. Bath, J. M. Orgogozo, and C. Tirilazad International Steering, "Tirilazad for acute ischaemic stroke," *Cochrane Database Syst Rev,* no. 4, pp. CD002087, 2001.
66. M. Zhang, K. Takahashi, E. M. Alicot, T. Vorup-Jensen, B. Kessler, S. Thiel, J. C. Jensenius, R. A. Ezekowitz, F. D. Moore, and M. C. Carroll, "Activation of the lectin pathway by natural IgM in a model of ischemia/reperfusion injury," *Journal of Immunology,* vol. 177, no. 7, pp. 4727-34, 2006.
67. M. Noris, and G. Remuzzi, "Overview of complement activation and regulation," *Seminars in Nephrology,* vol. 33, no. 6, pp. 479-92, 2013.
68. A. J. Nauta, M. R. Daha, C. van Kooten, and A. Roos, "Recognition and clearance of apoptotic cells: a role for complement and pentraxins," *Trends in Immunology,* vol. 24, no. 3, pp. 148-54, 2003.
69. D. Ricklin, and J. D. Lambris, "Complement in immune and inflammatory disorders: pathophysiological mechanisms," *Journal of Immunology,* vol. 190, no. 8, pp. 3831-8, 2013.

70. A. Klos, A. J. Tenner, K. O. Johswich, R. R. Ager, E. S. Reis, and J. Kohl, "The role of the anaphylatoxins in health and disease," *Molecular Immunology,* vol. 46, no. 14, pp. 2753-66, 2009.
71. R. F. Guo, and P. A. Ward, "Role of C5a in inflammatory responses," *Annual Review of Immunology,* vol. 23, pp. 821-52, 2005.
72. J. H. Hill, and P. A. Ward, "The phlogistic role of C3 leukotactic fragments in myocardial infarcts of rats," *Journal of Experimental Medicine,* vol. 133, no. 4, pp. 885-900, 1971.
73. T. Murohara, J. P. Guo, J. A. Delyani, and A. M. Lefer, "Cardioprotective effects of selective inhibition of the two complement activation pathways in myocardial ischemia and reperfusion injury," *Methods and Findings in Experimental and Clinical Pharmacology,* vol. 17, no. 8, pp. 499-507, 1995.
74. M. Zhang, Y. J. Hou, E. Cavusoglu, D. C. Lee, R. Steffensen, L. Yang, D. Bashari, J. Villamil, M. Moussa, G. Fernaine, J. C. Jensenius, J. D. Marmur, W. Ko, and K. Shevde, "MASP-2 activation is involved in ischemia-related necrotic myocardial injury in humans," *International Journal of Cardiology,* vol. 166, no. 2, pp. 499-504, 2013.
75. C. Costa, L. Zhao, Y. Shen, X. Su, L. Hao, S. P. Colgan, G. L. Stahl, T. Zhou, and Y. Wang, "Role of complement component C5 in cerebral ischemia/reperfusion injury," *Brain Research,* vol. 1100, no. 1, pp. 142-51, 2006.
76. M. R. Zamora, R. D. Davis, S. H. Keshavjee, L. Schulman, J. Levin, U. Ryan, and G. A. Patterson, "Complement inhibition attenuates human lung transplant reperfusion injury: a multicenter trial," *Chest,* vol. 116, no. 1 Suppl, pp. 46S, 1999.
77. B. H. Heijnen, I. H. Straatsburg, N. D. Padilla, G. J. Van Mierlo, C. E. Hack, and T. M. Van Gulik, "Inhibition of classical complement activation attenuates liver ischaemia and reperfusion injury in a rat model," *Clinical and Experimental Immunology,* vol. 143, no. 1, pp. 15-23, 2006.
78. W. Zhou, C. A. Farrar, K. Abe, J. R. Pratt, J. E. Marsh, Y. Wang, G. L. Stahl, and S. H. Sacks, "Predominant role for C5b-9 in renal ischemia/reperfusion injury," *Journal of Clinical Investigation,* vol. 105, no. 10, pp. 1363-71, 2000.
79. T. M. Woodruff, T. V. Arumugam, I. A. Shiels, R. C. Reid, D. P. Fairlie, and S. M. Taylor, "Protective effects of a potent C5a receptor antagonist on experimental acute limb ischemia-reperfusion in rats," *Journal of Surgical Research,* vol. 116, no. 1, pp. 81-90, 2004.
80. A. Vakeva, B. P. Morgan, I. Tikkanen, K. Helin, P. Laurila, and S. Meri, "Time course of complement activation and inhibitor expression after ischemic injury of rat myocardium," *American Journal of Pathology,* vol. 144, no. 6, pp. 1357-68, 1994.
81. M. L. Hart, K. A. Ceonzo, L. A. Shaffer, K. Takahashi, R. P. Rother, W. R. Reenstra, J. A. Buras, and G. L. Stahl, "Gastrointestinal ischemia-reperfusion injury is lectin complement pathway dependent without involving C1q," *Journal of Immunology,* vol. 174, no. 10, pp. 6373-80, 2005.
82. J. Mocco, W. J. Mack, A. F. Ducruet, S. A. Sosunov, M. E. Sughrue, B. G. Hassid, M. N. Nair, I. Laufer, R. J. Komotar, M. Claire, H. Holland, D. J. Pinsky, and E. S. Connolly, Jr., "Complement component C3 mediates inflammatory injury following focal cerebral ischemia," *Circulation Research,* vol. 99, no. 2, pp. 209-17, 2006.
83. R. K. Chan, S. I. Ibrahim, K. Takahashi, E. Kwon, M. McCormack, A. Ezekowitz, M. C. Carroll, F. D. Moore, Jr., and W. G. Austen, Jr., "The differing roles of the classical and mannose-binding lectin complement pathways in the events following skeletal muscle ischemia-reperfusion," *Journal of Immunology,* vol. 177, no. 11, pp. 8080-5, 2006.
84. M. Osthoff, M. Katan, F. Fluri, P. Schuetz, R. Bingisser, L. Kappos, A. J. Steck, S. T. Engelter, B. Mueller, M. Christ-Crain, and M. Trendelenburg, "Mannose-binding lectin deficiency is associated with smaller infarction size and favorable outcome in ischemic stroke patients," *PloS One,* vol. 6, no. 6, pp. e21338, 2011.
85. R. A. Matthijsen, J. P. Derikx, R. Steffensen, R. M. van Dam, C. H. Dejong, and W. A. Buurman, "Mannose-binding lectin null alleles are associated with preserved epithelial cell integrity following intestinal ischemia reperfusion in man," *Molecular Immunology,* vol. 46, no. 11-12, pp. 2244-8, 2009.
86. M. R. Weiser, J. P. Williams, F. D. Moore, Jr., L. Kobzik, M. Ma, H. B. Hechtman, and M. C. Carroll, "Reperfusion injury of ischemic skeletal muscle is mediated by natural antibody and complement," *Journal of Experimental Medicine,* vol. 183, no. 5, pp. 2343-8, 1996.
87. J. P. Williams, T. T. Pechet, M. R. Weiser, R. Reid, L. Kobzik, F. D. Moore, Jr., M. C. Carroll, and H. B. Hechtman, "Intestinal reperfusion injury is mediated by IgM and complement," *J Appl Physiol (1985),* vol. 86, no. 3, pp. 938-42, 1999.
88. J. M. Thurman, D. Ljubanovic, C. L. Edelstein, G. S. Gilkeson, and V. M. Holers, "Lack of a functional alternative complement pathway ameliorates ischemic acute renal failure in mice," *Journal of Immunology,* vol. 170, no. 3, pp. 1517-23, 2003.
89. A. Elvington, C. Atkinson, H. Zhu, J. Yu, K. Takahashi, G. L. Stahl, M. S. Kindy, and S. Tomlinson, "The alternative complement pathway propagates inflammation and injury in murine ischemic stroke," *Journal of Immunology,* vol. 189, no. 9, pp. 4640-7, 2012.
90. C. R. Amura, B. Renner, T. Lyubchenko, S. Faubel, P. L. Simonian, and J. M. Thurman, "Complement activation and toll-like receptor-2 signaling contribute to cytokine production after renal ischemia/reperfusion," *Molecular Immunology,* vol. 52, no. 3-4, pp. 249-57, 2012.

91. H. Lee, D. J. Green, L. Lai, Y. J. Hou, J. C. Jensenius, D. Liu, C. Cheong, C. G. Park, and M. Zhang, "Early complement factors in the local tissue immunocomplex generated during intestinal ischemia/reperfusion injury," *Molecular Immunology,* vol. 47, no. 5, pp. 972-81, 2010.
92. O. Hurtado, J. Serrano, M. Sobrado, A. P. Fernandez, I. Lizasoain, R. Martinez-Murillo, M. A. Moro, and A. Martinez, "Lack of adrenomedullin, but not complement factor H, results in larger infarct size and more extensive brain damage in a focal ischemia model," *Neuroscience,* vol. 171, no. 3, pp. 885-92, 2010.
93. C. Kyriakides, W. Austen, Jr., Y. Wang, J. Favuzza, L. Kobzik, F. D. Moore, Jr., and H. B. Hechtman, "Skeletal muscle reperfusion injury is mediated by neutrophils and the complement membrane attack complex," *American Journal of Physiology,* vol. 277, no. 6 Pt 1, pp. C1263-8, 1999.
94. W. G. Austen, Jr., C. Kyriakides, J. Favuzza, Y. Wang, L. Kobzik, F. D. Moore, Jr., and H. B. Hechtman, "Intestinal ischemia-reperfusion injury is mediated by the membrane attack complex," *Surgery,* vol. 126, no. 2, pp. 343-8, 1999.
95. K. Yamada, T. Miwa, J. Liu, M. Nangaku, and W. C. Song, "Critical protection from renal ischemia reperfusion injury by CD55 and CD59," *Journal of Immunology,* vol. 172, no. 6, pp. 3869-75, 2004.
96. T. Miwa, S. Sato, D. Gullipalli, M. Nangaku, and W. C. Song, "Blocking properdin, the alternative pathway, and anaphylatoxin receptors ameliorates renal ischemia-reperfusion injury in decay-accelerating factor and CD59 double-knockout mice," *Journal of Immunology,* vol. 190, no. 7, pp. 3552-9, 2013.
97. D. Z. Xu, S. B. Zaets, R. Chen, Q. Lu, H. Rajan, X. Yang, J. Zhang, E. Feketova, N. Bogdan, E. A. Deitch, and Y. Cao, "Elimination of C5aR prevents intestinal mucosal damage and attenuates neutrophil infiltration in local and remote organs," *Shock,* vol. 31, no. 5, pp. 493-9, 2009.
98. Q. Peng, K. Li, L. A. Smyth, G. Xing, N. Wang, L. Meader, B. Lu, S. H. Sacks, and W. Zhou, "C3a and C5a promote renal ischemia-reperfusion injury," *Journal of the American Society of Nephrology,* vol. 23, no. 9, pp. 1474-85, 2012.
99. M. C. Wu, F. H. Brennan, J. P. Lynch, S. Mantovani, S. Phipps, R. A. Wetsel, M. J. Ruitenberg, S. M. Taylor, and T. M. Woodruff, "The receptor for complement component C3a mediates protection from intestinal ischemia-reperfusion injuries by inhibiting neutrophil mobilization," *Proceedings of the National Academy of Sciences of the United States of America,* vol. 110, no. 23, pp. 9439-44, 2013.
100. A. Agostoni, L. Bergamaschini, G. Martignoni, M. Cicardi, and B. Marasini, "Treatment of acute attacks of hereditary angioedema with C1-inhibitor concentrate," *Annals of Allergy,* vol. 44, no. 5, pp. 299-301, 1980.
101. K. Fattouch, G. Bianco, G. Speziale, R. Sampognaro, C. Lavalle, F. Guccione, P. Dioguardi, and G. Ruvolo, "Beneficial effects of C1 esterase inhibitor in ST-elevation myocardial infarction in patients who underwent surgical reperfusion: a randomised double-blind study," *European Journal of Cardio-Thoracic Surgery,* vol. 32, no. 2, pp. 326-32, 2007.
102. T. G. Lehmann, M. Heger, S. Munch, M. Kirschfink, and E. Klar, "In vivo microscopy reveals that complement inhibition by C1-esterase inhibitor reduces ischemia/reperfusion injury in the liver," *Transplant International,* vol. 13 Suppl 1, pp. S547-50, 2000.
103. C. Storini, E. Rossi, V. Marrella, M. Distaso, R. Veerhuis, C. Vergani, L. Bergamaschini, and M. G. De Simoni, "C1-inhibitor protects against brain ischemia-reperfusion injury via inhibition of cell recruitment and inflammation," *Neurobiology of Disease,* vol. 19, no. 1-2, pp. 10-7, 2005.
104. G. A. Toomayan, L. E. Chen, H. X. Jiang, W. N. Qi, A. V. Seaber, M. M. Frank, and J. R. Urbaniak, "C1-esterase inhibitor and a novel peptide inhibitor improve contractile function in reperfused skeletal muscle," *Microsurgery,* vol. 23, no. 6, pp. 561-7, 2003.
105. A. Salvatierra, F. Velasco, M. Rodriguez, A. Alvarez, R. Lopez-Pedrera, R. Ramirez, J. Carracedo, F. Lopez-Rubio, J. Lopez-Pujol, and R. Guerrero, "C1-esterase inhibitor prevents early pulmonary dysfunction after lung transplantation in the dog," *American Journal of Respiratory and Critical Care Medicine,* vol. 155, no. 3, pp. 1147-54, 1997.
106. U. Klima, I. Kutschka, G. Warnecke, P. Kim, M. Struber, M. Kirschfink, and A. Haverich, "Improved right ventricular function after intracoronary administration of a C1 esterase inhibitor in a right heart transplantation model," *European Journal of Cardio-Thoracic Surgery,* vol. 18, no. 3, pp. 321-7, 2000.
107. C. Caliezi, S. Zeerleder, M. Redondo, B. Regli, H. U. Rothen, R. Zurcher-Zenklusen, R. Rieben, J. Devay, C. E. Hack, B. Lammle, and W. A. Wuillemin, "C1-inhibitor in patients with severe sepsis and septic shock: beneficial effect on renal dysfunction," *Critical Care Medicine,* vol. 30, no. 8, pp. 1722-8, 2002.
108. M. Buerke, H. Schwertz, W. Seitz, J. Meyer, and H. Darius, "Novel small molecule inhibitor of C1s exerts cardioprotective effects in ischemia-reperfusion injury in rabbits," *Journal of Immunology,* vol. 167, no. 9, pp. 5375-80, 2001.
109. V. I. Pavlov, M. O. Skjoedt, Y. Siow Tan, A. Rosbjerg, P. Garred, and G. L. Stahl, "Endogenous and natural complement inhibitor attenuates myocardial injury and arterial thrombogenesis," *Circulation,* vol. 126, no. 18, pp. 2227-35, 2012.
110. H. F. Weisman, T. Bartow, M. K. Leppo, H. C. Marsh, Jr., G. R. Carson, M. F. Concino, M. P. Boyle, K. H. Roux, M. L. Weisfeldt, and D. T. Fearon, "Soluble human complement receptor type 1: in vivo inhibitor of complement suppressing post-ischemic myocardial inflammation and necrosis," *Science,* vol. 249, no. 4965, pp. 146-51, 1990.

111. R. E. Chavez-Cartaya, G. P. DeSola, L. Wright, N. V. Jamieson, and D. J. White, "Regulation of the complement cascade by soluble complement receptor type 1. Protective effect in experimental liver ischemia and reperfusion," *Transplantation,* vol. 59, no. 7, pp. 1047-52, 1995.
112. M. Pemberton, G. Anderson, V. Vetvicka, D. E. Justus, and G. D. Ross, "Microvascular effects of complement blockade with soluble recombinant CR1 on ischemia/reperfusion injury of skeletal muscle," *Journal of Immunology,* vol. 150, no. 11, pp. 5104-13, 1993.
113. R. A. Schmid, A. Zollinger, T. Singer, S. Hillinger, J. R. Leon-Wyss, O. M. Schob, K. Hogasen, G. Zund, G. A. Patterson, and W. Weder, "Effect of soluble complement receptor type 1 on reperfusion edema and neutrophil migration after lung allotransplantation in swine," *Journal of Thoracic and Cardiovascular Surgery,* vol. 116, no. 1, pp. 90-7, 1998.
114. B. Tan, Y. J. Lan, and D. C. Zhang, "[Protective effects of recombinant SCR15-18 domain of human soluble complement receptor type 1 on myocardial ischemia and reperfusion injury]," *Zhonghua Xin Xue Guan Bing Za Zhi,* vol. 35, no. 11, pp. 1037-40, 2007.
115. S. Li, J. Xian, L. He, X. Luo, B. Tan, Y. Yang, G. Liu, and Z. Wang, "The protective effect of SCR(15-18) on cerebral ischemia-reperfusion injury," *Neurological Research,* vol. 33, no. 8, pp. 866-74, 2011.
116. D. G. Souza, D. Esser, R. Bradford, A. T. Vieira, and M. M. Teixeira, "APT070 (Mirococept), a membrane-localised complement inhibitor, inhibits inflammatory responses that follow intestinal ischaemia and reperfusion injury," *British Journal of Pharmacology,* vol. 145, no. 8, pp. 1027-34, 2005.
117. Y. Banz, O. M. Hess, S. C. Robson, E. Csizmadia, D. Mettler, P. Meier, A. Haeberli, S. Shaw, R. A. Smith, and R. Rieben, "Attenuation of myocardial reperfusion injury in pigs by Mirococept, a membrane-targeted complement inhibitor derived from human CR1," *Cardiovascular Research,* vol. 76, no. 3, pp. 482-93, 2007.
118. C. Duehrkop, Y. Banz, R. Spirig, S. Miescher, M. W. Nolte, M. Spycher, R. A. Smith, S. H. Sacks, and R. Rieben, "C1 esterase inhibitor reduces lower extremity ischemia/reperfusion injury and associated lung damage," *PloS One,* vol. 8, no. 8, pp. e72059, 2013.
119. S. Keshavjee, R. D. Davis, M. R. Zamora, M. de Perrot, and G. A. Patterson, "A randomized, placebo-controlled trial of complement inhibition in ischemia-reperfusion injury after lung transplantation in human beings," *Journal of Thoracic and Cardiovascular Surgery,* vol. 129, no. 2, pp. 423-8, 2005.
120. J. R. Pratt, M. J. Hibbs, A. J. Laver, R. A. Smith, and S. H. Sacks, "Effects of complement inhibition with soluble complement receptor-1 on vascular injury and inflammation during renal allograft rejection in the rat," *American Journal of Pathology,* vol. 149, no. 6, pp. 2055-66, 1996.
121. H. L. Lazar, P. M. Bokesch, F. van Lenta, C. Fitzgerald, C. Emmett, H. C. Marsh, Jr., U. Ryan, Obe, and T. P. C. S. S. G. the, "Soluble human complement receptor 1 limits ischemic damage in cardiac surgery patients at high risk requiring cardiopulmonary bypass," *Circulation,* vol. 110, no. 11 Suppl 1, pp. II274-9, 2004.
122. M. N. Busche, and G. L. Stahl, "Role of the complement components C5 and C3a in a mouse model of myocardial ischemia and reperfusion injury," *German Medical Science: GMS E-Journal,* vol. 8, 2010.
123. K. Wada, M. C. Montalto, and G. L. Stahl, "Inhibition of complement C5 reduces local and remote organ injury after intestinal ischemia/reperfusion in the rat," *Gastroenterology,* vol. 120, no. 1, pp. 126-33, 2001.
124. B. De Vries, R. A. Matthijsen, T. G. Wolfs, A. A. Van Bijnen, P. Heeringa, and W. A. Buurman, "Inhibition of complement factor C5 protects against renal ischemia-reperfusion injury: inhibition of late apoptosis and inflammation," *Transplantation,* vol. 75, no. 3, pp. 375-82, 2003.
125. M. Ferraresso, P. Macor, M. Valente, M. Della Barbera, F. D'Amelio, O. Borghi, E. Raschi, P. Durigutto, P. Meroni, and F. Tedesco, "Posttransplant ischemia-reperfusion injury in transplanted heart is prevented by a minibody to the fifth component of complement," *Transplantation,* vol. 86, no. 10, pp. 1445-51, 2008.
126. A. J. Fleisig, and E. D. Verrier, "Pexelizumab -- a C5 complement inhibitor for use in both acute myocardial infarction and cardiac surgery with cardiopulmonary bypass," *Expert Opinion on Biological Therapy,* vol. 5, no. 6, pp. 833-9, 2005.
127. S. K. Shernan, J. C. Fitch, N. A. Nussmeier, J. C. Chen, S. A. Rollins, C. F. Mojcik, K. J. Malloy, T. G. Todaro, T. Filloon, S. W. Boyce, D. M. Gangahar, M. Goldberg, L. J. Saidman, D. T. Mangano, and I. Pexelizumab Study, "Impact of pexelizumab, an anti-C5 complement antibody, on total mortality and adverse cardiovascular outcomes in cardiac surgical patients undergoing cardiopulmonary bypass," *Annals of Thoracic Surgery,* vol. 77, no. 3, pp. 942-9; discussion 949-50, 2004.
128. L. Testa, W. J. Van Gaal, R. Bhindi, G. G. Biondi-Zoccai, A. Abbate, P. Agostoni, I. Porto, F. Andreotti, F. Crea, and A. P. Banning, "Pexelizumab in ischemic heart disease: a systematic review and meta-analysis on 15,196 patients," *Journal of Thoracic and Cardiovascular Surgery,* vol. 136, no. 4, pp. 884-93, 2008.
129. G. M. Lin, "Pexelizumab, an Anti-C5 Complement Antibody for Primary Coronary Revascularization: A New Insight from Old Versions," *Cardiovascular & Hematological Disorders Drug Targets,* vol. 11, no. 2, pp. 97-101, 2011.
130. G. M. Lin, K. M. Chu, and C. L. Han, "Pexelizumab may be hazardous to those with ST-segment elevation myocardial infarction undergoing primary percutaneous interventions without using glycoprotein IIb-IIIa inhibitors," *International Journal of Cardiology,* vol. 146, no. 2, pp. 280-2, 2011.

131. L. Testa, M. Meco, S. Cirri, and F. Bedogni, "Pexelizumab and survival in cardiac surgery," *HSR Proc Intensive Care Cardiovasc Anesth,* vol. 3, no. 1, pp. 23-4, 2011.
132. C. Legendre, R. Sberro-Soussan, J. Zuber, M. Rabant, A. Loupy, M. O. Timsit, and D. Anglicheau, "Eculizumab in renal transplantation," *Transplant Rev (Orlando),* vol. 27, no. 3, pp. 90-2, 2013.
133. A. N. Barnett, E. Asgari, P. Chowdhury, S. H. Sacks, A. Dorling, and N. Mamode, "The use of eculizumab in renal transplantation," *Clinical Transplantation,* vol. 27, no. 3, pp. E216-29, 2013.
134. C. K. Johnson, and N. Leca, "Eculizumab use in kidney transplantation," *Curr Opin Organ Transplant,* vol. 20, no. 6, pp. 643-51, 2015.
135. C. W. Vogel, and D. C. Fritzinger, "Cobra venom factor: Structure, function, and humanization for therapeutic complement depletion," *Toxicon,* vol. 56, no. 7, pp. 1198-222, 2010.
136. P. R. Maroko, C. B. Carpenter, M. Chiariello, M. C. Fishbein, P. Radvany, J. D. Knostman, and S. L. Hale, "Reduction by cobra venom factor of myocardial necrosis after coronary artery occlusion," *Journal of Clinical Investigation,* vol. 61, no. 3, pp. 661-70, 1978.
137. Y. F. Mao, Q. H. Yu, X. F. Zheng, K. Liu, W. Q. Liang, Y. W. Wang, X. M. Deng, and L. Jiang, "Pre-treatment with Cobra venom factor alleviates acute lung injury induced by intestinal ischemia-reperfusion in rats," *European Review for Medical and Pharmacological Sciences,* vol. 17, no. 16, pp. 2207-17, 2013.
138. E. Figueroa, L. E. Gordon, P. W. Feldhoff, and H. A. Lassiter, "The administration of cobra venom factor reduces post-ischemic cerebral injury in adult and neonatal rats," *Neuroscience Letters,* vol. 380, no. 1-2, pp. 48-53, 2005.
139. W. B. Gorsuch, B. J. Guikema, D. C. Fritzinger, C. W. Vogel, and G. L. Stahl, "Humanized cobra venom factor decreases myocardial ischemia-reperfusion injury," *Molecular Immunology,* vol. 47, no. 2-3, pp. 506-10, 2009.
140. A. F. Ducruet, B. G. Hassid, W. J. Mack, S. A. Sosunov, M. L. Otten, D. J. Fusco, Z. L. Hickman, G. H. Kim, R. J. Komotar, J. Mocco, and E. S. Connolly, "C3a receptor modulation of granulocyte infiltration after murine focal cerebral ischemia is reperfusion dependent," *Journal of Cerebral Blood Flow and Metabolism,* vol. 28, no. 5, pp. 1048-58, 2008.
141. C. T. Esmon, "Does inflammation contribute to thrombotic events?," *Haemostasis,* vol. 30 Suppl 2, pp. 34-40, 2000.
142. C. J. Boos, and G. Y. Lip, "Blood clotting, inflammation, and thrombosis in cardiovascular events: perspectives," *Frontiers in Bioscience,* vol. 11, pp. 328-36, 2006.
143. S. Danese, A. Papa, S. Saibeni, A. Repici, A. Malesci, and M. Vecchi, "Inflammation and coagulation in inflammatory bowel disease: The clot thickens," *American Journal of Gastroenterology,* vol. 102, no. 1, pp. 174-86, 2007.
144. L. Armaganian, G. Kam, P. R. Eisenberg, K. B. Schechtman, and D. R. Abendschein, "Role of tissue factor-mediated coagulation in ischemia/ reperfusion-induced injury of Langendorf-perfused rabbit hearts," *Coronary Artery Disease,* vol. 11, no. 6, pp. 481-7, 2000.
145. L. Wang, H. M. Wang, J. L. Zhang, M. L. Ka, and Z. Sun, "[Damage of the expression of NF-kappa B, myeloperoxidase and tissue factor in liver tissue and disorder of blood coagulation following liver ischemia-reperfusion injury in rats]," *Zhonghua Gan Zang Bing Za Zhi,* vol. 13, no. 8, pp. 607-8, 2005.
146. J. Steffel, T. F. Luscher, and F. C. Tanner, "Tissue factor in cardiovascular diseases: molecular mechanisms and clinical implications," *Circulation,* vol. 113, no. 5, pp. 722-31, 2006.
147. F. Muller, N. J. Mutch, W. A. Schenk, S. A. Smith, L. Esterl, H. M. Spronk, S. Schmidbauer, W. A. Gahl, J. H. Morrissey, and T. Renne, "Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo," *Cell,* vol. 139, no. 6, pp. 1143-56, 2009.
148. A. P. Kaplan, K. Joseph, Y. Shibayama, S. Reddigari, B. Ghebrehiwet, and M. Silverberg, "The intrinsic coagulation/kinin-forming cascade: assembly in plasma and cell surfaces in inflammation," *Advances in Immunology,* vol. 66, pp. 225-72, 1997.
149. W. L. Chandler, M. C. Alessi, M. F. Aillaud, P. Henderson, P. Vague, and I. Juhan-Vague, "Clearance of tissue plasminogen activator (TPA) and TPA/plasminogen activator inhibitor type 1 (PAI-1) complex: relationship to elevated TPA antigen in patients with high PAI-1 activity levels," *Circulation,* vol. 96, no. 3, pp. 761-8, 1997.
150. R. Watanabe, H. Wada, Y. Miura, Y. Murata, Y. Watanabe, M. Sakakura, Y. Okugawa, T. Nakasaki, Y. Mori, M. Nishikawa, E. C. Gabazza, H. Shiku, and T. Nobori, "Plasma levels of total plasminogen activator inhibitor-I (PAI-I) and tPA/PAI-1 complex in patients with disseminated intravascular coagulation and thrombotic thrombocytopenic purpura," *Clinical and Applied Thrombosis/Hemostasis,* vol. 7, no. 3, pp. 229-33, 2001.
151. H. Bounameaux, and E. K. Kruithof, "On the association of elevated tPA/PAI-1 complex and von Willebrand factor with recurrent myocardial infarction," *Arteriosclerosis, Thrombosis, and Vascular Biology,* vol. 20, no. 8, pp. 1857-9, 2000.
152. A. Suehiro, and T. Koyama, "[Clinical usefulness of the measurement of plasma D-dimer levels]," *Rinsho Byori. Japanese Journal of Clinical Pathology,* vol. 39, no. 7, pp. 694-700, 1991.
153. M. Marci, A. Lozzi, R. Miconi, E. Panzini, M. Monaldo, and S. Raffa, "D-dimer assays in patients with carotid atherosclerosis in clinical practice," *Minerva Cardioangiologica,* vol. 48, no. 4-5, pp. 97-102, 2000.
154. J. A. Rennie, and D. Ogston, "Changes in coagulation factors following acute myocardial infarction in man," *Haemostasis,* vol. 5, no. 4, pp. 258-64, 1976.

155. N. D. Vaziri, S. C. Kennedy, D. Kennedy, and E. Gonzales, "Coagulation, fibrinolytic, and inhibitory proteins in acute myocardial infarction and angina pectoris," *American Journal of Medicine,* vol. 93, no. 6, pp. 651-7, 1992.
156. Y. Kobayashi, N. Yoshimura, H. Yamagishi, and T. Oka, "Role of tissue factor in ischemic reperfusion injury: (I). Tissue factor levels of liver tissue and serum after hepatic injury in rats," *Transplantation Proceedings,* vol. 30, no. 7, pp. 3726-7, 1998.
157. J. Sevastos, S. E. Kennedy, D. R. Davis, M. Sam, P. W. Peake, J. A. Charlesworth, N. Mackman, and J. H. Erlich, "Tissue factor deficiency and PAR-1 deficiency are protective against renal ischemia reperfusion injury," *Blood,* vol. 109, no. 2, pp. 577-83, 2007.
158. Y. Okada, B. R. Copeland, R. Fitridge, J. A. Koziol, and G. J. del Zoppo, "Fibrin contributes to microvascular obstructions and parenchymal changes during early focal cerebral ischemia and reperfusion," *Stroke,* vol. 25, no. 9, pp. 1847-53; discussion 1853-4, 1994.
159. X. S. Zhou, Y. F. Qiao, R. P. Wu, and R. S. Li, "Study on related indexes of the coagulation and fibrinolytic system after renal ischemia reperfusion injury in Wistar rats," *Saudi Medical Journal,* vol. 34, no. 6, pp. 579-83, 2013.
160. C. L. Lau, Y. Zhao, J. Kim, I. L. Kron, A. Sharma, Z. Yang, V. E. Laubach, J. Linden, G. Ailawadi, and D. J. Pinsky, "Enhanced fibrinolysis protects against lung ischemia-reperfusion injury," *Journal of Thoracic and Cardiovascular Surgery,* vol. 137, no. 5, pp. 1241-8, 2009.
161. A. K. Ajay, J. Saikumar, V. Bijol, and V. S. Vaidya, "Heterozygosity for fibrinogen results in efficient resolution of kidney ischemia reperfusion injury," *PloS One,* vol. 7, no. 9, pp. e45628, 2012.
162. S. Antoniak, M. Rojas, D. Spring, T. A. Bullard, E. D. Verrier, B. C. Blaxall, N. Mackman, and R. Pawlinski, "Protease-activated receptor 2 deficiency reduces cardiac ischemia/reperfusion injury," *Arteriosclerosis, Thrombosis, and Vascular Biology,* vol. 30, no. 11, pp. 2136-42, 2010.
163. C. Kleinschnitz, G. Stoll, M. Bendszus, K. Schuh, H. U. Pauer, P. Burfeind, C. Renne, D. Gailani, B. Nieswandt, and T. Renne, "Targeting coagulation factor XII provides protection from pathological thrombosis in cerebral ischemia without interfering with hemostasis," *Journal of Experimental Medicine,* vol. 203, no. 3, pp. 513-8, 2006.
164. M. Pham, C. Kleinschnitz, X. Helluy, A. J. Bartsch, M. Austinat, V. C. Behr, T. Renne, B. Nieswandt, G. Stoll, and M. Bendszus, "Enhanced cortical reperfusion protects coagulation factor XII-deficient mice from ischemic stroke as revealed by high-field MRI," *Neuroimage,* vol. 49, no. 4, pp. 2907-14, 2010.
165. J. H. Erlich, E. M. Boyle, J. Labriola, J. C. Kovacich, R. A. Santucci, C. Fearns, E. N. Morgan, W. Yun, T. Luther, O. Kojikawa, T. R. Martin, T. H. Pohlman, E. D. Verrier, and N. Mackman, "Inhibition of the tissue factor-thrombin pathway limits infarct size after myocardial ischemia-reperfusion injury by reducing inflammation," *American Journal of Pathology,* vol. 157, no. 6, pp. 1849-62, 2000.
166. K. Nakamura, Y. Kadotani, H. Ushigome, K. Akioka, M. Okamoto, Y. Ohmori, T. Yaoi, S. Fushiki, R. Yoshimura, and N. Yoshimura, "Antisense oligonucleotide for tissue factor inhibits hepatic ischemic reperfusion injury," *Biochemical and Biophysical Research Communications,* vol. 297, no. 3, pp. 433-41, 2002.
167. X. He, B. Han, M. Mura, L. Li, M. Cypel, A. Soderman, K. Picha, J. Yang, and M. Liu, "Anti-human tissue factor antibody ameliorated intestinal ischemia reperfusion-induced acute lung injury in human tissue factor knock-in mice," *PloS One,* vol. 3, no. 1, pp. e1527, 2008.
168. W. S. Thomas, E. Mori, B. R. Copeland, J. Q. Yu, J. H. Morrissey, and G. J. del Zoppo, "Tissue factor contributes to microvascular defects after focal cerebral ischemia," *Stroke,* vol. 24, no. 6, pp. 847-53; discussion 847, 1993.
169. J. W. Chen, J. L. Figueiredo, G. R. Wojtkiewicz, C. Siegel, Y. Iwamoto, D. E. Kim, M. W. Nolte, G. Dickneite, R. Weissleder, and M. Nahrendorf, "Selective factor XIIa inhibition attenuates silent brain ischemia: application of molecular imaging targeting coagulation pathway," *JACC: Cardiovascular Imaging,* vol. 5, no. 11, pp. 1127-38, 2012.
170. P. Kraft, T. Schwarz, L. Pochet, G. Stoll, and C. Kleinschnitz, "COU254, a specific 3-carboxamide-coumarin inhibitor of coagulation factor XII, does not protect mice from acute ischemic stroke," *Experimental & Translational Stroke Medicine,* vol. 2, no. 1, pp. 5, 2010.
171. P. Y. Leung, S. Hurst, M. A. Berny-Lang, N. G. Verbout, D. Gailani, E. I. Tucker, R. K. Wang, O. J. McCarty, and A. Gruber, "Inhibition of Factor XII-Mediated Activation of Factor XI Provides Protection Against Experimental Acute Ischemic Stroke in Mice," *Transl Stroke Res,* vol. 3, no. 3, pp. 381-9, 2012.
172. N. Yoshimura, Y. Kobayashi, K. Nakamura, H. Yamagishi, and T. Oka, "The effect of tissue factor pathway inhibitor on hepatic ischemic reperfusion injury of the rat," *Transplantation,* vol. 67, no. 1, pp. 45-53, 1999.
173. P. Golino, M. Ragni, P. Cirillo, A. Scognamiglio, A. Ravera, C. Buono, A. Guarino, O. Piro, C. Lambiase, F. Botticella, M. Ezban, M. Condorelli, and M. Chiariello, "Recombinant human, active site-blocked factor VIIa reduces infarct size and no-reflow phenomenon in rabbits," *American Journal of Physiology: Heart and Circulatory Physiology,* vol. 278, no. 5, pp. H1507-16, 2000.
174. S. T. Loubele, C. A. Spek, P. Leenders, R. van Oerle, H. L. Aberson, D. van der Voort, K. Hamulyak, L. C. Petersen, H. M. Spronk, and H. ten Cate, "Active site inhibited factor VIIa attenuates myocardial ischemia/reperfusion injury in mice," *Journal of Thrombosis and Haemostasis,* vol. 7, no. 2, pp. 290-8, 2009.

175. Y. Yamaguchi, K. Okabe, J. Liang, F. Matsumura, H. Ohshiro, K. Ishihara, T. Matsuda, M. Takeya, J. I. Kuratsu, K. Mori, S. Yamada, and M. Ogawa, "A synthetic selective inhibitor of factor Xa, DX-9065a, reduces monocyte chemoattractant protein-1 expression after ischemia-reperfusion injury in rat liver," *Digestive Diseases and Sciences,* vol. 44, no. 12, pp. 2568-76, 1999.
176. R. C. Becker, J. H. Alexander, L. K. Newby, H. Yang, Y. Barrett, P. Mohan, J. Wang, R. A. Harrington, and L. C. Wallentin, "Effect of apixaban, an oral and direct factor Xa inhibitor, on coagulation activity biomarkers following acute coronary syndrome," *Thrombosis and Haemostasis,* vol. 104, no. 5, pp. 976-83, 2010.
177. J. L. Mega, E. Braunwald, S. D. Wiviott, J. P. Bassand, D. L. Bhatt, C. Bode, P. Burton, M. Cohen, N. Cook-Bruns, K. A. Fox, S. Goto, S. A. Murphy, A. N. Plotnikov, D. Schneider, X. Sun, F. W. Verheugt, C. M. Gibson, and A. A. T. Investigators, "Rivaroxaban in patients with a recent acute coronary syndrome," *New England Journal of Medicine,* vol. 366, no. 1, pp. 9-19, 2012.
178. D. H. Fitchett, "Potential role of rivaroxaban in patients with acute coronary syndrome," *Drug Design, Development and Therapy,* vol. 6, pp. 349-57, 2012.
179. P. G. Steg, S. R. Mehta, J. W. Jukema, G. Y. Lip, C. M. Gibson, F. Kovar, P. Kala, A. Garcia-Hernandez, R. W. Renfurm, C. B. Granger, and R.-. Investigators, "RUBY-1: a randomized, double-blind, placebo-controlled trial of the safety and tolerability of the novel oral factor Xa inhibitor darexaban (YM150) following acute coronary syndrome," *European Heart Journal,* vol. 32, no. 20, pp. 2541-54, 2011.
180. S. Goldstein, E. R. Bates, D. L. Bhatt, C. Cao, D. Holmes, S. Kupfer, F. Martinez, J. Spaeder, J. I. Weitz, Z. Ye, F. Zannad, and A. investigators, "Phase 2 study of TAK-442, an oral factor Xa inhibitor, in patients following acute coronary syndrome," *Thrombosis and Haemostasis,* vol. 111, no. 6, pp. 1141-52, 2014.
181. A. J. Chong, T. H. Pohlman, C. R. Hampton, A. Shimamoto, N. Mackman, and E. D. Verrier, "Tissue factor and thrombin mediate myocardial ischemia-reperfusion injury," *Annals of Thoracic Surgery,* vol. 75, no. 2, pp. S649-55, 2003.
182. W. Elikowski, P. Psuja, K. Lewandowski, M. Przybyl, M. Wendland, D. Wroblewski, B. Jazienicki, L. Przybyl, and K. Zawilska, "[Low molecular weight heparin (Fraxiparine) as adjunctive therapy with thrombolysis for acute myocardial infarction: a pilot study with a one year follow up]," *Polskie Archiwum Medycyny Wewnetrznej,* vol. 95, no. 1, pp. 53-61, 1996.
183. E. Buccelletti, C. Spadaccio, M. Chello, D. Marsiliani, A. Carroccia, P. Iacomini, G. Calabro, N. Gentiloni Silveri, and F. Franceschi, "A critical review of the efficacy and safety in the use of low-molecular-weight heparin in acute ST-elevation myocardial infarction: a Bayesan approach," *European Review for Medical and Pharmacological Sciences,* vol. 15, no. 6, pp. 701-10, 2011.
184. N. Harada, K. Okajima, and M. Uchiba, "Dalteparin, a low molecular weight heparin, attenuates inflammatory responses and reduces ischemia-reperfusion-induced liver injury in rats," *Critical Care Medicine,* vol. 34, no. 7, pp. 1883-91, 2006.
185. Z. G. Zhang, Q. Z. Zhang, Y. N. Cheng, S. L. Ji, and G. H. Du, "Antagonistic effects of ultra-low-molecular-weight heparin against cerebral ischemia/reperfusion injury in rats," *Pharmacological Research,* vol. 56, no. 4, pp. 350-5, 2007.
186. A. F. Shorr, W. L. Jackson, J. H. Sherner, and L. K. Moores, "Differences between low-molecular-weight and unfractionated heparin for venous thromboembolism prevention following ischemic stroke: a metaanalysis," *Chest,* vol. 133, no. 1, pp. 149-55, 2008.
187. P. A. Sandercock, C. Counsell, and M. C. Tseng, "Low-molecular-weight heparins or heparinoids versus standard unfractionated heparin for acute ischaemic stroke," *Cochrane Database Syst Rev,* no. 3, pp. CD000119, 2008.
188. W. N. Lay, B. S. Rayner, T. Sabaretnam, G. A. Figtree, C. Jackson, S. N. Hunyor, and R. Bhindi, "Activated protein C improves left ventricular remodelling after ischemia-reperfusion injury in rats," *International Journal of Cardiology,* vol. 159, no. 3, pp. 246-8, 2012.
189. A. Mizutani, K. Okajima, M. Uchiba, and T. Noguchi, "Activated protein C reduces ischemia/reperfusion-induced renal injury in rats by inhibiting leukocyte activation," *Blood,* vol. 95, no. 12, pp. 3781-7, 2000.
190. J. P. Dillon, A. J. Laing, R. A. Cahill, G. C. O'Brien, J. T. Street, J. H. Wang, A. Mc Guinness, and H. P. Redmond, "Activated protein C attenuates acute ischaemia reperfusion injury in skeletal muscle," *Journal of Orthopaedic Research,* vol. 23, no. 6, pp. 1454-9, 2005.
191. M. Shibata, S. R. Kumar, A. Amar, J. A. Fernandez, F. Hofman, J. H. Griffin, and B. V. Zlokovic, "Anti-inflammatory, antithrombotic, and neuroprotective effects of activated protein C in a murine model of focal ischemic stroke," *Circulation,* vol. 103, no. 13, pp. 1799-805, 2001.
192. C. J. Wiedermann, and N. C. Kaneider, "A meta-analysis of controlled trials of recombinant human activated protein C therapy in patients with sepsis," *BMC Emergency Medicine,* vol. 5, pp. 7, 2005.
193. J. Thachil, C. H. Toh, M. Levi, and H. G. Watson, "The withdrawal of Activated Protein C from the use in patients with severe sepsis and DIC [Amendment to the BCSH guideline on disseminated intravascular coagulation]," *British Journal of Haematology,* vol. 157, no. 4, pp. 493-4, 2012.
194. A. Ozden, A. Sarioglu, N. C. Demirkan, A. Bilgihan, and E. Duzcan, "Antithrombin III reduces renal ischemia-reperfusion injury in rats," *Research in Experimental Medicine,* vol. 200, no. 3, pp. 195-203, 2001.

195. N. A. Zambas, C. D. Karkos, A. G. Kambaroudis, D. G. Karamanos, C. T. Spyridis, and T. S. Gerassimidis, "Protective effect of antithrombin III against lung and myocardial injury in lower-limb ischemia-reperfusion syndrome," *Annals of Vascular Surgery,* vol. 26, no. 4, pp. 566-70, 2012.
196. K. Okano, Y. Kokudo, K. Okajima, M. A. Hossain, K. Ishimura, S. Yachida, T. Tsubouchi, H. Wakabayashi, T. Maeba, and H. Maeta, "Protective effects of antithrombin III supplementation on warm ischemia and reperfusion injury in rat liver," *World Journal of Surgery,* vol. 20, no. 8, pp. 1069-75, 1996.
197. C. Koksal, A. K. Bozkurt, G. Sirin, D. Konukoglu, and N. Ustundag, "Aprotinin ameliorates ischemia/reperfusion injury in a rat hind limb model," *Vascular Pharmacology,* vol. 41, no. 4-5, pp. 125-9, 2004.
198. Z. Ozer, N. Sucu, D. Dusmez, L. Tamer, A. A. Altunkan, D. I. M, and U. Oral, "The effect of aprotinin on ischemia-reperfusion injury in the rabbit kidney," *Pharmacological Research,* vol. 44, no. 6, pp. 455-60, 2001.
199. S. M. Maksan, M. O. Maksan, M. M. Gebhard, C. Herfarth, and E. Klar, "Reduction of hepatic reperfusion injury by antithrombin III and aprotinin," *Transplant International,* vol. 13 Suppl 1, pp. S562-4, 2000.
200. D. T. Mangano, I. C. Tudor, C. Dietzel, G. Multicenter Study of Perioperative Ischemia Research, R. Ischemia, and F. Education, "The risk associated with aprotinin in cardiac surgery," *New England Journal of Medicine,* vol. 354, no. 4, pp. 353-65, 2006.
201. V. McMullan, and R. P. Alston, "III. Aprotinin and cardiac surgery: a sorry tale of evidence misused," *British Journal of Anaesthesia,* vol. 110, no. 5, pp. 675-8, 2013.
202. R. W. Colman, and A. H. Schmaier, "Contact system: a vascular biology modulator with anticoagulant, profibrinolytic, antiadhesive, and proinflammatory attributes," *Blood,* vol. 90, no. 10, pp. 3819-43, 1997.
203. Y. Wu, "Contact pathway of coagulation and inflammation," *Thromb J,* vol. 13, pp. 17, 2015.
204. C. Maas, C. Oschatz, and T. Renne, "The plasma contact system 2.0," *Seminars in Thrombosis and Hemostasis,* vol. 37, no. 4, pp. 375-81, 2011.
205. A. Yang, J. Dai, Z. Xie, R. W. Colman, Q. Wu, R. B. Birge, and Y. Wu, "High molecular weight kininogen binds phosphatidylserine and opsonizes urokinase plasminogen activator receptor-mediated efferocytosis," *Journal of Immunology,* vol. 192, no. 9, pp. 4398-408, 2014.
206. K. Joseph, B. G. Tholanikunnel, A. Bygum, B. Ghebrehiwet, and A. P. Kaplan, "Factor XII-independent activation of the bradykinin-forming cascade: Implications for the pathogenesis of hereditary angioedema types I and II," *Journal of Allergy and Clinical Immunology,* vol. 132, no. 2, pp. 470-5, 2013.
207. C. Golias, A. Charalabopoulos, D. Stagikas, K. Charalabopoulos, and A. Batistatou, "The kinin system--bradykinin: biological effects and clinical implications. Multiple role of the kinin system--bradykinin," *Hippokratia,* vol. 11, no. 3, pp. 124-8, 2007.
208. D. Regoli, S. Nsa Allogho, A. Rizzi, and F. J. Gobeil, "Bradykinin receptors and their antagonists," *European Journal of Pharmacology,* vol. 348, no. 1, pp. 1-10, 1998.
209. S. H. Ferreira, and J. R. Vane, "The disappearance of bradykinin and eledoisin in the circulation and vascular beds of the cat," *British Journal of Pharmacology and Chemotherapy,* vol. 30, no. 2, pp. 417-24, 1967.
210. K. D. Bhoola, C. D. Figueroa, and K. Worthy, "Bioregulation of kinins: kallikreins, kininogens, and kininases," *Pharmacological Reviews,* vol. 44, no. 1, pp. 1-80, 1992.
211. L. J. Murphey, D. L. Hachey, J. A. Oates, J. D. Morrow, and N. J. Brown, "Metabolism of bradykinin In vivo in humans: identification of BK1-5 as a stable plasma peptide metabolite," *Journal of Pharmacology and Experimental Therapeutics,* vol. 294, no. 1, pp. 263-9, 2000.
212. C. Albert-Weissenberger, A. L. Siren, and C. Kleinschnitz, "Ischemic stroke and traumatic brain injury: the role of the kallikrein-kinin system," *Progress in Neurobiology,* vol. 101-102, pp. 65-82, 2013.
213. T. Renne, M. Pozgajova, S. Gruner, K. Schuh, H. U. Pauer, P. Burfeind, D. Gailani, and B. Nieswandt, "Defective thrombus formation in mice lacking coagulation factor XII," *Journal of Experimental Medicine,* vol. 202, no. 2, pp. 271-81, 2005.
214. T. Iwaki, and F. J. Castellino, "Plasma levels of bradykinin are suppressed in factor XII-deficient mice," *Thrombosis and Haemostasis,* vol. 95, no. 6, pp. 1003-10, 2006.
215. Q. Cheng, E. I. Tucker, M. S. Pine, I. Sisler, A. Matafonov, M. F. Sun, T. C. White-Adams, S. A. Smith, S. R. Hanson, O. J. McCarty, T. Renne, A. Gruber, and D. Gailani, "A role for factor XIIa-mediated factor XI activation in thrombus formation in vivo," *Blood,* vol. 116, no. 19, pp. 3981-9, 2010.
216. X. Wang, P. L. Smith, M. Y. Hsu, D. Gailani, W. A. Schumacher, M. L. Ogletree, and D. A. Seiffert, "Effects of factor XI deficiency on ferric chloride-induced vena cava thrombosis in mice," *Journal of Thrombosis and Haemostasis,* vol. 4, no. 9, pp. 1982-8, 2006.
217. A. S. Revenko, D. Gao, J. R. Crosby, G. Bhattacharjee, C. Zhao, C. May, D. Gailani, B. P. Monia, and A. R. MacLeod, "Selective depletion of plasma prekallikrein or coagulation factor XII inhibits thrombosis in mice without increased risk of bleeding," *Blood,* vol. 118, no. 19, pp. 5302-11, 2011.

218. A. Girolami, E. Allemand, I. Bertozzi, N. Candeo, S. Marun, and B. Girolami, "Thrombotic events in patients with congenital prekallikrein deficiency: a critical evaluation of all reported cases," *Acta Haematologica,* vol. 123, no. 4, pp. 210-4, 2010.
219. E. U. Bojanini, A. Loaiza-Bonilla, and A. Pimentel, "Prekallikrein deficiency presenting as recurrent cerebrovascular accident: case report and review of the literature," *Case Rep Hematol,* vol. 2012, pp. 723204, 2012.
220. S. Pons, V. Griol-Charhbili, C. Heymes, P. Fornes, D. Heudes, A. Hagege, X. Loyer, P. Meneton, J. F. Giudicelli, J. L. Samuel, F. Alhenc-Gelas, and C. Richer, "Tissue kallikrein deficiency aggravates cardiac remodelling and decreases survival after myocardial infarction in mice," *European Journal of Heart Failure,* vol. 10, no. 4, pp. 343-51, 2008.
221. E. Gob, S. Reymann, F. Langhauser, M. K. Schuhmann, P. Kraft, I. Thielmann, K. Gobel, M. Brede, G. Homola, L. Solymosi, G. Stoll, C. Geis, S. G. Meuth, B. Nieswandt, and C. Kleinschnitz, "Blocking of plasma kallikrein ameliorates stroke by reducing thromboinflammation," *Annals of Neurology,* vol. 77, no. 5, pp. 784-803, 2015.
222. Q. Zhang, H. Ding, J. Yan, W. Wang, A. Ma, Z. Zhu, K. Cianflone, F. B. Hu, R. Hui, and D. W. Wang, "Plasma tissue kallikrein level is negatively associated with incident and recurrent stroke: a multicenter case-control study in China," *Annals of Neurology,* vol. 70, no. 2, pp. 265-73, 2011.
223. F. Langhauser, E. Gob, P. Kraft, C. Geis, J. Schmitt, M. Brede, K. Gobel, X. Helluy, M. Pham, M. Bendszus, P. Jakob, G. Stoll, S. G. Meuth, B. Nieswandt, K. R. McCrae, and C. Kleinschnitz, "Kininogen deficiency protects from ischemic neurodegeneration in mice by reducing thrombosis, blood-brain barrier damage, and inflammation," *Blood,* vol. 120, no. 19, pp. 4082-92, 2012.
224. D. G. Souza, E. S. Lomez, V. Pinho, J. B. Pesquero, M. Bader, J. L. Pesquero, and M. M. Teixeira, "Role of bradykinin B2 and B1 receptors in the local, remote, and systemic inflammatory responses that follow intestinal ischemia and reperfusion injury," *Journal of Immunology,* vol. 172, no. 4, pp. 2542-8, 2004.
225. P. H. Wang, M. A. Cenedeze, J. B. Pesquero, A. Pacheco-Silva, and N. O. Camara, "Influence of bradykinin B1 and B2 receptors in the immune response triggered by renal ischemia-reperfusion injury," *International Immunopharmacology,* vol. 6, no. 13-14, pp. 1960-5, 2006.
226. H. Yin, J. Chao, M. Bader, and L. Chao, "Differential role of kinin B1 and B2 receptors in ischemia-induced apoptosis and ventricular remodeling," *Peptides,* vol. 28, no. 7, pp. 1383-9, 2007.
227. M. Kakoki, R. W. McGarrah, H. S. Kim, and O. Smithies, "Bradykinin B1 and B2 receptors both have protective roles in renal ischemia/reperfusion injury," *Proceedings of the National Academy of Sciences of the United States of America,* vol. 104, no. 18, pp. 7576-81, 2007.
228. M. Groger, D. Lebesgue, D. Pruneau, J. Relton, S. W. Kim, J. Nussberger, and N. Plesnila, "Release of bradykinin and expression of kinin B2 receptors in the brain: role for cell death and brain edema formation after focal cerebral ischemia in mice," *Journal of Cerebral Blood Flow and Metabolism,* vol. 25, no. 8, pp. 978-89, 2005.
229. M. Austinat, S. Braeuninger, J. B. Pesquero, M. Brede, M. Bader, G. Stoll, T. Renne, and C. Kleinschnitz, "Blockade of bradykinin receptor B1 but not bradykinin receptor B2 provides protection from cerebral infarction and brain edema," *Stroke,* vol. 40, no. 1, pp. 285-93, 2009.
230. D. Wu, X. Lin, C. Bernloehr, T. Hildebrandt, and H. Doods, "Effects of a novel bradykinin B1 receptor antagonist and angiotensin II receptor blockade on experimental myocardial infarction in rats," *PloS One,* vol. 7, no. 12, pp. e51151, 2012.
231. C. Lagneux, M. Bader, J. B. Pesquero, P. Demenge, and C. Ribuot, "Detrimental implication of B1 receptors in myocardial ischemia: evidence from pharmacological blockade and gene knockout mice," *International Immunopharmacology,* vol. 2, no. 6, pp. 815-22, 2002.
232. C. Tschope, F. Spillmann, C. Altmann, M. Koch, D. Westermann, N. Dhayat, S. Dhayat, J. L. Bascands, L. Gera, S. Hoffmann, H. P. Schultheiss, and T. Walther, "The bradykinin B1 receptor contributes to the cardioprotective effects of AT1 blockade after experimental myocardial infarction," *Cardiovascular Research,* vol. 61, no. 3, pp. 559-69, 2004.
233. J. K. Relton, V. E. Beckey, W. L. Hanson, and E. T. Whalley, "CP-0597, a selective bradykinin B2 receptor antagonist, inhibits brain injury in a rat model of reversible middle cerebral artery occlusion," *Stroke,* vol. 28, no. 7, pp. 1430-6, 1997.
234. K. Arakawa, I. Takeyoshi, Y. Akao, O. Totsuka, K. Matsumoto, and Y. Morishita, "Bradykinin B2 receptor antagonist FR173657 ameliorates small bowel ischemia-reperfusion injury in dogs," *Digestive Diseases and Sciences,* vol. 50, no. 1, pp. 27-36, 2005.
235. N. Hashimoto, I. Takeyoshi, H. Tsutsumi, Y. Sunose, M. Tokumine, O. Totsuka, S. Ohwada, T. Yokoe, K. Matsumoto, and Y. Morishita, "Effects of a bradykinin B(2) receptor antagonist, FR173657, on pulmonary ischemia-reperfusion injury in dogs," *Journal of Heart and Lung Transplantation,* vol. 21, no. 9, pp. 1022-9, 2002.
236. N. Hashimoto, I. Takeyoshi, H. Tsutsumi, Y. Sunose, M. Tokumine, O. Totsuka, S. Ohwada, K. Matsumoto, and Y. Morishita, "Effects of a bradykinin B(2) receptor antagonist on ischemia-reperfusion injury in a canine lung transplantation model," *Journal of Heart and Lung Transplantation,* vol. 23, no. 5, pp. 606-13, 2004.
237. K. Nowak, S. Weih, S. Post, M. M. Gebhard, and P. Hohenberger, "Bradykinin in ischemia-reperfusion injury of the rat lung," *Journal of Physiology and Pharmacology,* vol. 58 Suppl 5, no. Pt 2, pp. 513-22, 2007.

238. L. R. Mendonca, E. E. Joviliano, F. S. Ramalho, L. N. Ramalho, P. R. Evora, and C. E. Piccinato, "Bradykinin Impairs and HOE 140 does not Protect Rat Hindlimb Skeletal Muscle Against Tourniquet-induced Reperfusion Injury," *Journal of Investigative Surgery,* pp. 1-7, 2015.
239. R. Chahine, A. Adam, N. Yamaguchi, R. Gaspo, D. Regoli, and R. Nadeau, "Protective effects of bradykinin on the ischaemic heart: implication of the B1 receptor," *British Journal of Pharmacology,* vol. 108, no. 2, pp. 318-22, 1993.
240. J. Lehmberg, J. Beck, A. Baethmann, and E. Uhl, "Bradykinin antagonists reduce leukocyte-endothelium interactions after global cerebral ischemia," *Journal of Cerebral Blood Flow and Metabolism,* vol. 23, no. 4, pp. 441-8, 2003.
241. H. Yoshida, J. J. Zhang, L. Chao, and J. Chao, "Kallikrein gene delivery attenuates myocardial infarction and apoptosis after myocardial ischemia and reperfusion," *Hypertension,* vol. 35, no. 1 Pt 1, pp. 25-31, 2000.
242. C. F. Xia, H. Yin, C. V. Borlongan, L. Chao, and J. Chao, "Kallikrein gene transfer protects against ischemic stroke by promoting glial cell migration and inhibiting apoptosis," *Hypertension,* vol. 43, no. 2, pp. 452-9, 2004.
243. C. Storini, L. Bergamaschini, R. Gesuete, E. Rossi, D. Maiocchi, and M. G. De Simoni, "Selective inhibition of plasma kallikrein protects brain from reperfusion injury," *Journal of Pharmacology and Experimental Therapeutics,* vol. 318, no. 2, pp. 849-54, 2006.
244. C. M. Pedersen, M. R. Schmidt, G. Barnes, H. E. Botker, R. K. Kharbanda, D. E. Newby, and N. L. Cruden, "Bradykinin does not mediate remote ischaemic preconditioning or ischaemia-reperfusion injury in vivo in man," *Heart,* vol. 97, no. 22, pp. 1857-61, 2011.
245. J. M. Balaguer, C. Yu, J. G. Byrne, S. K. Ball, M. R. Petracek, N. J. Brown, and M. Pretorius, "Contribution of endogenous bradykinin to fibrinolysis, inflammation, and blood product transfusion following cardiac surgery: a randomized clinical trial," *Clinical Pharmacology and Therapeutics,* vol. 93, no. 4, pp. 326-34, 2013.
246. R. Couture, N. Blaes, and J. P. Girolami, "Kinin receptors in vascular biology and pathology," *Current Vascular Pharmacology,* vol. 12, no. 2, pp. 223-48, 2014.
247. M. M. Krem, and E. Di Cera, "Evolution of enzyme cascades from embryonic development to blood coagulation," *Trends in Biochemical Sciences,* vol. 27, no. 2, pp. 67-74, 2002.
248. M. Dissing, H. Giordano, and R. DeLotto, "Autoproteolysis and feedback in a protease cascade directing Drosophila dorsal-ventral cell fate," *EMBO Journal,* vol. 20, no. 10, pp. 2387-93, 2001.
249. E. K. LeMosy, Y. Q. Tan, and C. Hashimoto, "Activation of a protease cascade involved in patterning the Drosophila embryo," *Proceedings of the National Academy of Sciences of the United States of America,* vol. 98, no. 9, pp. 5055-60, 2001.
250. J. S. Presanis, K. Hajela, G. Ambrus, P. Gal, and R. B. Sim, "Differential substrate and inhibitor profiles for human MASP-1 and MASP-2," *Molecular Immunology,* vol. 40, no. 13, pp. 921-9, 2004.
251. K. Hajela, M. Kojima, G. Ambrus, K. H. Wong, B. E. Moffatt, J. Ferluga, S. Hajela, P. Gal, and R. B. Sim, "The biological functions of MBL-associated serine proteases (MASPs)," *Immunobiology,* vol. 205, no. 4-5, pp. 467-75, 2002.
252. J. Dobo, B. Major, K. A. Kekesi, I. Szabo, M. Megyeri, K. Hajela, G. Juhasz, P. Zavodszky, and P. Gal, "Cleavage of kininogen and subsequent bradykinin release by the complement component: mannose-binding lectin-associated serine protease (MASP)-1," *PloS One,* vol. 6, no. 5, pp. e20036, 2011.
253. M. Takahashi, Y. Ishida, D. Iwaki, K. Kanno, T. Suzuki, Y. Endo, Y. Homma, and T. Fujita, "Essential role of mannose-binding lectin-associated serine protease-1 in activation of the complement factor D," *Journal of Experimental Medicine,* vol. 207, no. 1, pp. 29-37, 2010.
254. G. Oroszlan, E. Kortvely, D. Szakacs, A. Kocsis, S. Dammeier, A. Zeck, M. Ueffing, P. Zavodszky, G. Pal, P. Gal, and J. Dobo, "MASP-1 and MASP-2 Do Not Activate Pro-Factor D in Resting Human Blood, whereas MASP-3 Is a Potential Activator: Kinetic Analysis Involving Specific MASP-1 and MASP-2 Inhibitors," *Journal of Immunology,* 2015.
255. A. Krarup, R. Wallis, J. S. Presanis, P. Gal, and R. B. Sim, "Simultaneous activation of complement and coagulation by MBL-associated serine protease 2," *PloS One,* vol. 2, no. 7, pp. e623, 2007.
256. K. C. Gulla, K. Gupta, A. Krarup, P. Gal, W. J. Schwaeble, R. B. Sim, C. D. O'Connor, and K. Hajela, "Activation of mannan-binding lectin-associated serine proteases leads to generation of a fibrin clot," *Immunology,* vol. 129, no. 4, pp. 482-95, 2010.
257. M. J. Polley, and R. L. Nachman, "Human platelet activation by C3a and C3a des-arg," *Journal of Experimental Medicine,* vol. 158, no. 2, pp. 603-15, 1983.
258. P. J. Sims, and T. Wiedmer, "The response of human platelets to activated components of the complement system," *Immunology Today,* vol. 12, no. 9, pp. 338-42, 1991.
259. P. J. Sims, E. M. Faioni, T. Wiedmer, and S. J. Shattil, "Complement proteins C5b-9 cause release of membrane vesicles from the platelet surface that are enriched in the membrane receptor for coagulation factor Va and express prothrombinase activity," *Journal of Biological Chemistry,* vol. 263, no. 34, pp. 18205-12, 1988.
260. T. W. Muhlfelder, J. Niemetz, D. Kreutzer, D. Beebe, P. A. Ward, and S. I. Rosenfeld, "C5 chemotactic fragment induces leukocyte production of tissue factor activity: a link between complement and coagulation," *Journal of Clinical Investigation,* vol. 63, no. 1, pp. 147-50, 1979.

261. K. Ikeda, K. Nagasawa, T. Horiuchi, T. Tsuru, H. Nishizaka, and Y. Niho, "C5a induces tissue factor activity on endothelial cells," *Thrombosis and Haemostasis,* vol. 77, no. 2, pp. 394-8, 1997.
262. F. Tedesco, M. Pausa, E. Nardon, M. Introna, A. Mantovani, and A. Dobrina, "The cytolytically inactive terminal complement complex activates endothelial cells to express adhesion molecules and tissue factor procoagulant activity," *Journal of Experimental Medicine,* vol. 185, no. 9, pp. 1619-27, 1997.
263. U. Amara, M. A. Flierl, D. Rittirsch, A. Klos, H. Chen, B. Acker, U. B. Bruckner, B. Nilsson, F. Gebhard, J. D. Lambris, and M. Huber-Lang, "Molecular intercommunication between the complement and coagulation systems," *Journal of Immunology,* vol. 185, no. 9, pp. 5628-36, 2010.
264. M. Huber-Lang, J. V. Sarma, F. S. Zetoune, D. Rittirsch, T. A. Neff, S. R. McGuire, J. D. Lambris, R. L. Warner, M. A. Flierl, L. M. Hoesel, F. Gebhard, J. G. Younger, S. M. Drouin, R. A. Wetsel, and P. A. Ward, "Generation of C5a in the absence of C3: a new complement activation pathway," *Nature Medicine,* vol. 12, no. 6, pp. 682-7, 2006.
265. B. Ghebrehiwet, M. Silverberg, and A. P. Kaplan, "Activation of the classical pathway of complement by Hageman factor fragment," *Journal of Experimental Medicine,* vol. 153, no. 3, pp. 665-76, 1981.
266. B. Ghebrehiwet, B. P. Randazzo, J. T. Dunn, M. Silverberg, and A. P. Kaplan, "Mechanisms of activation of the classical pathway of complement by Hageman factor fragment," *Journal of Clinical Investigation,* vol. 71, no. 5, pp. 1450-6, 1983.
267. O. A. Hamad, P. H. Nilsson, M. Lasaosa, D. Ricklin, J. D. Lambris, B. Nilsson, and K. N. Ekdahl, "Contribution of chondroitin sulfate A to the binding of complement proteins to activated platelets," *PloS One,* vol. 5, no. 9, pp. e12889, 2010.
268. Y. Endo, N. Nakazawa, D. Iwaki, M. Takahashi, M. Matsushita, and T. Fujita, "Interactions of ficolin and mannose-binding lectin with fibrinogen/fibrin augment the lectin complement pathway," *Journal of Innate Immunity,* vol. 2, no. 1, pp. 33-42, 2010.
269. J. Wojta, C. Kaun, G. Zorn, M. Ghannadan, A. W. Hauswirth, W. R. Sperr, G. Fritsch, D. Printz, B. R. Binder, G. Schatzl, J. Zwirner, G. Maurer, K. Huber, and P. Valent, "C5a stimulates production of plasminogen activator inhibitor-1 in human mast cells and basophils," *Blood,* vol. 100, no. 2, pp. 517-23, 2002.
270. J. Wojta, K. Huber, and P. Valent, "New aspects in thrombotic research: complement induced switch in mast cells from a profibrinolytic to a prothrombotic phenotype," *Pathophysiology of Haemostasis and Thrombosis,* vol. 33, no. 5-6, pp. 438-41, 2003.
271. V. J. Christiansen, P. J. Sims, and K. K. Hamilton, "Complement C5b-9 increases plasminogen binding and activation on human endothelial cells," *Arteriosclerosis, Thrombosis, and Vascular Biology,* vol. 17, no. 1, pp. 164-71, 1997.
272. R. C. Wiggins, P. C. Giclas, and P. M. Henson, "Chemotactic activity generated from the fifth component of complement by plasma kallikrein of the rabbit," *Journal of Experimental Medicine,* vol. 153, no. 6, pp. 1391-404, 1981.
273. R. G. DiScipio, "The activation of the alternative pathway C3 convertase by human plasma kallikrein," *Immunology,* vol. 45, no. 3, pp. 587-95, 1982.
274. P. S. Hiemstra, M. R. Daha, and B. N. Bouma, "Activation of factor B of the complement system by kallikrein and its light chain," *Thrombosis Research,* vol. 38, no. 5, pp. 491-503, 1985.
275. Z. Shariat-Madar, F. Mahdi, and A. H. Schmaier, "Assembly and activation of the plasma kallikrein/kinin system: a new interpretation," *International Immunopharmacology,* vol. 2, no. 13-14, pp. 1841-9, 2002.
276. F. Mahdi, Z. S. Madar, C. D. Figueroa, and A. H. Schmaier, "Factor XII interacts with the multiprotein assembly of urokinase plasminogen activator receptor, gC1qR, and cytokeratin 1 on endothelial cell membranes," *Blood,* vol. 99, no. 10, pp. 3585-96, 2002.
277. R. N. Puri, E. J. Gustafson, F. X. Zhou, H. Bradford, R. F. Colman, and R. W. Colman, "Inhibition of thrombin-induced platelet aggregation by high molecular weight kininogen," *Transactions of the Association of American Physicians,* vol. 100, pp. 232-40, 1987.
278. R. W. Colman, R. N. Puri, E. J. Gustafson, F. X. Zhou, and H. Bradford, "Inhibition of thrombin-induced platelet aggregation by high molecular weight kininogen," *Advances in Experimental Medicine and Biology,* vol. 247A, pp. 349-51, 1989.
279. A. A. Hasan, S. Amenta, and A. H. Schmaier, "Bradykinin and its metabolite, Arg-Pro-Pro-Gly-Phe, are selective inhibitors of alpha-thrombin-induced platelet activation," *Circulation,* vol. 94, no. 3, pp. 517-28, 1996.
280. L. J. Murphey, H. A. Malave, J. Petro, I. Biaggioni, D. W. Byrne, D. E. Vaughan, J. M. Luther, M. Pretorius, and N. J. Brown, "Bradykinin and its metabolite bradykinin 1-5 inhibit thrombin-induced platelet aggregation in humans," *Journal of Pharmacology and Experimental Therapeutics,* vol. 318, no. 3, pp. 1287-92, 2006.
281. L. O. Mosnier, X. V. Yang, and J. H. Griffin, "Activated protein C mutant with minimal anticoagulant activity, normal cytoprotective activity, and preservation of thrombin activable fibrinolysis inhibitor-dependent cytoprotective functions," *Journal of Biological Chemistry,* vol. 282, no. 45, pp. 33022-33, 2007.
282. S. Reitsma, D. W. Slaaf, H. Vink, M. A. van Zandvoort, and M. G. oude Egbrink, "The endothelial glycocalyx: composition, functions, and visualization," *Pflügers Archiv. European Journal of Physiology,* vol. 454, no. 3, pp. 345-59, 2007.

283. J. M. Tarbell, and M. Y. Pahakis, "Mechanotransduction and the glycocalyx," *Journal of Internal Medicine,* vol. 259, no. 4, pp. 339-50, 2006.
284. W. A. Wuillemin, H. te Velthuis, Y. T. Lubbers, C. P. de Ruig, E. Eldering, and C. E. Hack, "Potentiation of C1 inhibitor by glycosaminoglycans: dextran sulfate species are effective inhibitors of in vitro complement activation in plasma," *Journal of Immunology,* vol. 159, no. 4, pp. 1953-60, 1997.
285. E. Migliorini, D. Thakar, J. Kuhnle, R. Sadir, D. P. Dyer, Y. Li, C. Sun, B. F. Volkman, T. M. Handel, L. Coche-Guerente, D. G. Fernig, H. Lortat-Jacob, and R. P. Richter, "Cytokines and growth factors cross-link heparan sulfate," *Open Biol,* vol. 5, no. 8, 2015.
286. U. Jung, and K. Ley, "Regulation of E-selectin, P-selectin, and intercellular adhesion molecule 1 expression in mouse cremaster muscle vasculature," *Microcirculation,* vol. 4, no. 2, pp. 311-9, 1997.
287. Y. Sugama, C. Tiruppathi, K. offakidevi, T. T. Andersen, J. W. Fenton, 2nd, and A. B. Malik, "Thrombin-induced expression of endothelial P-selectin and intercellular adhesion molecule-1: a mechanism for stabilizing neutrophil adhesion," *Journal of Cell Biology,* vol. 119, no. 4, pp. 935-44, 1992.
288. J. A. Lopez, "The platelet glycoprotein Ib-IX complex," *Blood Coagulation and Fibrinolysis,* vol. 5, no. 1, pp. 97-119, 1994.
289. Y. Wallez, and P. Huber, "Endothelial adherens and tight junctions in vascular homeostasis, inflammation and angiogenesis," *Biochimica et Biophysica Acta,* vol. 1778, no. 3, pp. 794-809, 2008.
290. M. G. Lampugnani, M. Corada, L. Caveda, F. Breviario, O. Ayalon, B. Geiger, and E. Dejana, "The molecular organization of endothelial cell to cell junctions: differential association of plakoglobin, beta-catenin, and alpha-catenin with vascular endothelial cadherin (VE-cadherin)," *Journal of Cell Biology,* vol. 129, no. 1, pp. 203-17, 1995.
291. D. Ivanov, M. Philippova, J. Antropova, F. Gubaeva, O. Iljinskaya, E. Tararak, V. Bochkov, P. Erne, T. Resink, and V. Tkachuk, "Expression of cell adhesion molecule T-cadherin in the human vasculature," *Histochemistry and Cell Biology,* vol. 115, no. 3, pp. 231-42, 2001.
292. P. Telo, F. Breviario, P. Huber, C. Panzeri, and E. Dejana, "Identification of a novel cadherin (vascular endothelial cadherin-2) located at intercellular junctions in endothelial cells," *Journal of Biological Chemistry,* vol. 273, no. 28, pp. 17565-72, 1998.
293. M. Furuse, and S. Tsukita, "Claudins in occluding junctions of humans and flies," *Trends in Cell Biology,* vol. 16, no. 4, pp. 181-8, 2006.
294. W. A. Muller, "Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response," *Trends in Immunology,* vol. 24, no. 6, pp. 327-34, 2003.
295. T. Okamoto, M. Akiyama, M. Takeda, E. C. Gabazza, T. Hayashi, and K. Suzuki, "Connexin32 is expressed in vascular endothelial cells and participates in gap-junction intercellular communication," *Biochemical and Biophysical Research Communications,* vol. 382, no. 2, pp. 264-8, 2009.
296. M. P. Soares, and F. H. Bach, "C1q receptors and endothelial cell activation," *Journal of Laboratory and Clinical Medicine,* vol. 133, no. 6, pp. 520-2, 1999.
297. J. H. Reinders, P. G. de Groot, J. J. Sixma, and J. A. van Mourik, "Storage and secretion of von Willebrand factor by endothelial cells," *Haemostasis,* vol. 18, no. 4-6, pp. 246-61, 1988.
298. J. O. Utgaard, F. L. Jahnsen, A. Bakka, P. Brandtzaeg, and G. Haraldsen, "Rapid secretion of prestored interleukin 8 from Weibel-Palade bodies of microvascular endothelial cells," *Journal of Experimental Medicine,* vol. 188, no. 9, pp. 1751-6, 1998.
299. J. L. Platt, G. M. Vercellotti, B. J. Lindman, T. R. Oegema, Jr., F. H. Bach, and A. P. Dalmasso, "Release of heparan sulfate from endothelial cells. Implications for pathogenesis of hyperacute rejection," *Journal of Experimental Medicine,* vol. 171, no. 4, pp. 1363-8, 1990.
300. S. Saadi, and J. L. Platt, "Transient perturbation of endothelial integrity induced by natural antibodies and complement," *Journal of Experimental Medicine,* vol. 181, no. 1, pp. 21-31, 1995.
301. O. Morel, P. Ohlmann, E. Epailly, B. Bakouboula, F. Zobairi, L. Jesel, N. Meyer, M. P. Chenard, J. M. Freyssinet, P. Bareiss, J. P. Mazzucotelli, and F. Toti, "Endothelial cell activation contributes to the release of procoagulant microparticles during acute cardiac allograft rejection," *Journal of Heart and Lung Transplantation,* vol. 27, no. 1, pp. 38-45, 2008.
302. J. Zhang, A. F. Defelice, J. P. Hanig, and T. Colatsky, "Biomarkers of endothelial cell activation serve as potential surrogate markers for drug-induced vascular injury," *Toxicologic Pathology,* vol. 38, no. 6, pp. 856-71, 2010.
303. M. Rehm, D. Bruegger, F. Christ, P. Conzen, M. Thiel, M. Jacob, D. Chappell, M. Stoeckelhuber, U. Welsch, B. Reichart, K. Peter, and B. F. Becker, "Shedding of the endothelial glycocalyx in patients undergoing major vascular surgery with global and regional ischemia," *Circulation,* vol. 116, no. 17, pp. 1896-906, 2007.
304. E. Rahbar, J. C. Cardenas, G. Baimukanova, B. Usadi, R. Bruhn, S. Pati, S. R. Ostrowski, P. I. Johansson, J. B. Holcomb, and C. E. Wade, "Endothelial glycocalyx shedding and vascular permeability in severely injured trauma patients," *Journal of Translational Medicine,* vol. 13, pp. 117, 2015.

305. D. Chappell, M. Jacob, K. Hofmann-Kiefer, M. Rehm, U. Welsch, P. Conzen, and B. F. Becker, "Antithrombin reduces shedding of the endothelial glycocalyx following ischaemia/reperfusion," *Cardiovascular Research,* vol. 83, no. 2, pp. 388-96, 2009.
306. H. H. Lipowsky, L. Gao, and A. Lescanic, "Shedding of the endothelial glycocalyx in arterioles, capillaries, and venules and its effect on capillary hemodynamics during inflammation," *American Journal of Physiology: Heart and Circulatory Physiology,* vol. 301, no. 6, pp. H2235-45, 2011.
307. S. Devaraj, D. Y. Xu, and I. Jialal, "C-reactive protein increases plasminogen activator inhibitor-1 expression and activity in human aortic endothelial cells: implications for the metabolic syndrome and atherothrombosis," *Circulation,* vol. 107, no. 3, pp. 398-404, 2003.
308. M. Noutsias, B. Seeberg, H. P. Schultheiss, and U. Kuhl, "Expression of cell adhesion molecules in dilated cardiomyopathy: evidence for endothelial activation in inflammatory cardiomyopathy," *Circulation,* vol. 99, no. 16, pp. 2124-31, 1999.
309. M. H. Wilhelmi, R. G. Leyh, M. Wilhelmi, and A. Haverich, "Upregulation of endothelial adhesion molecules in hearts with congestive and ischemic cardiomyopathy: immunohistochemical evaluation of inflammatory endothelial cell activation," *European Journal of Cardio-Thoracic Surgery,* vol. 27, no. 1, pp. 122-7, 2005.
310. S. Nakamura, H. Ohtani, Y. Watanabe, K. Fukushima, T. Matsumoto, A. Kitano, K. Kobayashi, and H. Nagura, "In situ expression of the cell adhesion molecules in inflammatory bowel disease. Evidence of immunologic activation of vascular endothelial cells," *Laboratory Investigation,* vol. 69, no. 1, pp. 77-85, 1993.
311. P. S. Seifert, J. L. Catalfamo, and J. Dodds, "Complement activation after aortic endothelial injury," *Seminars in Thrombosis and Hemostasis,* vol. 12, no. 4, pp. 280-3, 1986.
312. S. Tsuji, K. Kaji, and S. Nagasawa, "Activation of the alternative pathway of human complement by apoptotic human umbilical vein endothelial cells," *Journal of Biochemistry,* vol. 116, no. 4, pp. 794-800, 1994.
313. C. Mold, and C. A. Morris, "Complement activation by apoptotic endothelial cells following hypoxia/reoxygenation," *Immunology,* vol. 102, no. 3, pp. 359-64, 2001.
314. K. Joseph, Y. Shibayama, B. Ghebrehiwet, and A. P. Kaplan, "Factor XII-dependent contact activation on endothelial cells and binding proteins gC1qR and cytokeratin 1," *Thrombosis and Haemostasis,* vol. 85, no. 1, pp. 119-24, 2001.
315. B. Ghebrehiwet, C. CebadaMora, L. Tantral, J. Jesty, and E. I. Peerschke, "gC1qR/p33 serves as a molecular bridge between the complement and contact activation systems and is an important catalyst in inflammation," *Advances in Experimental Medicine and Biology,* vol. 586, pp. 95-105, 2006.
316. J. Anrather, V. Csizmadia, C. Brostjan, M. P. Soares, F. H. Bach, and H. Winkler, "Inhibition of bovine endothelial cell activation in vitro by regulated expression of a transdominant inhibitor of NF-kappa B," *Journal of Clinical Investigation,* vol. 99, no. 4, pp. 763-72, 1997.
317. X. Feng, M. G. Tonnesen, E. I. Peerschke, and B. Ghebrehiwet, "Cooperation of C1q receptors and integrins in C1q-mediated endothelial cell adhesion and spreading," *Journal of Immunology,* vol. 168, no. 5, pp. 2441-8, 2002.
318. B. Ghebrehiwet, X. Feng, R. Kumar, and E. I. Peerschke, "Complement component C1q induces endothelial cell adhesion and spreading through a docking/signaling partnership of C1q receptors and integrins," *International Immunopharmacology,* vol. 3, no. 3, pp. 299-310, 2003.
319. K. E. Foreman, A. A. Vaporciyan, B. K. Bonish, M. L. Jones, K. J. Johnson, M. M. Glovsky, S. M. Eddy, and P. A. Ward, "C5a-induced expression of P-selectin in endothelial cells," *Journal of Clinical Investigation,* vol. 94, no. 3, pp. 1147-55, 1994.
320. K. S. Kilgore, J. P. Shen, B. F. Miller, P. A. Ward, and J. S. Warren, "Enhancement by the complement membrane attack complex of tumor necrosis factor-alpha-induced endothelial cell expression of E-selectin and ICAM-1," *Journal of Immunology,* vol. 155, no. 3, pp. 1434-41, 1995.
321. K. S. Kilgore, E. Schmid, T. P. Shanley, C. M. Flory, V. Maheswari, N. L. Tramontini, H. Cohen, P. A. Ward, H. P. Friedl, and J. S. Warren, "Sublytic concentrations of the membrane attack complex of complement induce endothelial interleukin-8 and monocyte chemoattractant protein-1 through nuclear factor-kappa B activation," *American Journal of Pathology,* vol. 150, no. 6, pp. 2019-31, 1997.
322. R. Hattori, K. K. Hamilton, R. P. McEver, and P. J. Sims, "Complement proteins C5b-9 induce secretion of high molecular weight multimers of endothelial von Willebrand factor and translocation of granule membrane protein GMP-140 to the cell surface," *Journal of Biological Chemistry,* vol. 264, no. 15, pp. 9053-60, 1989.
323. S. Saadi, R. A. Holzknecht, C. P. Patte, D. M. Stern, and J. L. Platt, "Complement-mediated regulation of tissue factor activity in endothelium," *Journal of Experimental Medicine,* vol. 182, no. 6, pp. 1807-14, 1995.
324. J. Friedl, M. Puhlmann, D. L. Bartlett, S. K. Libutti, E. N. Turner, M. F. Gnant, and H. R. Alexander, "Induction of permeability across endothelial cell monolayers by tumor necrosis factor (TNF) occurs via a tissue factor-dependent mechanism: relationship between the procoagulant and permeability effects of TNF," *Blood,* vol. 100, no. 4, pp. 1334-9, 2002.

325. A. M. ElKeeb, M. E. Collier, A. Maraveyas, and C. Ettelaie, "Accumulation of tissue factor in endothelial cells induces cell apoptosis, mediated through p38 and p53 activation," *Thrombosis and Haemostasis,* vol. 114, no. 2, pp. 364-78, 2015.
326. M. Usui, N. Kuriyama, M. Kisawada, T. Hamada, S. Mizuno, H. Sakurai, M. Tabata, H. Imai, K. Okamoto, S. Uemoto, and S. Isaji, "Tissue factor expression demonstrates severe sinusoidal endothelial cell damage during rejection after living-donor liver transplantation," *Journal of Hepato-Biliary-Pancreatic Surgery,* vol. 16, no. 4, pp. 513-20, 2009.
327. M. Laposata, D. K. Dovnarsky, and H. S. Shin, "Thrombin-induced gap formation in confluent endothelial cell monolayers in vitro," *Blood,* vol. 62, no. 3, pp. 549-56, 1983.
328. D. Anrather, M. T. Millan, A. Palmetshofer, S. C. Robson, C. Geczy, A. J. Ritchie, F. H. Bach, and B. M. Ewenstein, "Thrombin activates nuclear factor-kappaB and potentiates endothelial cell activation by TNF," *Journal of Immunology,* vol. 159, no. 11, pp. 5620-8, 1997.
329. H. J. Wei, Y. H. Li, G. Y. Shi, S. L. Liu, P. C. Chang, C. H. Kuo, and H. L. Wu, "Thrombomodulin domains attenuate atherosclerosis by inhibiting thrombin-induced endothelial cell activation," *Cardiovascular Research,* vol. 92, no. 2, pp. 317-27, 2011.
330. J. E. Kaplan, D. G. Moon, L. K. Weston, F. L. Minnear, P. J. Del Vecchio, J. M. Shepard, and J. W. Fenton, 2nd, "Platelets adhere to thrombin-treated endothelial cells in vitro," *American Journal of Physiology,* vol. 257, no. 2 Pt 2, pp. H423-33, 1989.
331. J. M. Li, R. S. Podolsky, M. J. Rohrer, B. S. Cutler, M. T. Massie, M. R. Barnard, and A. D. Michelson, "Adhesion of activated platelets to venous endothelial cells is mediated via GPIIb/IIIa," *Journal of Surgical Research,* vol. 61, no. 2, pp. 543-8, 1996.
332. G. Conforti, C. Dominguez-Jimenez, E. Ronne, G. Hoyer-Hansen, and E. Dejana, "Cell-surface plasminogen activation causes a retraction of in vitro cultured human umbilical vein endothelial cell monolayer," *Blood,* vol. 83, no. 4, pp. 994-1005, 1994.
333. I. Guevara-Lora, K. Stalinska, B. Augustynek, and A. Labedz-Maslowska, "Influence of kinin peptides on monocyte-endothelial cell adhesion," *Journal of Cellular Biochemistry,* vol. 115, no. 11, pp. 1985-95, 2014.
334. V. S. Carl, E. E. Moore, F. A. Moore, and E. T. Whalley, "Involvement of bradykinin B1 and B2 receptors in human PMN elastase release and increase in endothelial cell monolayer permeability," *Immunopharmacology,* vol. 33, no. 1-3, pp. 325-9, 1996.
335. P. Jungmann, M. Wilhelmi, H. Oberleithner, and C. Riethmuller, "Bradykinin does not induce gap formation between human endothelial cells," *Pflügers Archiv. European Journal of Physiology,* vol. 455, no. 6, pp. 1007-16, 2008.
336. O. D. Ratnoff, and I. H. Lepow, "Some properties of an esterase derived from preparations of the first component of complement," *Journal of Experimental Medicine,* vol. 106, no. 2, pp. 327-43, 1957.
337. G. J. Arlaud, A. Reboul, R. B. Sim, and M. G. Colomb, "Interaction of C1-inhibitor with the C1r and C1s subcomponents in human C1," *Biochimica et Biophysica Acta,* vol. 576, no. 1, pp. 151-62, 1979.
338. S. Chesne, C. L. Villiers, G. J. Arlaud, M. B. Lacroix, and M. G. Colomb, "Fluid-phase interaction of C1 inhibitor (C1 Inh) and the subcomponents C1r and C1s of the first component of complement, C1," *Biochemical Journal,* vol. 201, no. 1, pp. 61-70, 1982.
339. F. K. Kerr, A. R. Thomas, L. C. Wijeyewickrema, J. C. Whisstock, S. E. Boyd, D. Kaiserman, A. Y. Matthews, P. I. Bird, N. M. Thielens, V. Rossi, and R. N. Pike, "Elucidation of the substrate specificity of the MASP-2 protease of the lectin complement pathway and identification of the enzyme as a major physiological target of the serpin, C1-inhibitor," *Molecular Immunology,* vol. 45, no. 3, pp. 670-7, 2008.
340. E. W. Nielsen, C. Waage, H. Fure, O. L. Brekke, G. Sfyroera, J. D. Lambris, and T. E. Mollnes, "Effect of supraphysiologic levels of C1-inhibitor on the classical, lectin and alternative pathways of complement," *Molecular Immunology,* vol. 44, no. 8, pp. 1819-26, 2007.
341. H. Jiang, E. Wagner, H. Zhang, and M. M. Frank, "Complement 1 inhibitor is a regulator of the alternative complement pathway," *Journal of Experimental Medicine,* vol. 194, no. 11, pp. 1609-16, 2001.
342. P. M. Jansen, B. Eisele, I. W. de Jong, A. Chang, U. Delvos, F. B. Taylor, Jr., and C. E. Hack, "Effect of C1 inhibitor on inflammatory and physiologic response patterns in primates suffering from lethal septic shock," *Journal of Immunology,* vol. 160, no. 1, pp. 475-84, 1998.
343. I. Gigli, J. W. Mason, R. W. Colman, and K. F. Austen, "Interaction of plasma kallikrein with the C1 inhibitor," *Journal of Immunology,* vol. 104, no. 3, pp. 574-81, 1970.
344. M. Schapira, A. de Agostini, and R. W. Colman, "C1 inhibitor: the predominant inhibitor of plasma kallikrein," *Methods in Enzymology,* vol. 163, pp. 179-85, 1988.
345. S. Ravindran, T. E. Grys, R. A. Welch, M. Schapira, and P. A. Patston, "Inhibition of plasma kallikrein by C1-inhibitor: role of endothelial cells and the amino-terminal domain of C1-inhibitor," *Thrombosis and Haemostasis,* vol. 92, no. 6, pp. 1277-83, 2004.
346. E. W. Nielsen, J. Morrissey, J. O. Olsen, and B. Osterud, "Factor VIIa in patients with C1-inhibitor deficiency," *Thrombosis and Haemostasis,* vol. 74, no. 4, pp. 1103-6, 1995.

347. E. W. Nielsen, H. T. Johansen, K. Hogasen, W. Wuillemin, C. E. Hack, and T. E. Mollnes, "Activation of the complement, coagulation, fibrinolytic and kallikrein-kinin systems during attacks of hereditary angioedema," *Scandinavian Journal of Immunology,* vol. 44, no. 2, pp. 185-92, 1996.
348. K. Joseph, T. E. Tholanikunnel, and A. P. Kaplan, "Treatment of episodes of hereditary angioedema with C1 inhibitor: serial assessment of observed abnormalities of the plasma bradykinin-forming pathway and fibrinolysis," *Annals of Allergy, Asthma, and Immunology,* vol. 104, no. 1, pp. 50-4, 2010.
349. L. G. Huisman, J. M. van Griensven, and C. Kluft, "On the role of C1-inhibitor as inhibitor of tissue-type plasminogen activator in human plasma," *Thrombosis and Haemostasis,* vol. 73, no. 3, pp. 466-71, 1995.
350. E. W. Brown, S. Ravindran, and P. A. Patston, "The reaction between plasmin and C1-inhibitor results in plasmin inhibition by the serpin mechanism," *Blood Coagulation and Fibrinolysis,* vol. 13, no. 8, pp. 711-4, 2002.
351. A. P. Dalmasso, and J. L. Platt, "Prevention of complement-mediated activation of xenogeneic endothelial cells in an in vitro model of xenograft hyperacute rejection by C1 inhibitor," *Transplantation,* vol. 56, no. 5, pp. 1171-6, 1993.
352. S. Cai, and A. E. Davis, 3rd, "Complement regulatory protein C1 inhibitor binds to selectins and interferes with endothelial-leukocyte adhesion," *Journal of Immunology,* vol. 171, no. 9, pp. 4786-91, 2003.
353. S. Zeerleder, C. Caliezi, G. van Mierlo, A. Eerenberg-Belmer, I. Sulzer, C. E. Hack, and W. A. Wuillemin, "Administration of C1 inhibitor reduces neutrophil activation in patients with sepsis," *Clinical and Diagnostic Laboratory Immunology,* vol. 10, no. 4, pp. 529-35, 2003.
354. F. Lu, S. M. Fernandes, and A. E. Davis, 3rd, "The role of the complement and contact systems in the dextran sulfate sodium-induced colitis model: the effect of C1 inhibitor in inflammatory bowel disease," *American Journal of Physiology: Gastrointestinal and Liver Physiology,* vol. 298, no. 6, pp. G878-83, 2010.
355. M. Buerke, T. Murohara, and A. M. Lefer, "Cardioprotective effects of a C1 esterase inhibitor in myocardial ischemia and reperfusion," *Circulation,* vol. 91, no. 2, pp. 393-402, 1995.
356. N. Akita, H. Nakase, Y. Kanemoto, T. Kaido, T. Nishioka, and T. Sakaki, "[The effect of C 1 esterase inhibitor on ischemia: reperfusion injury in the rat brain]," *No to Shinkei. Brain and Nerve,* vol. 53, no. 7, pp. 641-4, 2001.
357. R. F. Saidi, B. Rajeshkumar, A. Shariftabrizi, K. Dresser, and O. Walter, "Human C1 inhibitor attenuates liver ischemia-reperfusion injury and promotes liver regeneration," *Journal of Surgical Research,* vol. 187, no. 2, pp. 660-6, 2014.
358. E. W. Nielsen, T. E. Mollnes, J. M. Harlan, and R. K. Winn, "C1-inhibitor reduces the ischaemia-reperfusion injury of skeletal muscles in mice after aortic cross-clamping," *Scandinavian Journal of Immunology,* vol. 56, no. 6, pp. 588-92, 2002.
359. F. Lu, A. K. Chauhan, S. M. Fernandes, M. T. Walsh, D. D. Wagner, and A. E. Davis, 3rd, "The effect of C1 inhibitor on intestinal ischemia and reperfusion injury," *American Journal of Physiology: Gastrointestinal and Liver Physiology,* vol. 295, no. 5, pp. G1042-9, 2008.
360. M. Scherer, S. Demertzis, F. Langer, A. Moritz, and H. J. Schafers, "C1-esterase inhibitor reduces reperfusion injury after lung transplantation," *Annals of Thoracic Surgery,* vol. 73, no. 1, pp. 233-8; discussion 238-9, 2002.
361. M. Kunschak, W. Engl, F. Maritsch, F. S. Rosen, G. Eder, G. Zerlauth, and H. P. Schwarz, "A randomized, controlled trial to study the efficacy and safety of C1 inhibitor concentrate in treating hereditary angioedema," *Transfusion,* vol. 38, no. 6, pp. 540-9, 1998.
362. M. A. Riedl, J. A. Bernstein, H. Li, A. Reshef, W. Lumry, D. Moldovan, H. Farkas, R. Levy, J. Baker, Y. Hardiman, M. C. Totoritis, A. Relan, M. Cicardi, and I. Study, "Recombinant human C1-esterase inhibitor relieves symptoms of hereditary angioedema attacks: phase 3, randomized, placebo-controlled trial," *Annals of Allergy, Asthma, and Immunology,* vol. 112, no. 2, pp. 163-169 e1, 2014.
363. M. A. Riedl, D. S. Hurewitz, R. Levy, P. J. Busse, D. Fitts, and I. Kalfus, "Nanofiltered C1 esterase inhibitor (human) for the treatment of acute attacks of hereditary angioedema: an open-label trial," *Annals of Allergy, Asthma, and Immunology,* vol. 108, no. 1, pp. 49-53, 2012.
364. M. Heeres, T. Visser, K. J. van Wessem, A. H. Koenderman, P. F. Strengers, L. Koenderman, and L. P. Leenen, "The effect of C1-esterase inhibitor on systemic inflammation in trauma patients with a femur fracture - The CAESAR study: study protocol for a randomized controlled trial," *Trials,* vol. 12, pp. 223, 2011.
365. A. A. Vo, A. Zeevi, J. Choi, K. Cisneros, M. Toyoda, J. Kahwaji, A. Peng, R. Villicana, D. Puliyanda, N. Reinsmoen, M. Haas, and S. C. Jordan, "A phase I/II placebo-controlled trial of C1-inhibitor for prevention of antibody-mediated rejection in HLA sensitized patients," *Transplantation,* vol. 99, no. 2, pp. 299-308, 2015.
366. T. Laumonier, A. J. Walpen, C. F. Maurus, P. J. Mohacsi, K. M. Matozan, E. Y. Korchagina, N. V. Bovin, B. Vanhove, J. D. Seebach, and R. Rieben, "Dextran sulfate acts as an endothelial cell protectant and inhibits human complement and natural killer cell-mediated cytotoxicity against porcine cells," *Transplantation,* vol. 76, no. 5, pp. 838-43, 2003.
367. T. Laumonier, P. J. Mohacsi, K. M. Matozan, Y. Banz, A. Haeberli, E. Y. Korchagina, N. V. Bovin, B. Vanhove, and R. Rieben, "Endothelial cell protection by dextran sulfate: a novel strategy to prevent acute vascular rejection in xenotransplantation," *American Journal of Transplantation,* vol. 4, no. 2, pp. 181-7, 2004.
368. Y. Banz, T. Gajanayake, K. Matozan, Z. Yang, and R. Rieben, "Dextran sulfate modulates MAP kinase signaling and reduces endothelial injury in a rat aortic clamping model," *Journal of Vascular Surgery,* vol. 50, no. 1, pp. 161-70, 2009.

369. C. Duehrkop, J. Denoyelle, S. Shaw, and R. Rieben, "Use of dextran sulfate in tourniquet-induced skeletal muscle reperfusion injury," *Journal of Surgical Research,* vol. 187, no. 1, pp. 150-61, 2014.
370. S. Zeerleder, T. Mauron, B. Lammle, and W. A. Wuillemin, "Effect of low-molecular weight dextran sulfate on coagulation and platelet function tests," *Thrombosis Research,* vol. 105, no. 5, pp. 441-6, 2002.
371. N. Eto, I. Kojima, N. Uesugi, R. Inagi, T. Miyata, T. Fujita, R. J. Johnson, S. J. Shankland, and M. Nangaku, "Protection of endothelial cells by dextran sulfate in rats with thrombotic microangiopathy," *Journal of the American Society of Nephrology,* vol. 16, no. 10, pp. 2997-3005, 2005.
372. Y. Banz, R. Rieben, C. Zobrist, P. Meier, S. Shaw, J. Lanz, T. Carrel, and P. Berdat, "Addition of dextran sulfate to blood cardioplegia attenuates reperfusion injury in a porcine model of cardiopulmonary bypass," *European Journal of Cardio-Thoracic Surgery,* vol. 34, no. 3, pp. 653-60, 2008.
373. T. Mauron, B. Lammle, and W. A. Wuillemin, "Influence of low molecular weight heparin and low molecular weight dextran sulfate on the inhibition of coagulation factor XIa by serpins," *Thrombosis and Haemostasis,* vol. 80, no. 1, pp. 82-6, 1998.
374. Y. Takada, and A. Takada, "Influence of dextran sulfate and tranexamic acid on the activation of plasminogen by urokinase," *Thrombosis Research,* vol. 16, no. 5-6, pp. 865-9, 1979.
375. H. Tsuda, T. Miyata, S. Iwanaga, and T. Yamamoto, "Analysis of intrinsic fibrinolysis in human plasma induced by dextran sulfate," *Thrombosis and Haemostasis,* vol. 67, no. 4, pp. 440-4, 1992.
376. L. A. Miles, Z. Rothschild, and J. H. Griffin, "Dextran sulfate-dependent fibrinolysis in whole human plasma," *Journal of Laboratory and Clinical Medicine,* vol. 101, no. 2, pp. 214-25, 1983.
377. D. L. Tankersley, B. M. Alving, and J. S. Finlayson, "Activation of factor XII by dextran sulfate: the basis for an assay of factor XII," *Blood,* vol. 62, no. 2, pp. 448-56, 1983.
378. H. Hasegawa, H. Nagata, M. Yamauchi, T. Murakoshi, Y. Oguma, H. Takei, T. Seya, and M. Murao, "Role of dextran sulfate in urokinase therapy and evaluation of the effects by estimation of plasmin inhibitor, fibrinogenolytic degradation products, and fibrinolytic degradation products," *Japanese Heart Journal,* vol. 23, no. 3, pp. 339-47, 1982.
379. E. Holmer, K. Kurachi, and G. Soderstrom, "The molecular-weight dependence of the rate-enhancing effect of heparin on the inhibition of thrombin, factor Xa, factor IXa, factor XIa, factor XIIa and kallikrein by antithrombin," *Biochemical Journal,* vol. 193, no. 2, pp. 395-400, 1981.
380. J. S. Rosenberg, P. W. McKenna, and R. D. Rosenberg, "Inhibition of human factor IXa by human antithrombin," *Journal of Biological Chemistry,* vol. 250, no. 23, pp. 8883-8, 1975.
381. J. Y. Chan, C. E. Burrowes, F. M. Habal, and H. Z. Movat, "The inhibition of activated factor XII (Hageman factor) by antithrombin III: the effect of other plasma proteinase inhibitors," *Biochemical and Biophysical Research Communications,* vol. 74, no. 1, pp. 150-8, 1977.
382. C. E. Burrowes, F. M. Habal, and H. Z. Movat, "The inhibition of human plasma kallikrein by antithrombin III," *Thrombosis Research,* vol. 7, no. 1, pp. 175-83, 1975.
383. P. K. Anonick, B. Wolf, and S. L. Gonias, "Regulation of plasmin, miniplasmin, and streptokinase-plasmin complex by alpha 2-antiplasmin, alpha 2-macroglobulin, and antithrombin III in the presence of heparin," *Thrombosis Research,* vol. 59, no. 3, pp. 449-62, 1990.
384. D. Ogston, J. Murray, and G. P. Crawford, "Inhibition of the activated Cls subunit of the first component of complement by antithrombin III in the presence of heparin," *Thrombosis Research,* vol. 9, no. 3, pp. 217-222, 1976.
385. D. Chappell, F. Brettner, N. Doerfler, M. Jacob, M. Rehm, D. Bruegger, P. Conzen, B. Jacob, and B. F. Becker, "Protection of glycocalyx decreases platelet adhesion after ischaemia/reperfusion: an animal study," *European Journal of Anaesthesiology,* vol. 31, no. 9, pp. 474-81, 2014.
386. D. Karamanos, C. Karkos, A. Kambaroudis, M. Kritsepi, S. Papadopoulos, C. Spyridis, and T. Gerassimidis, "The effect of Antithrombin-III on routine hematological and biochemical parameters in an experimental animal model of skeletal muscle ischemia-reperfusion injury," *Hippokratia,* vol. 18, no. 3, pp. 234-9, 2014.
387. B. Eisele, M. Lamy, L. G. Thijs, H. O. Keinecke, H. P. Schuster, F. R. Matthias, F. Fourrier, H. Heinrichs, and U. Delvos, "Antithrombin III in patients with severe sepsis. A randomized, placebo-controlled, double-blind multicenter trial plus a meta-analysis on all randomized, placebo-controlled, double-blind trials with antithrombin III in severe sepsis," *Intensive Care Medicine,* vol. 24, no. 7, pp. 663-72, 1998.
388. F. Fourrier, M. Jourdain, and A. Tournoys, "Clinical trial results with antithrombin III in sepsis," *Critical Care Medicine,* vol. 28, no. 9 Suppl, pp. S38-43, 2000.
389. D. Rublee, S. M. Opal, W. Schramm, H. O. Keinecke, and S. Knaub, "Quality of life effects of antithrombin III in sepsis survivors: results from the KyberSept trial [ISRCTN22931023]," *Critical Care (London, England),* vol. 6, no. 4, pp. 349-56, 2002.
390. A. Afshari, J. Wetterslev, J. Brok, and A. Moller, "Antithrombin III in critically ill patients: systematic review with meta-analysis and trial sequential analysis," *BMJ,* vol. 335, no. 7632, pp. 1248-51, 2007.
391. G. Dickneite, "Influence of C1-inhibitor on inflammation, edema and shock," *Behring Institute Mitteilungen,* no. 93, pp. 299-305, 1993.

392. R. Giebler, U. Schmidt, S. Koch, J. Peters, and R. Scherer, "Combined antithrombin III and C1-esterase inhibitor treatment decreases intravascular fibrin deposition and attenuates cardiorespiratory impairment in rabbits exposed to Escherichia coli endotoxin," *Critical Care Medicine,* vol. 27, no. 3, pp. 597-604, 1999.
393. H. Yamaguchi, H. Weidenbach, H. Luhrs, M. M. Lerch, G. Dickneite, and G. Adler, "Combined treatment with C1 esterase inhibitor and antithrombin III improves survival in severe acute experimental pancreatitis," *Gut,* vol. 40, no. 4, pp. 531-5, 1997.

Paper I

Inhalation anesthesia of rats: influence of the fraction of inspired oxygen on limb ischemia/reperfusion injury

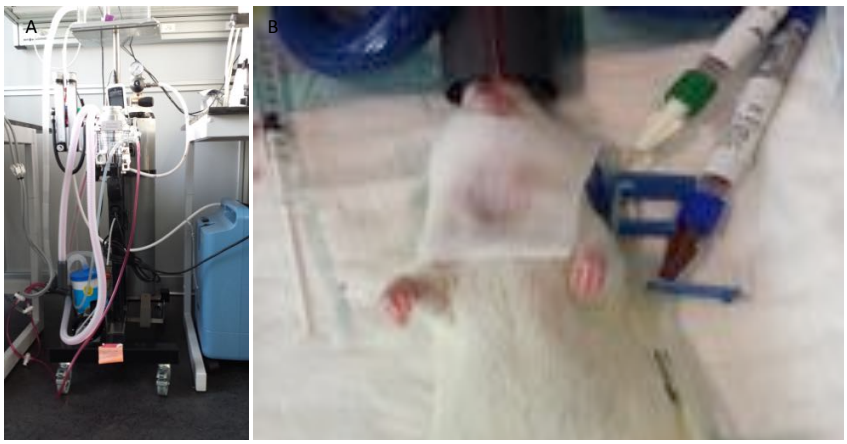
S Zhang[1,2], C Duehrkop[3], J Plock[4] and R Rieben[1]

[1]Department of Clinical Research, University of Bern, Switzerland; [2]Graduate School for Cellular and Biomedical Sciences, University of Bern, Switzerland; [3]Department of Immunology, University of Uppsala, Uppsala, Sweden; [4]Division of Hand, Plastic and Reconstructive Surgery, University Hospital Zurich, Switzerland

Status: Published in Lab Anim. 2015 Sep 7. Doi:10.1177/0023677215604531. [Epub ahead of print].

Aim: The objective was to investigate the influence of the fractions of inspired oxygen (FiO_2) on rat hind limb IRI and to refine the used inhalation anesthesia.

Conclusion: A refined inhalation anesthesia setting using 40% FiO2, reflecting more or less the clinical situation, leads to a more severe and more physiologically relevant reperfusion injury than higher FiO2. Oxidative stress did not correlate with FiO2 and seemed to have no influence on reperfusion injury.



Figures: (A) The anesthetic equipments used in our study, including isoflurane bottle, oxygen generator, nitrogen bottle, and pipes. (B) Rats under anesthesia in a standard nose mask.

Lab Anim OnlineFirst, published on September 7, 2015 as doi:10.1177/0023677215604531

Original Article

Laboratory Animals
0(0) 1–13

DOI: 10.1177/0023677215604531
la.sagepub.com

Inhalation anesthesia of rats: influence of the fraction of inspired oxygen on limb ischemia/reperfusion injury

S Zhang[1,2], C Duehrkop[3], JA Plock[4] and R Rieben[1]

Abstract

Inhalation anesthesia with isoflurane is a well-established and safe method used in small laboratory animals. In most cases oxygen is used as a carrier gas for isoflurane, but room air or mixtures of oxygen with air or nitrous oxide are also being used. Anesthesia is therefore administered using different fractions of inspired oxygen (FiO_2), and this may have consequences for the outcome of experiments. The aim of the present study was to investigate the influence of FiO_2 on rat hind limb ischemia/reperfusion injury and to refine the used inhalation anesthesia. Male Wistar rats were subjected to 3.5 h of ischemia and 2 h of reperfusion, and divided into three groups according to FiO_2 in the O_2/air/isoflurane anesthesia gas mixture: 40%, 60%, and 100% O_2. Normal, healthy rats were used as controls. Muscle edema and creatine kinase MM, a marker for myocyte necrosis, were significantly increased with 40% FiO_2 as compared with 100% FiO_2 ($P < 0.05$). Partial pressure of oxygen, oxygen saturation, and oxyhemoglobin were significantly higher in the 100% O_2 group as compared with 40% O_2. No significant differences were detected for other parameters, such as the oxidative stress markers malondialdehyde and superoxide dismutase. We conclude that a refined inhalation anesthesia setting using 40% FiO_2, reflecting more or less the clinical situation, leads to a more severe and more physiologically relevant reperfusion injury than higher FiO_2. Oxidative stress did not correlate with FiO_2 and seemed to have no influence on reperfusion injury.

Keywords

refinement, ischemia/reperfusion injury, fraction of inspired oxygen, rat

Inhalation anesthesia systems using isoflurane are often used in laboratory animal studies because they allow for a safe and easily adjustable anesthesia. In many studies,[1–3] the carrier gas for isoflurane is oxygen. However, room air or a mixture of oxygen and air or nitrous oxide have also been used,[4–6] resulting in differences in the fraction of inspired oxygen (FiO_2) in experimental rodent anesthesia protocols. FiO_2 is the fraction or percentage of oxygen in the anesthesia gas mixture inhaled by the patient or laboratory animal. Room air is composed of 21% oxygen and 79% nitrogen, which is equivalent to an FiO_2 of 21%, while pure oxygen has an FiO_2 of 100%. We investigated whether different FiO_2 in the anesthesia gas mixture might influence the outcome of our rat hind limb ischemia/reperfusion injury (IRI) model.

Reperfusion injury of the lower extremity is a common clinical consequence of surgical interventions performed under tourniquet application as well as the treatment of peripheral vascular diseases such as thrombotic occlusion and embolism. Basically, IRI is caused by prolonged lack or massive restriction of

[1]Department of Clinical Research, University of Bern, Bern, Switzerland
[2]Graduate School for Cellular and Biomedical Sciences, University of Bern, Bern, Switzerland
[3]Department of Immunology, University of Uppsala, Uppsala, Sweden
[4]Division of Plastic Surgery and Hand Surgery, University Hospital Zurich, Zurich, Switzerland

Corresponding author:
Robert Rieben, Department of Clinical Research, University of Bern, Murtenstrasse 50, PO Box 44, CH-3008 Bern, Switzerland.
Email: robert.rieben@dkf.unibe.ch

blood supply to a tissue or organ, followed by re-establishment of the circulation of oxygenated blood. Paradoxically, reperfusion can aggravate ischemic tissue damage, causing an inflammatory reaction which may lead to both local injury and systemic complications as seen in clinical studies,[7,8] and experimentally in rat hind limb IRI models.[9,10]

During IRI, a complex cascade of inflammatory reactions occurs, which have been reviewed in detail previously.[11,12] During the ischemic stage, nutrient depletion and hypoxia may lead to neoepitopes being expressed particularly in the affected endothelium, which may be recognized by preformed antibodies circulating in the blood. This triggers the classical and lectin pathways of complement activation, as shown in mouse models of intestinal IRI.[13,14] Deposition of complement proteins on the endothelial surface was shown to be involved in the vascular leakage in human intestinal IRI[15] and in a mouse mesenteric IRI model.[16] Complement activation also leads to anaphylatoxin C5a formation, which is a potent chemoattractant for proinflammatory cells. It also initiates transforming the endothelium from an anticoagulant and anti-inflammatory to a procoagulant and proinflammatory state. Models of rat liver, renal and intestinal IRI showed that to be the case.[17–19]

It has also been proposed that reintroducing oxygen during the reperfusion phase may be a triggering factor for an overproduction of reactive oxygen species (ROS), which are generated by activated endothelial cells.[20,21] Overproduced ROS thus causes oxidative stress. For instance, in rat liver and cerebral IRI studies,[22,23] oxidative stress has been demonstrated to occur and plays an important role in the pathophysiology of reperfusion injury, and experimental treatments based on ROS-scavenging have shown beneficial effects.[24–26] However, in a recent clinical study on kidney and heart reperfusion injury, biomarkers of oxidative damage were not released upon reperfusion, so the causal involvement of ROS and oxidative stress in IRI has been questioned.[27] Additionally, tissue oxygen tension (tpO_2) decreased during ischemia and recovered to baseline levels during reperfusion in a skeletal muscle IRI model.[28] However, so far no studies have investigated whether the oxidative stress, blood oxygenation and tpO_2 may be linked to FiO_2 in inhalation anesthesia. Nor is it clear if they affect experimental reperfusion injury.

To address the above issues, and to refine FiO_2 in small animal inhalation anesthesia, a rat hind limb IRI model was used, in which FiO_2 was varied from 40% to 100%. The effect of FiO_2 on reperfusion injury was assessed by measuring edema formation, muscle viability, creatine kinase MM (CK-MM) in plasma, deposition of complement and fibrin in tissue.

Materials and methods

Ethics statement

Animal housing and experimental protocols were approved by the Cantonal Veterinary Office, Bern, Switzerland, under license No. BE 05/12, and were in accordance with Swiss Animal Protection Law and conform to Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals used for Scientific Purposes, as well as to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Animals

Adult male Wistar rats aged 7–9 weeks, weighing between 250 and 350 g, were purchased from Janvier Labs (Le Genest, France) and used in all the experiments. The rats were kept in groups of three animals in clear 1500 cm^2 Euro-standard type IV S cages (Tecniplast, Buguggiate, Italy) with overall dimensions of $48 \times 37.5 \times 21$ cm (length × width × height). Unrestricted feeding was with standard pelleted maintenance diet (Provimi Kliba, Kaiseraugst, Switzerland) and water ad libitum. Poplar wood chips were used as bedding material (Lignocel, Provimi Kliba). Paper tissue, a piece of poplar wood with a size of $20 \times 3.5 \times 3.5$ cm (length × width × height) and a black polyvinyl chloride (PVC) tube of 16 cm length and 7.3 cm diameter were provided as environmental enrichment. Cages were individually ventilated at $20 \pm 2\,^{\circ}C$ and 45–65% relative humidity with a circadian rhythm of 12/12 h. During the light cycle the animals were exposed to a light intensity of 200 lux.

Experimental design

Animals were subjected to 3.5 h of ischemia and 2 h of reperfusion, and divided into three groups according to FiO_2 in the anesthesia gas mixture (O_2/air/isoflurane): group 1 ($n = 7$) 40% O_2, group 2 ($n = 6$) 60% O_2, and group 3 ($n = 8$) 100% O_2. Four normal, healthy rats were used as controls for comparison. No substances were administrated to the animals. The numbers of animals per group were calculated based on the paper 'Guidelines for the design and statistical analysis of experiments using laboratory animals'[29] and the ARRIVE guidelines.[30] Data on edema formation (wet/dry ratio) of previous experiments[31] have revealed a difference in the means between experimental and control groups of 1.1 and a standard deviation of 0.5. Sample size calculations based on these data have resulted in at least six animals per group. Due to technical problems with blood gas analysis for some

animals, the n numbers had to be increased to $n=7$ for the 40% and $n=8$ for the 100% FiO_2 groups. For normal rats (control group), we observed a very small standard derivation (<1%) in our earlier study.[31] We therefore decided to limit this group to $n=4$ in order to keep the number of animals to a minimum.

Anesthesia and monitoring

Anesthesia was induced by 3% isoflurane in the oxygen/air mixture according to the experimental group in a clear, polystyrene induction chamber. Anesthesia throughout the whole IRI procedure was then maintained with 2% isoflurane in the same oxygen/air mixture in a standard rat nose mask (Provet, Lyssach, Switzerland). The animals were placed on a heating pad and rectal temperature kept at $37 \pm 1\,^{\circ}C$. Basic parameters (heart rate, breath rate, pulse distension, breath distension and oxygen saturation [sO_2]) were continuously monitored and recorded every 5 min using a MouseOx plus system (Starr Life Sciences Corp, Oakmont, PA, USA) and MouseOx plus software version 1.4.9.

Surgical procedure

All animals were placed supine, and the fur on their neck and on both hind limbs was shaved. The left carotid artery was cannulated with a polyethylene tubing (product code: 800/100/200; Portex, Smiths Medical, Labhardt AG, Basel, Switzerland) for blood sampling. The cannula was flushed with approximately 1 mL of heparin solution (100 IE/mL in saline; Laboratory Dr G Bichsel AG, Interlaken, Switzerland) prior to insertion in the carotid artery and filled with heparin solution after each blood sampling to prevent clogging. Care was taken to prevent heparinization of the animals.

Baseline measurements for blood gas and blood chemistry analyses and tpO_2 in the gastrocnemius muscle (details below) were performed before surgery. The used rat hind limb IRI model was performed as described earlier.[32,33] In brief, two microvascular clamps (B1-V; S&T, Neuhausen, Switzerland) were used to block arterial blood supply proximal to the deep femoral artery branch while a tourniquet, connected to a weight of 450 g, was placed underneath the femoral artery, vein and the sciatic nerve around the thigh as high as possible. The rats underwent 3.5 h of ischemia followed by reperfusion for 2 h and stayed under isoflurane anesthesia during the whole time. At the end, the rats were sacrificed by exsanguination and tissue samples from the soleus and gastrocnemius muscles as well as the lung were taken for further analysis.

Measurement of muscle and lung edema

To assess edema formation in the muscle and lung, two pieces of tissue from the gastrocnemius muscle in the contralateral and reperfused legs, as well as the lung, were taken, washed in phosphate-buffered saline (PBS), blotted dry and weighed immediately to get the wet weight. The pieces were then dried at 80 °C overnight until constant dry weights were obtained. The ratio of wet:dry weight was regarded as an indicator for edema formation.

Muscle viability

Muscle viability was determined using MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, M2128; Sigma-Aldrich, St Louis, MO, USA). The principle is that mitochondrial dehydrogenases of living cells reduce the MTT tetrazolium compound to its purple formazan product. Briefly, muscle tissue was incubated with 0.1 mg MTT/mL PBS at 37 °C overnight, then transferred to 100% isopropanol at 37 °C for 6 h. At the end, optical density (OD) of the supernatant was read at 570 nm (reference wavelength 690 nm, Infinite M1000 spectrophotometer; Tecan, Männedorf, Switzerland). OD/mg dry weight was used to quantitate muscle viability.

CK-MM in plasma

To assess myocyte damage, CK-MM was measured in EDTA plasma using an enzyme-linked immunosorbent assay (ELISA) kit (2112-2; Life Diagnostics Inc, West Chester, PA, USA). The assay was performed according to the manufacturer's protocol and the samples were analyzed in duplicate at baseline and at the end of reperfusion. Concentrations were determined by comparing with standards provided with the kit.

Histopathological examination

To assess histopathological changes, one piece of gastrocnemius muscle from both the contralateral and reperfused legs, as well a piece from the lung, were washed in PBS, blotted dry and fixed in 4% formalin until paraffin embedding and staining with hematoxylin and eosin (H&E). Hemorrhage, neutrophil infiltration, myocyte damage, and tissue edema were examined by a professional pathologist who was blinded to this study.

Immunofluorescence

To detect deposition of antibodies, complement components and fibrin, as well as heparan sulfate expression, immunofluorescence staining was performed. Briefly, the gastrocnemius muscle from both the

contralateral and reperfused legs was taken and stored at –25 °C for further use. Sections with 5 µm thickness were fixed in acetone and rehydrated in tris-buffered saline (TBS). Primary antibodies were added and incubated at 4 °C overnight, followed by incubation of secondary antibodies and 4′,6-diamidino-2-phenylindole (DAPI; Boehringer, Roche Diagnostics, Indianapolis, IN, USA) for staining of the nuclei at room temperature for 1 h. Finally, the slides were mounted and covered. The following primary antibodies were used: goat anti-rat IgM (3020-08; Southern Biotech, Birmingham, AL, USA), goat anti-rat IgG (3030-08; Southern Biotech), rabbit anti-human C3b/c (A0062; Dako, Baar, Switzerland), goat anti-human C6 (A307; Quidel, San Diego, CA, USA), rabbit anti-human fibrinogen (F0111; Dako), and mouse anti-heparan sulfate (370255; Amsbio, Abingdon, UK). Secondary antibodies and conjugates were donkey anti-goat Alexa488 (C 2306; Sigma-Aldrich), goat anti-mouse FITC (115-097-020; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and streptavidin-Cy3 (S6402; Sigma-Aldrich). Images were taken with a fluorescent microscope (Leica DMI 4000B; Leica Microsystems Schweiz, Heerbrugg, Switzerland) and analyzed by ImageJ (NIH, Bethesda, MD, USA) and GraphPad Prism 6 software (GraphPad Software Inc, San Diego, CA, USA).

Blood gas and blood chemistry analyses

Samples of 150 µL heparinized arterial blood were taken from the carotid catheter at baseline, end of ischemia, 1 h of reperfusion and 2 h of reperfusion. Partial pressures of oxygen and carbon dioxide (pO_2, pCO_2), sO_2, pH, base excess (BE), the concentration of total hemoglobin (tHb), oxyhemoglobin (O_2Hb), and deoxyhemoglobin (HHb), the hematocrit (Hct), the concentrations of the electrolytes Na^+, K^+, Ca^{2+}, HCO_3^- and Cl^-, as well as concentrations of glucose (Glu), lactate (Lac), and osmotic concentration (Osm) were measured on a Cobas b 123 POC blood gas analyzer (Roche, Basel, Switzerland).

Tissue oxygen tension

To monitor oxygen delivery and transport from vessels to tissue during IRI, tpO_2 was measured by inserting an oxygen probe (CC8, Licox; Integra Neurosciences, Plainsboro, NJ, USA) and a temperature probe (CC1.SB, Licox; Integra Neurosciences) into the gastrocnemius muscle through a 16 gauge introducer catheter (393229, BD Venflon Pro Safety; BD Europe, Allschwil, Switzerland). The probes were connected to a digital Licox monitor (Licox CMP; Integra Neurosciences). Time points at which tpO_2 was recorded were baseline, 1 h of ischemia, 2 h of ischemia, 3 h of ischemia, 3.5 h of ischemia, 1 h of reperfusion, and 2 h of reperfusion.

Oxidative stress

The oxidative stress markers, malondialdehyde (MDA) formed from lipid peroxidation, and superoxide dismutase (SOD), were determined in plasma by colorimetric and fluorometric methods, respectively, using a lipid peroxidation (118970; Abcam, Cambridge, UK) and an SOD assay kit (19160; Sigma-Aldrich). The assays were performed according to the manufacturers' protocols. Concentration of MDA was determined by interpolation from a standard curve, while SOD activity was calculated using the equation provided by the manufacturer.

Statistical analysis

All data are presented as a mean ± standard deviation (SD). Statistical analyses were done by one-way analysis of variance (ANOVA), followed by two post hoc tests with GraphPad Prism 6 software: Bonferroni's (to compare a set of selected means) and Dunnett's (to compare means with the control group). To compare different inter-group time points (such as breath rate and heart rate, blood gas analysis, tpO_2 and oxidative stress), the areas under the curve (AUC) were calculated for each experiment, and the groups were compared by one-way ANOVA with Bonferroni's post hoc test. P values <0.05 were considered statistically significant.

Results

Edema formation

In all groups, ischemia and reperfusion led to significantly higher wet/dry ratios – and thus edema formation – in the reperfused limbs as compared with the contralateral ones as well as with the normal, healthy controls. A tendency for increased edema formation after 3.5 h of ischemia and 2 h of reperfusion was observed with decreasing FiO_2, with a significant difference between the 40% and 100% O_2 groups. The observed wet/dry ratios were 6.0 ± 0.62 in the 40% O_2 group versus 5.4 ± 0.36 in the 100% O_2 group, $P = 0.049$ (Figure 1a). No edema was formed in the contralateral limbs of all three groups when compared with the hind limbs of the normal, healthy rats.

To assess distant organ damage, wet/dry ratios of the lungs were also measured, but no edema formation was found in the lungs in this model with 2 h of reperfusion (Figure 1b).

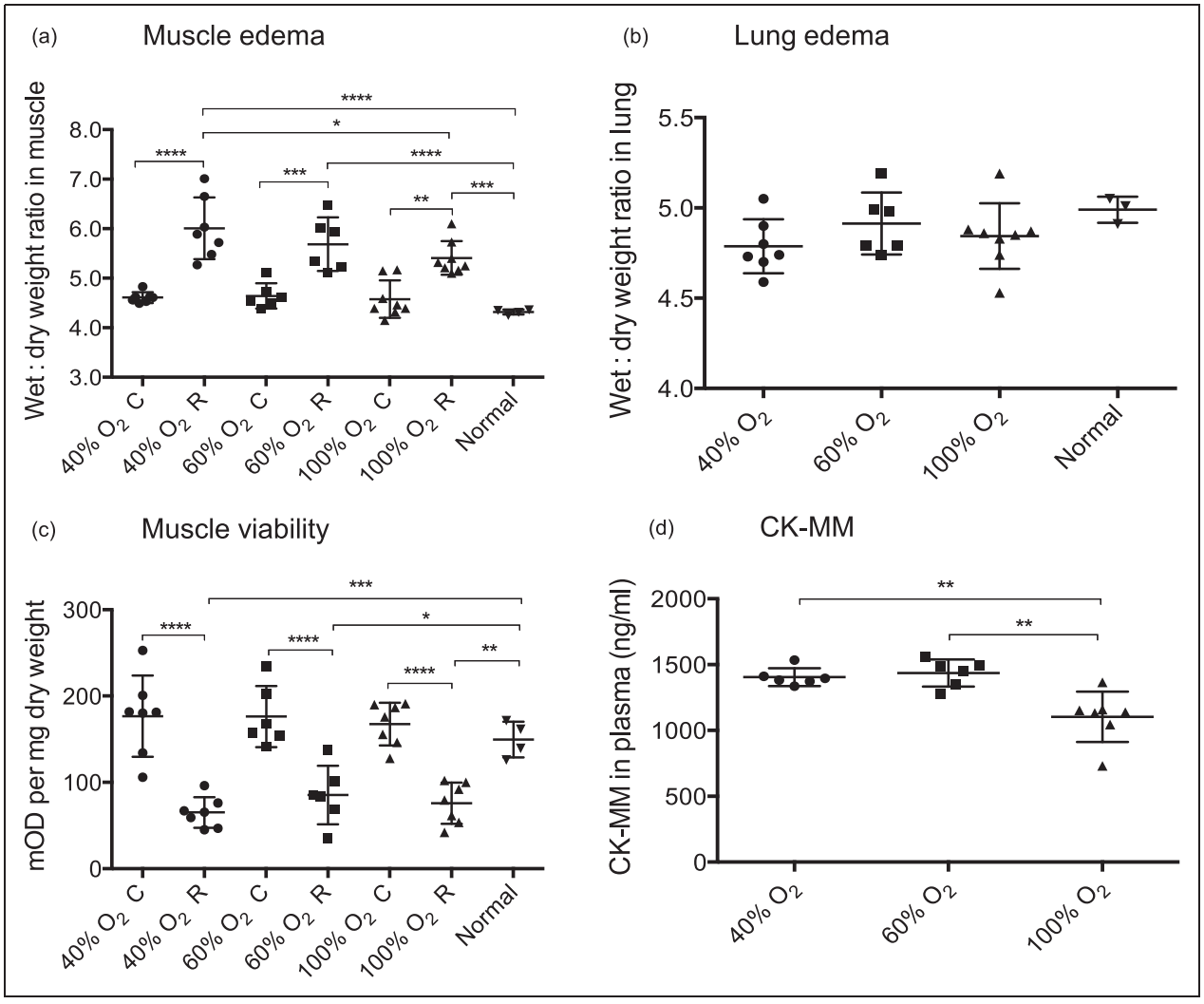


Figure 1. Edema formation in the muscle (a) and lung (b), muscle viability (c), and creatine kinase MM (CK-MM) in plasma (d). Error bars indicate mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. C: contralateral limb; R: reperfused limb.

Muscle viability

Muscle viability was reduced in the reperfused limbs of all three experimental groups as compared with the normal controls as shown in Figure 1c. However, no significant differences were found between experimental groups. No reduction of muscle viability was found in the contralateral limbs as compared with the normal controls.

CK-MM in plasma

CK-MM levels in plasma at the end of reperfusion – indicating muscle cell necrosis – were 1405 ± 68.2 ng/mL in the 40%, 1436 ± 103.0 ng/mL in the 60%, and 1103 ± 190.8 ng/mL in the 100% O_2 groups. The values in the 40% and 60% O_2 groups were significantly elevated as compared with the 100% O_2 group ($P = 0.0031$ and $P = 0.0013$, respectively; Figure 1d). Baseline values were all below the detection limit of the ELISA kit, i.e. less than 90 ng/mL.

Histopathological findings

Histopathological evaluation by H&E staining revealed intermediate damage to the gastrocnemius muscle tissue after 2 h of reperfusion. Myocyte destruction and muscular edema were observed in each experimental group to a similar extent, while only low levels of neutrophil infiltration were seen (Figures 2a–d). No hemorrhage was observed in all three experimental groups. Moreover, no histopathological changes were detectable in the lungs after 2 h of reperfusion (data not shown).

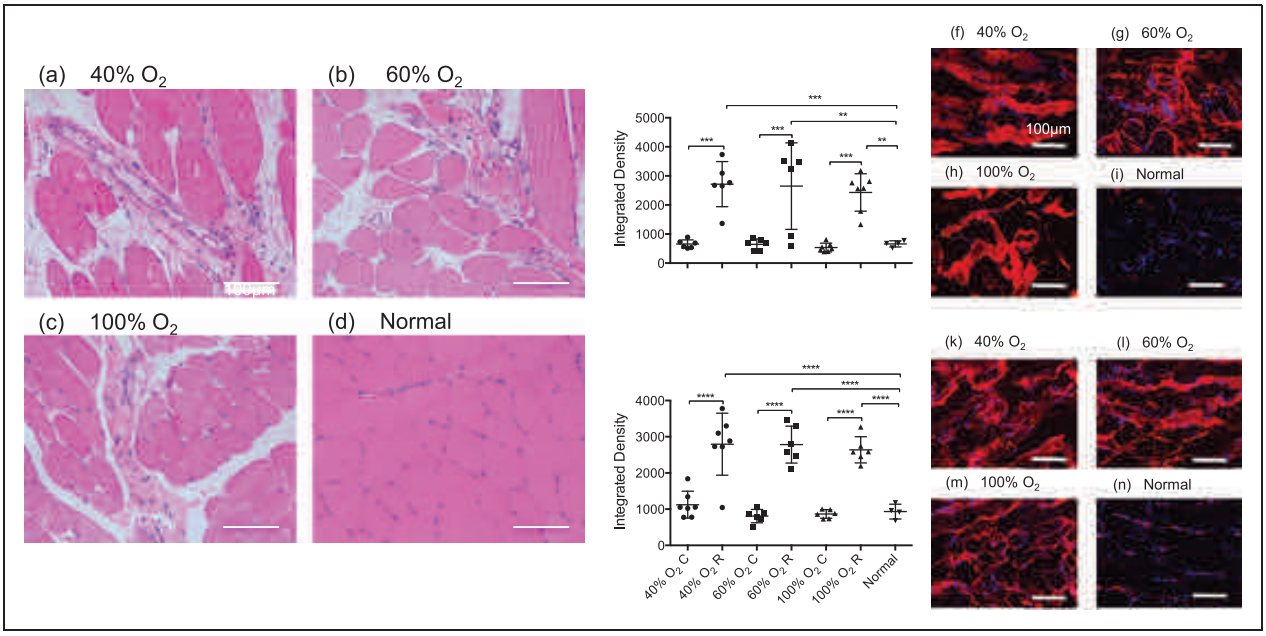


Figure 2. Representative images of hematoxylin–eosin stained muscle tissue in the 40% O_2 (a), 60% O_2 (b), 100% O_2 (d) groups and normal controls (d), as well as deposition of IgG – quantitative analysis (e) and representative images (f–i), and IgM – quantitative analysis (j) and representative images (k–n). Error bars indicate mean ± SD. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. C: contralateral limb; R: reperfused limb. Scale bar represents 100 µm.

Deposition of antibodies and complement

Depositions of IgG and IgM, and the complement proteins C3b/c and C6 were detected by immunofluorescence. Fluorescence intensities were quantified as integrated density by Image J and compared between groups. Both IgG and IgM deposits were significantly increased in the reperfused limbs of all experimental groups as compared with the normal controls ($P < 0.0001$; Figures 2e–n). For complement, an FiO_2 of 40% and 60% led to significantly higher deposition of C3b/c and C6, as compared with the normal control group ($P < 0.0001$), whereas deposition of complement was not significantly elevated in the rats with an FiO_2 of 100% (Figures 3a–j). However, no significant differences were found in the tissue of reperfused limbs between the 40%, 60% and 100% FiO_2 groups for deposition of antibodies and complement. Similarly, no significant differences were found for these markers between the contralateral limbs and the normal controls.

Deposition of fibrin and expression of heparan sulfate

Deposition of fibrin was measured as a marker of the activation of the coagulation system. A significantly higher level of fibrin deposition was observed in the tissue of the reperfused limbs in the 40% and 60% FiO_2 groups as compared with the normal controls ($P = 0.0016$ and $P = 00036$, respectively; Figures 3k–o), but this was not the case for the 100% FiO_2 group. Expression of heparan sulfate in tissue is known to be reduced as a consequence of inflammation.[34,35] Both the 40% and 100% O_2 inhalations led to significantly lower levels of expression of heparan sulfate in the tissue of the reperfused legs as compared with the normal controls ($P = 0.0004$ and $P = 0.0112$ respectively; Figures 3p–t). However, no significant differences were detected for the above markers in the reperfused tissue between the experimental groups, as well as between the contralateral limbs and the normal controls.

Breath rate and heart rate

All animals showed an increase in breath rates at the beginning of ischemia. By assessing AUC over the whole duration of the experiments, a significantly higher breath rate was observed in the 40% FiO_2 group as compared with both the 60% and 100% FiO_2 groups ($P = 0.0157$ and $P = 0.0461$, respectively; Figure 4). For heart rates, no significant intra-group changes over time or inter-group differences (as assessed by AUC) were observed.

Blood gas and blood chemistry analyses

Arterial blood gas and blood chemistry analyses were performed to assess the effect of FiO_2 on blood

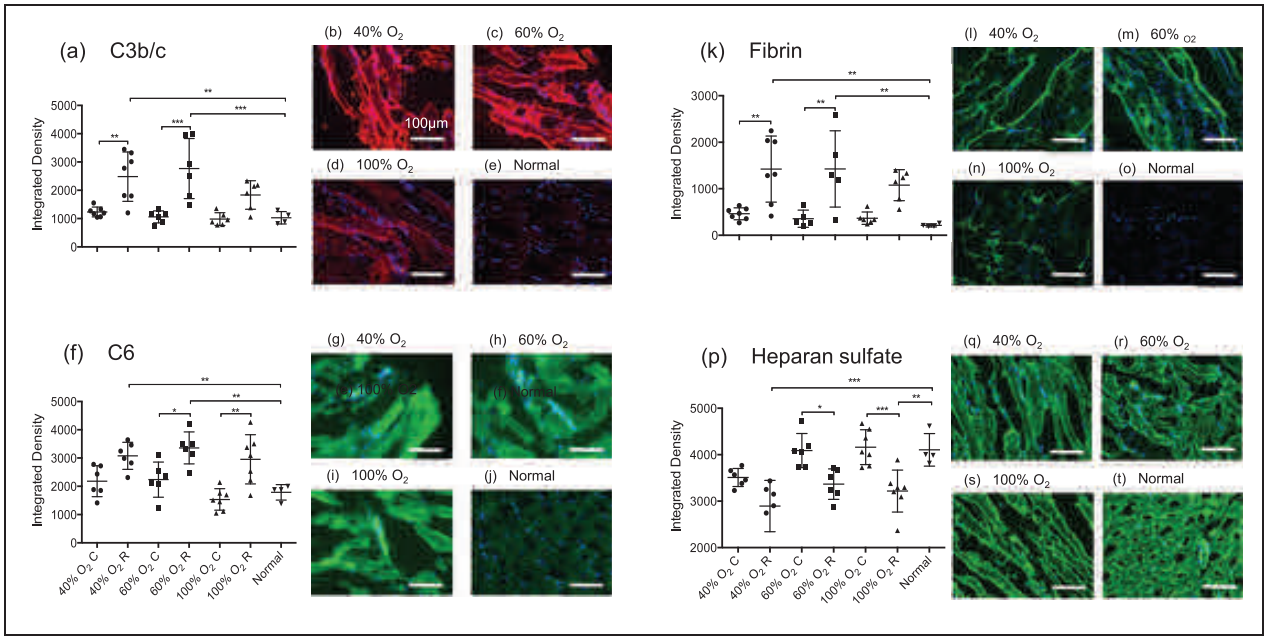


Figure 3. Deposition of C3b/c (a–e), C6 (f–j), fibrin (k–o), and heparan sulfate expression (p–t). Error bars indicate mean ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. C: contralateral limb; R: reperfused limb. Scale bar represents 100 μm.

oxygenation and related parameters. For inter-group comparison, AUC data were calculated for all parameters over time. As shown in Figures 5a–d, an FiO_2 of 100% led to significantly higher levels of pO_2, sO_2 and O_2Hb over time as compared with 40% ($P < 0.01$), while HHb was reduced significantly ($P < 0.01$) in the 100% as compared with the 40% group. No significant inter-group differences were found for the rest of the parameters over time by comparing AUC data. However, we found increased concentrations of K^+ in each group, while concentrations of Ca^{2+} and Glu dropped over time (supplementary Table 1, see http://lan.sagepub.com).

Tissue oxygen tension

Tissue oxygen tension was measured every hour to assess oxygen delivery from the blood to the tissue under different inhaled oxygen concentrations. At baseline tpO_2 was between 20 and 39 mmHg, it then dropped to below 10 mmHg during ischemia and rose again during reperfusion. However, significant differences between groups for tpO_2 were not observed over time (Figure 6).

Oxidative stress

Analysis of oxidative stress markers was performed to investigate the influence of different inspired oxygen concentrations on MDA levels and SOD activities in citrate plasma. No statistically significant inter-group differences were found for concentrations of MDA or SOD activities by comparing AUC data (Figure 7a, right panel, ANOVA with Bonferroni's post hoc test). Also intra-group analyses of MDA values over time did not reveal significant changes (Figure 7a, left panel, ANOVA with Dunnett's post hoc test versus baseline). However, the SOD activities showed a trend for reduction over time in all experimental groups, with the value at 2 h of reperfusion being significantly lower than baseline in the 40% O_2 group ($P = 0.026$, Figure 7b).

Discussion

In this study a rat hind limb IRI model was used in which FiO_2 was varied from 40% to 100% in order to assess the effect of inhaled oxygen in isoflurane anesthesia on reperfusion injury. A more severe muscle edema formation and a significantly higher level of CK-MM in plasma were detected in the 40% FiO_2 as compared with the 100% FiO_2 group. Clinically, edema formation and plasma levels of CK-MM, reflecting myocyte necrosis, are the most important factors to determine the extent of reperfusion injury. These tissue injury data are supported by the finding of C3b/c and fibrin deposition, indicating activation of the complement and coagulation systems, respectively. In the 40% and 60% FiO_2 groups significantly higher C3b/c and fibrin depositions were found in the reperfused limbs as compared with the contralateral ones

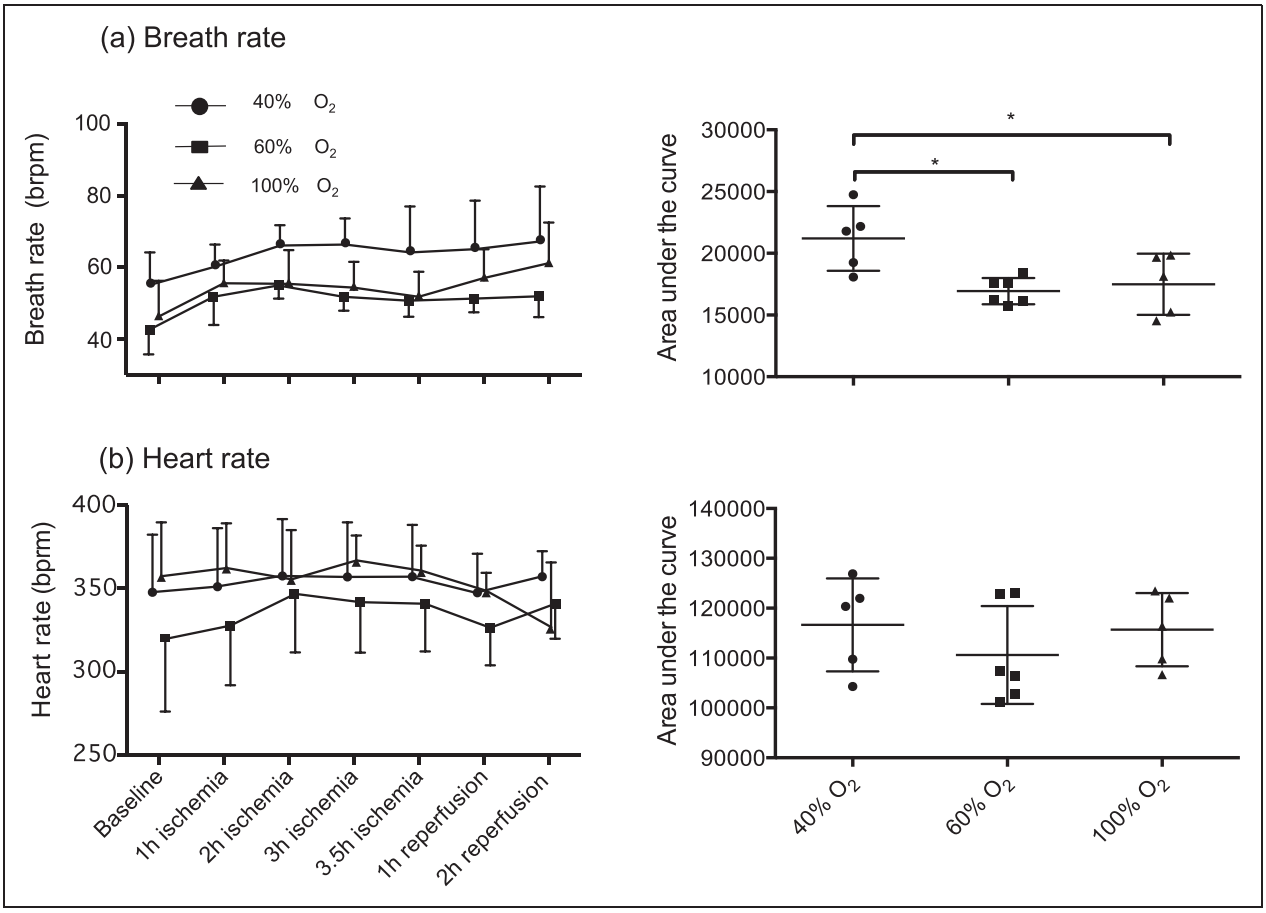


Figure 4. Breath rate and heart rate. Values of breath rate (a) and heart rate (b) over time are shown on the left panels for each group. The corresponding areas under the curve are presented on the right panels. Error bars indicate mean ± SD. $*P < 0.05$.

and the normal controls, whereas this was not the case in the 100% FiO_2 group. As expected, pO_2, sO_2, O_2Hb were significantly higher in the 100% FiO_2 group than in the group with 40% FiO_2. In addition, breath rate was significantly elevated in the 40% FiO_2 group as compared with the other two groups. For the rest of the parameters, no significant inter-group differences were detected.

Muscle edema was assessed as a basic parameter to evaluate the severity of reperfusion injury. Edema is caused by increased vascular permeability due to substantial inflammation.[33,36] Vascular leakage has been linked to endothelial cell activation, which can be caused by the deposition of complement[37] or by the presence of cytokines and chemokines.[38,39] In our study, muscle edema was more pronounced in the rats which underwent hind limb ischemia and reperfusion while breathing 40% O_2 as compared with the rats breathing 100% O_2. This is in line with a study of rat intestinal IRI[40] and a canine model of myocardial infarction and reperfusion,[41] both of which have shown that an FiO_2 of 100% attenuated reperfusion injury. Also CK-MM, the predominant CK isoenzyme in skeletal muscle and an indicator for muscle injury, was less present in the plasma of rats breathing 100% O_2 as compared with 40% and 60% O_2, providing further evidence for reduced muscle damage in the 100% O_2 group.

Higher FiO_2 did not lead to higher tpO_2 in the muscle tissue at the end of reperfusion. This finding seems to be in contrast to an earlier report, which showed that tpO_2 was significantly elevated over normoxic conditions (21% O_2) with FiO_2 between 30% and 100% in normovolemic rats.[42] Normoxic conditions were not used in this study because in our (unpublished) experience too many animals died when using room air/isoflurane anesthesia for hind limb IRI experiments, presumably due to the long duration (>6 h) of anesthesia. It is also possible that in our study the tpO_2 of 40%, 60% and 100% FiO_2 was higher than at normoxic conditions. Furthermore, breath rate was significantly elevated in the 40% FiO_2 group as compared with both the 60% and 100% O_2 groups. This can be explained by the reduced acidosis at the end of

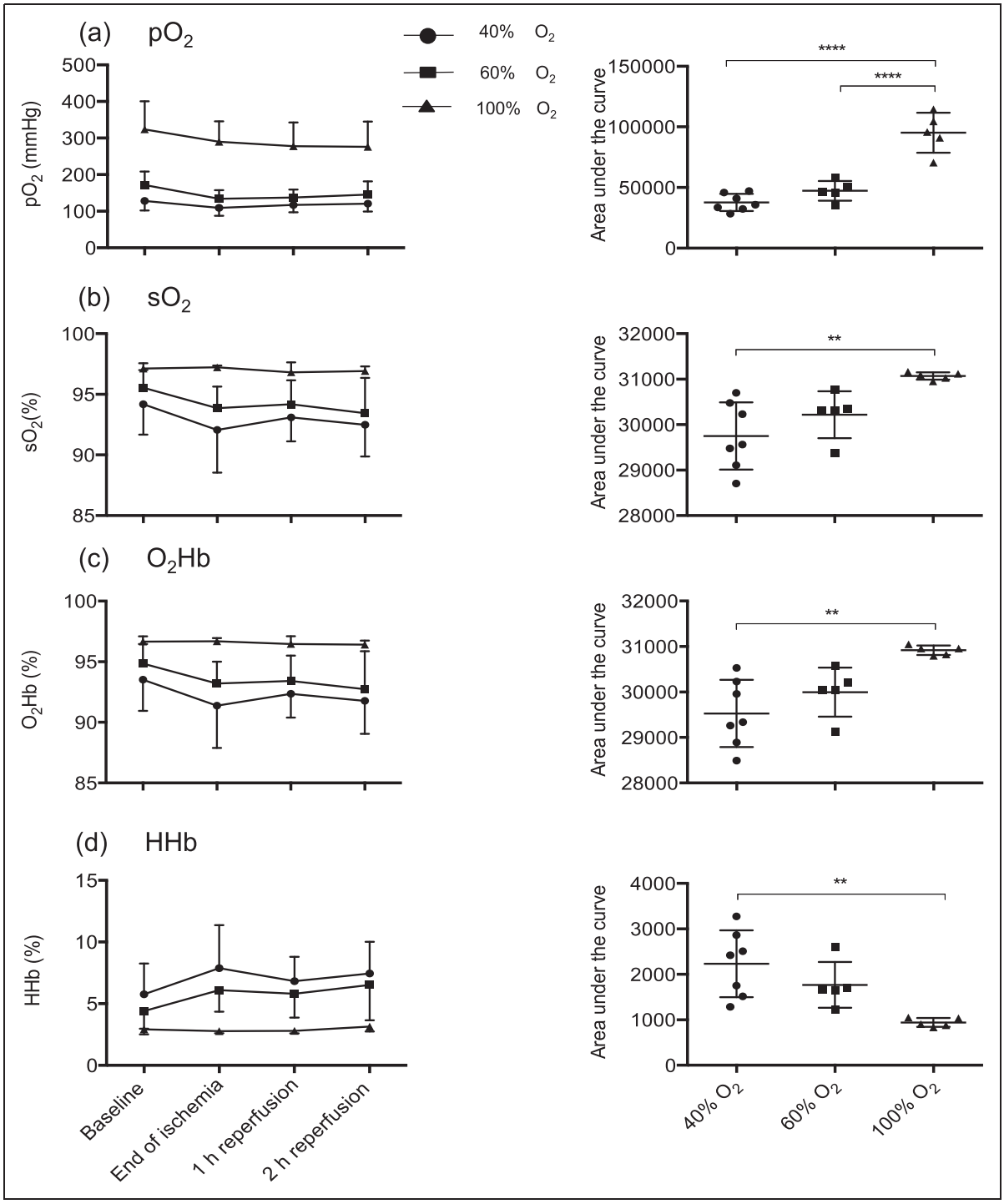


Figure 5. Blood gas and blood chemistry analyses. Values of pO_2 (a), sO_2 (b), O_2Hb (c), and HHb (d) over time are shown on the left panels for each group, while the corresponding areas under the curve are presented on the right panels. Error bars indicate mean ± SD. $^{**}P < 0.01$; $^{****}P < 0.0001$. pO_2: partial pressure of oxygen, sO_2: oxygen saturation, O_2Hb: oxyhemoglobin, HHB: deoxyhemoglobin.

reperfusion (higher pH, supplementary Table 1, see http://lan.sagepub.com) in the 40% FiO2 group as compared with the other two groups. So these animals breathed at an increase rate to fully exhale their acidosis. Lower oxygen and increased metabolic acids are the trigger for an increased breath rate.

We did not find any significantly elevated concentrations of MDA in plasma, nor did we find significantly

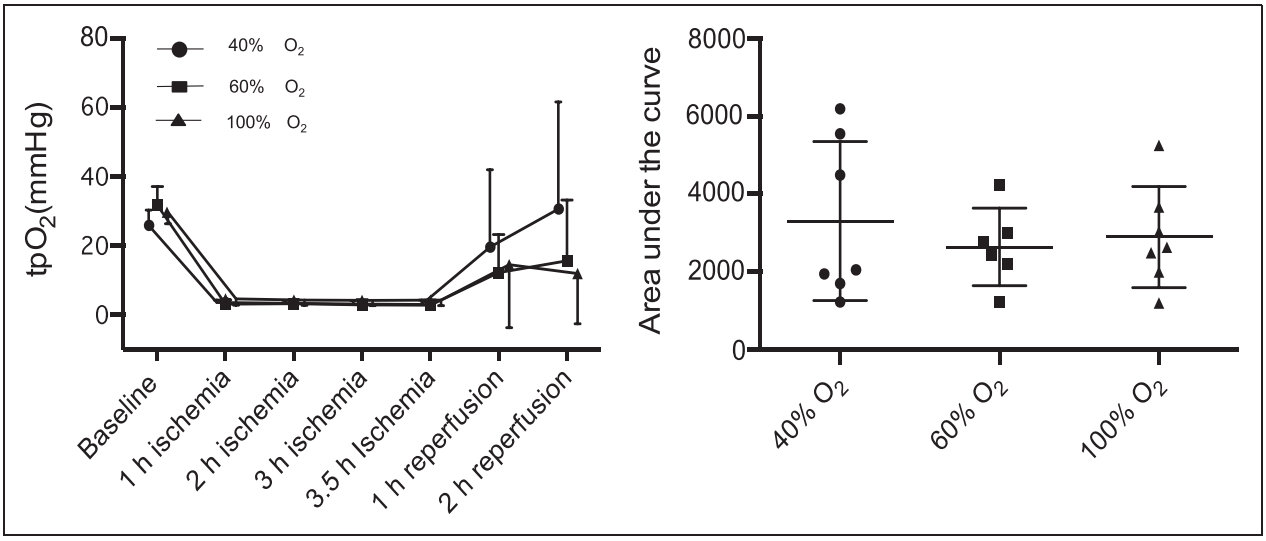


Figure 6. Tissue oxygen tension (tpO_2). Data of tpO_2 over time are shown on the left panel, while the corresponding areas under the curve are displayed on the right panel. Error bars indicate mean ± SD.

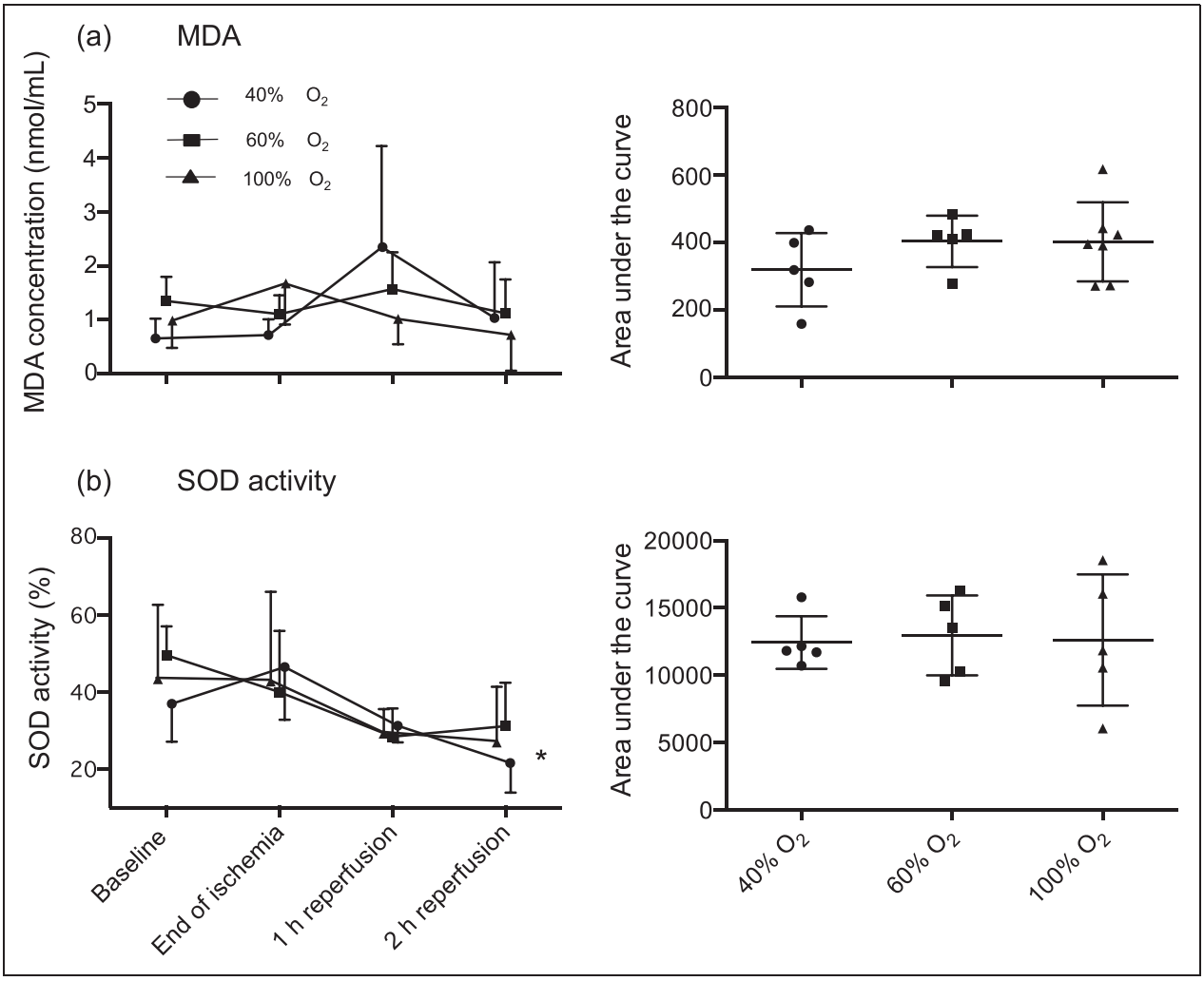


Figure 7. MDA concentration and SOD activity in plasma. Concentrations of MDA (a) and levels of SOD activity (b) are displayed on the left panels, while the corresponding areas under the curve are shown on the right panels. Error bars indicate mean ± SD. *$P < 0.05$. MDA: malondialdehyde, SOD: superoxide dismutase.

reduced SOD activities in the experimental groups, with the exception of the 2 h reperfusion value in the 40% FiO_2 group. These results are consistent with a recently published clinical study,[27] as well as earlier data from rat IRI studies,[40,43] which question the role of oxidative stress in IRI. Yet our data are different from those in previous skeletal muscle IRI studies in rabbits.[44,45] The controversial results might be due to differences in animal species and/or experimental techniques.

Taken together, different FiO_2 in the O_2/air/isoflurane mixture indeed affected the outcome of the rat hind limb IRI model in this study. An FiO_2 of 40% led to more severe reperfusion injury than inhalation of higher oxygen concentrations. The FiO_2 of 40% represents more or less the clinical conditions for standard anesthesia during surgical interventions and it is more physiological than the often used 100% FiO_2 setting in rodent models. The latter in fact seems to provide a certain protection from reperfusion injury. Perhaps the high pO_2 value (200–400 mmHg) in the 100% O_2 group causes a decreased vasodilator response during hind limb reperfusion,[46,47] and this leads to slower reperfusion, which has been shown to reduce IRI.[33]

Conclusions

Our data suggest that anesthesia with an FiO_2 of 40%, reflecting more or less the situation in a clinical setting, leads to more severe limb IRI than the use of higher FiO_2. Using an FiO_2 of 40% for rat anesthesia, in particular for IRI experiments, may therefore represent refinement of the animal experimentation model with more clinical relevance. In addition, we found no link between FiO_2 and the occurrence of oxidative stress and no indication for an important role of oxidative stress in our IRI model.

Acknowledgements

We wish to thank Anjan Bongoni (Department of Clinical Research, University of Bern) for support and helpful discussions, as well as Dr Yara Banz and her team at the Institute of Pathology for preparation of histological slides and examination of histopathological changes. This study was performed with the support of the Microscopy Imaging Center (MIC), University of Bern.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

Swiss National Science Foundation, grants no. 32003B_135272 and 320030_156193 were awarded to RR. The Chinese Scholarship Council in Switzerland awarded grant support to SZ.

References

1. Iwata A, Morrison ML and Roth MB. Iodide protects heart tissue from reperfusion injury. *PLoS One* 2014; 9: e112458.
2. Xu Z, Alloush J, Beck E and Weisleder N. A murine model of myocardial ischemia–reperfusion injury through ligation of the left anterior descending artery. *J Vis Exp* 2014; 86: e51329.
3. Xiong L, Zheng Y, Wu M, et al. Preconditioning with isoflurane produces dose-dependent neuroprotection via activation of adenosine triphosphate-regulated potassium channels after focal cerebral ischemia in rats. *Anesth Analg* 2003; 96: 233–237.
4. van Bruggen N, Thibodeaux H, Palmer JT, et al. VEGF antagonism reduces edema formation and tissue damage after ischemia/reperfusion injury in the mouse brain. *J Clin Invest* 1999; 104: 1613–1620.
5. Lee HT, Kim M, Kim M, et al. Isoflurane protects against renal ischemia and reperfusion injury and modulates leukocyte infiltration in mice. *Am J Physiol Renal Physiol* 2007; 293: F713–F722.
6. Carraretto AR, Vianna Filho PT, Castiglia YM, et al. Do propofol and isoflurane protect the kidney against ischemia/reperfusion injury during transient hyperglycemia? *Acta Cir Bras* 2013; 28: 161–166.
7. Edrees WK, Lau LL, Young IS, et al. The effect of lower limb ischaemia–reperfusion on intestinal permeability and the systemic inflammatory response. *Eur J Vasc Endovasc Surg* 2003; 25: 330–335.
8. Lin L, Wang L, Bai Y, et al. Pulmonary gas exchange impairment following tourniquet deflation: a prospective, single-blind clinical trial. *Orthopedics* 2010; 33: 395.
9. Yassin MM, Harkin DW, Barros D'Sa AA, Halliday MI and Rowlands BJ. Lower limb ischemia–reperfusion injury triggers a systemic inflammatory response and multiple organ dysfunction. *World J Surg* 2002; 26: 115–121.
10. Seekamp A, Mulligan MS, Till GO, et al. Role of beta 2 integrins and ICAM-1 in lung injury following ischemia–reperfusion of rat hind limbs. *Am J Pathol* 1993; 143: 464–472.
11. Eltzschig HK and Eckle T. Ischemia and reperfusion – from mechanism to translation. *Nat Med* 2011; 17: 1391–1401.
12. Kumar P, Shen Q, Pivetti CD, Lee ES, Wu MH and Yuan SY. Molecular mechanisms of endothelial hyperpermeability: implications in inflammation. *Expert Rev Mol Med* 2009; 11: e19.
13. Zhang M, Takahashi K, Alicot EM, et al. Activation of the lectin pathway by natural IgM in a model of ischemia/reperfusion injury. *J Immunol* 2006; 177: 4727–4734.
14. Lee H, Green DJ, Lai L, et al. Early complement factors in the local tissue immunocomplex generated during intestinal ischemia/reperfusion injury. *Mol Immunol* 2010; 47: 972–981.

15. Grootjans J, Lenaerts K, Derikx JP, et al. Human intestinal ischemia–reperfusion-induced inflammation characterized: experiences from a new translational model. *Am J Pathol* 2010; 176: 2283–2291.
16. Kannan L, Kis-Toth K, Yoshiya K, et al. R-spondin3 prevents mesenteric ischemia/reperfusion-induced tissue damage by tightening endothelium and preventing vascular leakage. *Proc Natl Acad Sci USA* 2013; 110: 14348–14353.
17. Mizutani A, Okajima K, Uchiba M, et al. Antithrombin reduces ischemia/reperfusion-induced renal injury in rats by inhibiting leukocyte activation through promotion of prostacyclin production. *Blood* 2003; 101: 3029–3036.
18. Harada N, Okajima K, Kushimoto S, Isobe H and Tanaka K. Antithrombin reduces ischemia/reperfusion injury of rat liver by increasing the hepatic level of prostacyclin. *Blood* 1999; 93: 157–164.
19. Tsuboi H, Naito Y, Katada K, et al. Role of the thrombin/protease-activated receptor 1 pathway in intestinal ischemia–reperfusion injury in rats. *Am J Physiol Gastrointest Liver Physiol* 2007; 292: G678–G683.
20. Carden DL and Granger DN. Pathophysiology of ischaemia–reperfusion injury. *J Pathol* 2000; 190: 255–266.
21. Balyasnikova IV, Visintine DJ, Gunnerson HB, et al. Propofol attenuates lung endothelial injury induced by ischemia–reperfusion and oxidative stress. *Anesth Analg* 2005; 100: 929–936.
22. Haga S, Remington SJ, Morita N, Terui K and Ozaki M. Hepatic ischemia induced immediate oxidative stress after reperfusion and determined the severity of the reperfusion-induced damage. *Antioxid Redox Signal* 2009; 11: 2563–2572.
23. Nita DA, Nita V, Spulber S, et al. Oxidative damage following cerebral ischemia depends on reperfusion – a biochemical study in rat. *J Cell Mol Med* 2001; 5: 163–170.
24. Katsumi H, Fukui K, Sato K, et al. Pharmacokinetics and preventive effects of platinum nanoparticles as reactive oxygen species scavengers on hepatic ischemia/reperfusion injury in mice. *Metallomics* 2014; 6: 1050–1056.
25. Al Rouq F and El Eter E. PPAR-gamma activator induces neuroprotection in hypercholesterolemic rats subjected to global cerebral ischemia/reperfusion injury: in vivo and in vitro inhibition of oxidative stress. *Exp Gerontol* 2014; 51: 1–7.
26. Yun X, Maximov VD, Yu J, Zhu H, Vertegel AA and Kindy MS. Nanoparticles for targeted delivery of antioxidant enzymes to the brain after cerebral ischemia and reperfusion injury. *J Cereb Blood Flow Metab* 2013; 33: 583–592.
27. de Vries DK, Kortekaas KA, Tsikas D, et al. Oxidative damage in clinical ischemia/reperfusion injury: a reappraisal. *Antioxid Redox Signal* 2013; 19: 535–545.
28. Troitzsch D, Vogt S, Abdul-Khaliq H and Moosdorf R. Muscle tissue oxygen tension and oxidative metabolism during ischemia and reperfusion. *J Surg Res* 2005; 128: 9–14.
29. Festing MF and Altman DG. Guidelines for the design and statistical analysis of experiments using laboratory animals. *ILAR J* 2002; 43: 244–258.
30. Kilkenny C, Browne WJ, Cuthill IC, Emerson M and Altman DG. Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. *J Pharmacol Pharmacother* 2010; 1: 94–99.
31. Duehrkop C and Rieben R. Refinement of tourniquet-induced peripheral ischemia/reperfusion injury in rats: comparison of 2 h vs 24 h reperfusion. *Lab Anim* 2014; 48: 143–154.
32. Duehrkop C, Banz Y, Spirig R, et al. C1 esterase inhibitor reduces lower extremity ischemia/reperfusion injury and associated lung damage. *PLoS One* 2013; 8: e72059.
33. Dick F, Li J, Giraud MN, Kalka C, Schmidli J and Tevaearai H. Basic control of reperfusion effectively protects against reperfusion injury in a realistic rodent model of acute limb ischemia. *Circulation* 2008; 118: 1920–1928.
34. Celie JW, Rutjes NW, Keuning ED, et al. Subendothelial heparan sulfate proteoglycans become major L-selectin and monocyte chemoattractant protein-1 ligands upon renal ischemia/reperfusion. *Am J Pathol* 2007; 170: 1865–1878.
35. Tong M, Tuk B, Hekking IM, et al. Heparan sulfate glycosaminoglycan mimetic improves pressure ulcer healing in a rat model of cutaneous ischemia–reperfusion injury. *Wound Repair Regen* 2011; 19: 505–514.
36. Menger MD, Rucker M and Vollmar B. Capillary dysfunction in striated muscle ischemia/reperfusion: on the mechanisms of capillary 'no-reflow'. *Shock* 1997; 8: 2–7.
37. Saadi S and Platt JL. Transient perturbation of endothelial integrity induced by natural antibodies and complement. *J Exp Med* 1995; 181: 21–31.
38. Hung TH, Charnock-Jones DS, Skepper JN and Burton GJ. Secretion of tumor necrosis factor-alpha from human placental tissues induced by hypoxia-reoxygenation causes endothelial cell activation in vitro: a potential mediator of the inflammatory response in preeclampsia. *Am J Pathol* 2004; 164: 1049–1061.
39. Maheshwari A, Christensen RD, Calhoun DA, Dimmitt RA and Lacson A. Circulating CXC–chemokine concentrations in a murine intestinal ischemia–reperfusion model. *Fetal Pediatr Pathol* 2004; 23: 145–157.
40. Sukhotnik I, Brod V, Lurie M, et al. The effect of 100% oxygen on intestinal preservation and recovery following ischemia–reperfusion injury in rats. *Crit Care Med* 2009; 37: 1054–1061.
41. Kelly RF, Hursey TL, Parrillo JE and Schaer GL. Effect of 100% oxygen administration on infarct size and left ventricular function in a canine model of myocardial infarction and reperfusion. *Am Heart J* 1995; 130: 957–965.
42. Dyson A, Stidwill R, Taylor V and Singer M. The impact of inspired oxygen concentration on tissue oxygenation during progressive haemorrhage. *Intensive Care Med* 2009; 35: 1783–1791.
43. Da Silveira M and Yoshida WB. Trimetazidine and N-acetylcysteine in attenuating hind-limb ischemia and

reperfusion injuries: experimental study in rats. *Int Angiol* 2009; 28: 412–417.
44. Nanobashvili J, Neumayer C, Fuegl A, et al. Combined L-arginine and antioxidative vitamin treatment mollifies ischemia–reperfusion injury of skeletal muscle. *J Vasc Surg* 2004; 39: 868–877.
45. Bolcal C, Yildirim V, Doganci S, et al. Protective effects of antioxidant medications on limb ischemia reperfusion injury. *J Surg Res* 2007; 139: 274–279.
46. Crawford P, Good PA, Gutierrez E, et al. Effects of supplemental oxygen on forearm vasodilation in humans. *J Appl Physiol (1985)* 1997; 82: 1601–1606.
47. Bredle DL, Bradley WE, Chapler CK and Cain SM. Muscle perfusion and oxygenation during local hyperoxia. *J Appl Physiol (1985)* 1988; 65: 2057–2062.

Paper II

Effects of C1 INH on endothelial cell activation in a rat hind limb ischemia / reperfusion injury model

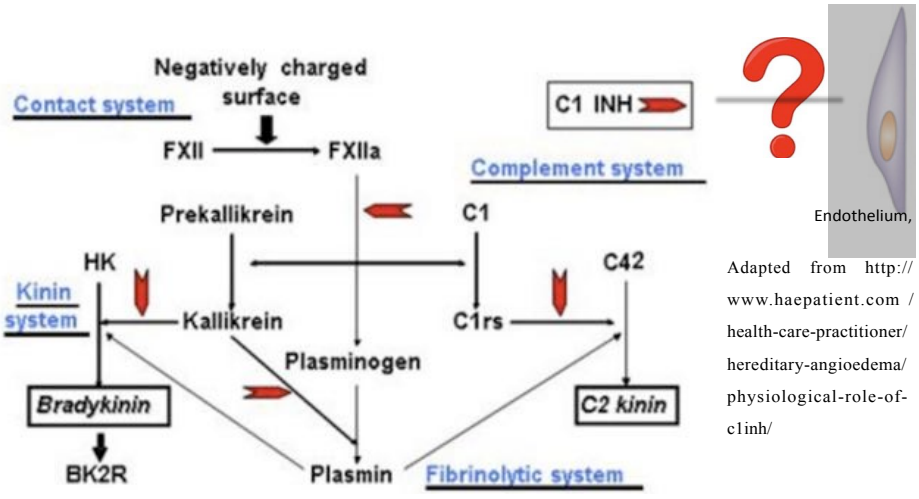
Shengye Zhang[1,2], Jane Shaw-Boden[1], Yara Banz[3], Anjan K. Bongoni[4], Adriano Taddeo[1,5], Rolf Spirig[6], Peter J. Cowan[4,7] and Robert Rieben[1]

[1]Department of Clinical Research, University of Bern, Bern, Switzerland; [2]Graduate School for Cellular and Biomedical Sciences, University of Bern, Bern, Switzerland; [3]Institute of Pathology, University of Bern, Bern, Switzerland; [4]Immunology Research Centre, St. Vincent's Hospital, Melbourne, Australia;[5]Division of Plastic and Hand Surgery, Inselspital, Bern, Switzerland; [6]CSL Behring AG, Bern, Switzerland; [7]Department of Medicine, University of Melbourne, Melbourne, Australia.

Status: In submission for publication to Mediators of Inflammation

Aim: The aim of this study was to investigate the effect of C1 INH on reperfusion injury and in particular on endothelial cell activation.

Conclusion: Pretreatment with both 50 and 100 IU/kg C1 inhibitor (C1 INH) attenuated reperfusion injury of rat hind limbs. Pretreatment with 100 IU/kg also preserved the endothelial heparan sulfate layer as well as the natural, pro-fibrinolytic phenotype of the endothelium. C1 INH may therefore be a promising strategy to prevent I/R injury in the clinical setting of elective surgery on extremities, including replantation or transplantation.



Adapted from http://www.haepatient.com/health-care-practitioner/hereditary-angioedema/physiological-role-of-c1inh/

Effects of C1 INH on endothelial cell activation in a rat hind limb ischemia / reperfusion injury model

Shengye Zhang[1,2], Jane Shaw-Boden[1], Yara Banz[3], Anjan K. Bongoni[4], Adriano Taddeo[1,5], Rolf Spirig[6], Peter J. Cowan[4,7] and Robert Rieben[1]

[1]Department of Clinical Research, University of Bern, Bern, Switzerland

[2]Graduate School for Cellular and Biomedical Sciences, University of Bern, Bern, Switzerland

[3]Institute of Pathology, University of Bern, Bern, Switzerland

[4]Immunology Research Centre, St. Vincent's Hospital, Melbourne, Australia

[5]Division of Plastic and Hand Surgery, Inselspital, Bern, Switzerland

[6]CSL Behring AG, Bern, Switzerland

[7]Department of Medicine, University of Melbourne, Melbourne, Australia

Corresponding author

Robert Rieben, Department of Clinical Research, University of Bern, Murtenstrasse 50, CH-3008 Bern, Switzerland.

Phone: +41 31 632 96 69

Fax: +41 31 632 75 94

Email: robert.rieben@dkf.unibe.ch

Abstract

We investigated the effect of C1 inhibitor on endothelial cell activation in rat hind limb reperfusion injury with 2h ischemia and 24h reperfusion. The animals were divided into five groups according to intravenous treatment 5 min before ischemia: 1) 50 IU/kg C1 inhibitor (n=5), 2) 100 IU/kg C1 inhibitor (n=7), 3) vehicle control (n=5), 4) non-treated control (n=7), and 5) normal, healthy control without intervention (n=4). Edema formation was reduced by C1 inhibitor, mirrored by improved histological injury scores and preserved muscle viability. Pretreatment with 100 IU/kg C1 inhibitor also significantly reduced heparan sulfate shedding and expression of plasminogen activator inhibitor-1 (PAI-1). In addition, plasma levels of high-mobility group box 1 (HMGB1) and deposition of IgM, complement fragments C4d and C5b-9, and fibrin were significantly decreased compared with non-treated controls. We conclude that pretreatment with both 50 and 100 IU/kg C1 inhibitor attenuated reperfusion injury of rat hind limbs. Pretreatment with 100 IU/kg also preserved the endothelial heparan sulfate layer as well as the natural, pro-fibrinolytic phenotype of the endothelium. Prevention of endothelial cell activation by C1 INH may therefore be a promising strategy to prevent I/R injury in the clinical setting of elective surgery on extremities, including replantation or transplantation.

1. Introduction

Reperfusion of temporarily ischemic tissue is essential to preserve viability and organ function. However, it is often associated with an inflammatory response, which augments tissue injury in excess of that initiated by ischemia alone. This phenomenon, termed ischemia/reperfusion (I/R) injury, is commonly encountered in myocardial and cerebral infarction, trauma, transplantation, tourniquet application in a surgical setting, as well as in peripheral vascular diseases (PAD). Several studies [1-3] showed that activation of the complement and coagulation systems are involved in the pathogenesis of I/R injury. Endothelial cell (EC) activation is also known to be involved in I/R injury, but so far no clinically applicable treatment strategies exist to prevent activation of EC in order to reduce reperfusion injury.

Activation of EC is characterized by shedding of the glycocalyx and expression or release of damage associated molecular patterns (DAMPs), with consequent activation of the plasma cascade systems. Moreover, adhesion molecules are upregulated on the endothelial surface, proinflammatory cytokines are released, and inflammatory cells adhere to the endothelium and translocate into the surrounding tissue. Shedding of the glycocalyx in I/R injury was first demonstrated by Czarnowska et al [4]. Later, Rehm et al [5] reported an elevated plasma concentration of syndecan-1 and heparan sulfate in patients after cardiac ischemia on cardiopulmonary bypass. Endothelial glycocalyx shedding was also documented in trauma patients [6], I/R of isolated guinea pig hearts [7], and vascular inflammation in rats [8]. One of the DAMPs involved in I/R injury is high-mobility group box 1 (HMGB-1), which is released by necrotic or damaged cells and triggers an inflammatory response in reperfusion injury of kidneys in mice [9], as well as hearts in mice and rats [10-12]. With exposure of DAMPs and expression of neo-epitopes [13, 14], the complement system may be activated via all three pathways, leading to formation of the anaphylatoxins C3a and C5a as well as the membrane attack complex C5b-9. The latter has been shown to alter signal transduction in EC [15], mediate apoptosis [16], and increase vascular permeability [17, 18]. During ischemia, adhesion molecules like E-selectin, P-selectin, and VCAM-1 can be upregulated to induce endothelial-leukocyte adhesion and endothelial-platelet aggregation [19-21]. As shown in myocardial infarction studies [22, 23], an increased production and secretion of plasminogen activator inhibitor-1 (PAI-1) by activated ECs plays a key role in the transition of the endothelium from a resting, anti-coagulant and pro-fibrinolytic state to an activated, pro-coagulant and anti-fibrinolytic state.

C1 inhibitor (C1 INH), a member of the serine protease inhibitor family, is an important regulator of all three plasma cascade systems, namely complement, coagulation, and kallikrein / kinin. Its beneficial effect in I/R injury models was documented in liver and hind limbs in rats [24, 25], kidney in swine [26], and brain in mice [27]. The favorable effect of C1 INH in reperfusion injury may not only result from serine protease inhibition alone, but also from attenuation of leukocyte-mediated inflammation [27-29]. Cai and Davis [30] reported that C1 INH could interfere with leukocyte-EC interaction by binding to P- and E-selectin.

The direct effect of C1 INH on EC activation in I/R injury has not been investigated so far. In the current study we hypothesized that C1 INH would reduce the extent of reperfusion injury by preventing EC activation, and tested this hypothesis in a rat hind limb I/R injury model.

2. Materials and Methods

2.1. Animals.

Male Wistar rats (wild type, bred at the central animal facility, University of Bern), weighing between 250g and 320g, were used in this study. All animals were housed under standard conditions with water and food ad lib. All animal experiments were performed in accordance with the terms of the Swiss animal protection law and were approved by the animal experimentation committee of the cantonal veterinary service (Canton of Bern, Switzerland). Experimental protocols were refined according to the 3R principles and state-of-the-art anesthesia and pain management were used to minimize the number of animals and to reduce the exposure of the animals to stress and pain during the experiments.

2.2. Reagents.

C1 esterase inhibitor (Berinert) and the respective vehicle (10 mg/ml glycine, 2.9 mg/ml sodium citrate, 8.5 mg/ml sodium chloride, pH 7.0) were provided by CSL Behring (CSL Behring GmbH, Marburg, Germany and CSL Behring, AG, Bern, Switzerland, respectively).

2.3. Experimental groups.

Rats were divided into five groups according to intravenous pretreatment 5 min before induction of ischemia. The experimental group 1 (n=5) received a dose of 50 IU/kg of human C1 INH, given as intravenous injection of 250-320 µl of a 50 IU/ml solution into the tail vein. Experimental group 2 (n=7) received a dose of 100 IU/kg of human C1 INH. The control group 1 (n=5) received C1 INH vehicle (glycine-citrate buffer, 250-320 µl). Control group 2 (n=7,

non-treated group) underwent ischemia/reperfusion injury without any treatment. Control group 3 (n=4, normal) consisted of normal healthy rats without any form of intervention.

2.4. Experimental procedure.

Anesthesia was induced with 3% isoflurane in 50% oxygen / air in a clear, polystyrene induction box and later maintained at 2% isoflurane using a standard rat nose mask (Provet, Lyssach, Switzerland). Analgesia was provided by 0.05 mg/kg of buprenorphine (Temgesic, Reckitt Benckiser, Switzerland AG), injected subcutaneously 30 minutes prior to the surgical intervention. The animals were placed on a heating pad and the rectal temperature kept at 37 ± 1°C. A Mouse-Ox plus System (Starr Life Sciences, Oakmont, PA, USA) was used to monitor and record rectal temperature, heart- and breath rates and arterial oxygen saturation throughout the anesthesia. Following groin incision, femoral artery, vein, and nerve were isolated and the femoral artery was clamped by two microvascular clamps (B1-V, S&T, Neuhausen, Switzerland) proximal to the deep femoral artery branch. To block collateral circulation, a tourniquet, connected to a weight of 450 g, was placed underneath the femoral vessels around the thigh as high as possible, without blocking the femoral vein. The rats underwent 2h ischemia followed by 24h reperfusion by releasing the vascular clamps and the tourniquet. At the onset of reperfusion, the rats were allowed to wake up and another dose of 0.05 mg/kg of buprenorphine was given to provide adequate analgesia. At the end of the 24h reperfusion period, the rats were anesthetized again and sacrificed by exsanguination during organ removal in deep anesthesia. Blood was collected and tissue samples from the gastrocnemius muscle were taken for further analysis.

2.5. Estimation of edema formation in muscle.

To assess edema formation in the affected gastrocnemius muscle, two pieces of muscle tissue in contralateral and reperfused limbs were taken, rinsed with PBS, blotted dry and weighed immediately to get the wet weight. The muscle pieces were then dried at 80°C overnight until a constant dry weight was obtained. The ratio of wet weight to dry weight (w/d ratio) was calculated as an indicator of edema formation within the muscle.

2.6. Muscle viability assay.

To assess muscle viability, an MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, M2128, Sigma) assay was performed. This assay relies on mitochondrial dehydrogenases, present in living cells, which can convert the MTT tetrazolium compound to its purple formazan dye. Dead cells are not able to catalyze this reaction. In brief, a piece of muscle tissue was incubated with 0.1 mg MTT/ml in PBS at 37°C for 2h, thereafter transferred to 100% isopropanol at 37°C overnight to extract the purple formazan dye from the tissue. Finally, the optical density of supernatant was measured at 570/690 nm (Infinite M1000 spectrophotometer, Tecan, Männedorf, Switzerland) and OD/mg dry muscle weight was used as a parameter for muscle viability and percent viability was calculated as: $\frac{OD\ /\ mg\ reperfused\ limb}{OD\ /\ mg\ contralateral\ limb} * 100$.

2.7. Evaluation of histopathological changes.

To evaluate histopathological changes after reperfusion injury, a piece of gastrocnemius muscle was taken, rinsed with PBS, blotted dry and fixed in 4% buffered formalin followed by paraffin embedding and hematoxylin / eosin (H&E) staining. Hemorrhage, neutrophil infiltration, myocyte damage, and edema were examined in a blinded manner by a broad certified pathologist familiar with I/R injury. A score of 0-2 was allocated to each of the four parameters, resulting in a total histological injury score ranging from 0-8.

2.8. Immunofluorescence.

Deposition of antibody, complement components and coagulation markers, and expression of heparan sulfate (HS), plasminogen activator inhibitor-1 (PAI-1), E-selectin, and vascular cell adhesion molecule-1 (VCAM-1) were all analyzed by immunofluorescence. In brief, muscle tissue samples from both legs were rinsed in PBS, blotted dry, embedded in OCT matrix (Tissue-Tek, Sakura Finetek Europe B.V., Leiden, The Netherlands), and stored at —25°C until cryosections were made and stained. The following antibodies were used: Goat anti-rat biotinylated-IgM (3020-08; Southern Biotech, Birmingham, AL, USA), rabbit anti-rat C1r (bs-15086R, Bioss Inc., Woburn, Massachusetts, USA), rabbit anti-rat C4d (HP8034, Hycult Biotech, Uden, Netherland), mouse anti-rat C5b-9 (mAb 2A1, Hycult Biotech), rabbit anti-human fibrin (F0111, Dako, Glostrup, Denmark), mouse anti-HS (370255, Amsbio, Abingdon, UK), rabbit anti-rat PAI-1 (sc-8979, Santa Cruz Biotechnology, Inc., Texas, USA), rabbit anti-human E-selectin (bs-1273R, Bioss Inc.), and rabbit anti-rat VCAM-1 (bs-0920R, Bioss Inc.). The polyclonal anti-human antibodies are known to be cross-reactive with the respective rat

antigens. The secondary antibodies and conjugates were sheep anti-rabbit IgG Cy3 (C 2306; Sigma, St. Louis, MO, USA), goat anti-mouse IgG alexa488 (A-21121; Thermo Fisher Scientific), goat anti-mouse IgM FITC (115-097-020; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and streptavidin-Cy3 (S6402; Sigma). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Boehringer, Roche Diagnostics, Indianapolis, IN, USA). Images were taken with a fluorescent microscope (Leica DMI 4000B, Leica Microsystems Schweiz AG, Heerbrugg, Switzerland) and analyzed by ImageJ (National Institutes of Health, Bethesda, MD, USA) and GraphPad Prism 6 software (GraphPad, San Diego, CA, USA). Fluorescence intensities or mean gray values were used for statistical comparison between groups. If the staining was observed throughout the tissue (IgM, fibrin, HS, and PAI-1), the whole area of the image was selected and analyzed, and integrated density was determined. When the signal was present specifically in blood vessels (C1r, C4d, C5b-9, E-selectin, and VCAM-1), only blood vessels were selected and the mean gray value was used to express the strength of the staining.

2.9. ELISA for C1 INH and HMGB-1.

To determine the levels of human C1 INH and rat HMGB-1 in plasma, a home-made sandwich-ELISA was performed. In brief, capture antibodies and HRP-conjugated detection antibodies were used, followed by TMB (3,3',5,5'-Tetramethylbenzidine) for color development, stopped by 1 mol/L H_2SO_4. At the end, optical density was measured at 450/650 nm for C1 INH and 450/540 nm for HMGB-1 (Infinite M1000 spectrophotometer, Tecan). Goat anti-human C1 INH (CL20023AP; Cedarlane, Canada) and HRP conjugated goat anti-human C1 INH (CL20023HP, Cedarlane) were used as capture and detection antibodies, respectively. Rabbit anti-rat HMGB-1 (H9539; Sigma-Aldrich) and HRP conjugated rabbit anti-rat HMGB-1 (ab128129; Abcam, Cambridge, UK) were used as capture and detection antibodies, respectively.

2.10. Statistical analysis.

All data are presented as a mean ± standard deviation (SD). Statistical analyses were performed by GraphPad Prism 6 software using one-way analysis of variance (ANOVA) followed by Fisher's LSD post hoc test to compare means of all groups. For comparing human C1 INH concentrations in plasma at the end of ischemia, unpaired t test was used. In the figures of this paper, statistical significance is only given for experimental groups 1 and 2 and control groups 1 and 2 to preserve visibility. P values <0.05 were considered statistically significant.

3. Results

3.1 Edema formation, muscle viability, histological evaluation.

Upon administration, edema formation in the reperfused limbs was significantly reduced both with 50 IU/kg (w/d ratio 5.07 ± 0.48, p=0.002) and 100 IU/kg (5.05 ± 0.50, p=0.001), as well as with the vehicle control group (5.21 ± 0.25, p=0.014), when compared with the non-treated group (5.69 ± 0.40) (Figure 1a). The protective effect of C1 INH was further evidenced by increased muscle viability (50 IU/kg C1 INH: 92.7 ± 9.8%, p= 0.004; 100 IU/kg C1 INH: 96.7 ± 14.5%, p=0.001) versus 60.6 ± 25.1% in the non-treated group (Figure 1b). Muscle viability of tissue from normal, healthy controls was 102.1 ± 16.6%. Histologically, a significantly higher injury score was found in the non-treated group (3.7 ± 1.5) compared to C1 INH treated groups (50 IU/kg: 1.0 ± 0.8, p<0.0001; 100 IU/kg: 2.1 ± 1.1, p=0.001) (Figure 1c-d). The histological injury score in the 50 IU/kg C1 INH pretreatment group was also significantly lower than the ones obtained for 100 IU/kg C1 INH (p=0.030) and vehicle (2.8 ± 1.1, p=0.002). No overt edema formation or tissue injury could be detected in tissue samples of the contralateral limbs, which did not undergo I/R, and in the limbs of normal, healthy rats without any intervention.

3.2. Deposition of IgM, C1r, C4d, and C5b-9.

To investigate the effect of C1 INH on natural antibody deposition and complement activation, IgM, C1r, C4d, and membrane attack complex C5b-9 were stained in the gastrocnemius muscle tissue by immunofluorescence. Deposition of all these proteins was significantly increased in the reperfused muscle tissue of the non-treated group compared to normal, healthy controls. Pre-treatment of animals with 100 IU/kg C1 INH led to a significant reduction of IgM, C4d, and C5b-9 deposits compared to the non-treated animals (p<0.0001, p<0.0001, and p=0.002, respectively), while a dose of 50 IU/kg C1 INH only led to a significant reduction of C4d deposition (p=0.008). In contrast, C1r deposition was not decreased by C1 INH administration. Compared to the non-treated group, tissue deposition of IgM and C4d was also significantly reduced in the vehicle group (p=0.012 and p=0.011, respectively, Figure 2).

3.3. Deposition of fibrin in tissue.

To assess the role of the coagulation system in the model, we performed tissue staining for fibrin deposition. Compared to normal, healthy controls, strong fibrin deposition was observed in the reperfused limbs of non-treated and vehicle-treated animals. This was significantly reduced in animals treated with 100 IU/kg C1 INH (p=0.002 vs. non-treated and p<0.0001 vs.

vehicle). In addition, pre-treatment with 50 IU/kg C1 INH led to a significant reduction of fibrin deposition compared to the vehicle-treated animals (p=0.006, Figure 3).

3.4 Human C1 INH in plasma.

Human C1 INH plasma concentrations were measured at baseline, end of ischemia, and end of reperfusion. The highest levels were found at the end of ischemia and correspond to the expected maximum plasma concentrations: 2.6 ± 1.3 IU/ml in the rats treated with 100 IU/kg and 1.2 ± 0.4 IU/ml when 50 IU/kg were given. After 24h of reperfusion no detectable human C1 INH was present in the plasma anymore (Figure 4).

3.5 HMGB-1 in plasma.

At the end of reperfusion, HMGB-1 in plasma was significantly reduced by 50 IU/kg C1 INH (27.3 ± 3.8 ng/ml, p=0.003), 100 IU/kg C1 INH (24.3 ± 4.0 ng/ml, p<0.0001), and vehicle treatment (28.9 ± 2.1 ng/ml, p=0.014) compared to the non-treated animals (34.7 ± 4.8 ng/ml, Figure 5).

3.6. Expression of heparan sulfate, PAI-1, E-selectin, and VCAM-1 in tissue.

To assess EC activation and C1 INH-mediated EC protection, staining for HS and PAI-1, as well as the adhesion molecules E-selectin and VCAM-1 was carried out. Compared with healthy controls, expression of HS was significantly reduced in muscle tissue of reperfused as well as contralateral limbs of non-treated and vehicle-treated rats (Figure 6a). Pre-treatment of rats with 100 IU/kg C1 INH significantly reduced I/R-induced HS shedding compared to the non-treated group (p=0.0001 and p=0.001, respectively), both between reperfused limbs and between contralateral limbs. 100IU/kg C1 INH also led to a significant reduction of HS shedding in the reperfused limbs compared with the vehicle group (p=0.028). Furthermore, a significant increase of HS staining compared to non-treated controls was also observed with a dose of 50 IU/kg (p=0.022 and p=0.007, respectively, Figure 6a), both between reperfused limbs and between contralateral limbs. For PAI-1 expression, a reciprocal pattern to HS was observed, with increased levels compared with normal, healthy controls in the reperfused as well as contralateral limbs of the vehicle group (p=0.001 and p=0.01, respectively). An increase of PAI-1 expression compared with healthy controls was also visible in the non-treated group, both in the reperfused and contralateral limbs, but did not reach statistical significance (p = 0.113 and 0.153, respectively. Figure 6b). C1 INH-treatment led to a significant reduction of PAI-1 expression compared to vehicle-treated animals, both in reperfused limbs of 50 IU/kg and

100 IU/kg groups (p=0.001 and p=0.0001, respectively) and in contralateral limbs of these two groups (p=0.006 and p=0.002). However, differences between PAI-1 expression in non-treated and C1 INH-treated animals were not significant (Figure 6b).

Expression of E-selectin and VCAM-1 was significantly increased in reperfused limbs of the experimental groups compared with normal animals without any intervention. No increase of expression was observed in contralateral limbs. A tendency of lower E-selectin expression was observed in reperfused limbs of animals treated with 50IU/kg C1 INH when compared with non-treated animals (p=0.060). However, no statistical differences were detected between these groups (Figure 6c-d).

4. Discussion

This study provides evidence that treatment with C1 INH limits the extent of reperfusion injury in a rat hind limb I/R injury model by preserving the natural anticoagulant and pro-fibrinolytic phenotype of the endothelium. C1 INH significantly reduced edema formation and histological injury, and preserved muscle viability in tissue. On a cellular level, C1 INH reduced shedding of HS, expression of PAI-1, release of HMGB-1, deposition of antibody, complement and fibrin. All of these markers are linked to EC activation either directly or indirectly during I/R injury.

Binding of natural antibodies and complement activation have been shown to be involved in inflammatory responses following reperfusion [31, 32]. Our study confirms this by demonstrating that deposition of IgM, C1r, C4d, and C5b-9 was significantly increased in reperfused limbs of the non-treated I/R injury group. Pre-treatment with 100 IU/kg C1 INH reduced deposition of IgM, C4d, and C5b-9, consistent with earlier data from other groups obtained via in vitro [33], ex-vivo [34], and in vivo experiments [35]. Deposition of the C1 complex, measured as C1r, was not affected by C1 INH administration. This is in line with earlier data of our own group [25] and suggests that the protective role of C1 INH may not depend on inhibition of C1 activation [36].

As reported earlier in reperfusion injury studies in brain and liver [37, 38], fibrin deposition was significantly increased in reperfused limbs of the I/R injury group without treatment as well as the vehicle-treatment group. Pretreatment of the rats with 100 IU/kg C1 INH clearly reduced fibrin deposition, suggesting either a direct effect of C1 INH treatment on the coagulation cascade or an indirect effect via prevention of EC activation.

Shedding of heparan sulfate proteoglycans and up-regulation of PAI-1 are key events in EC activation and transition to a pro-coagulant and anti-fibrinolytic phenotype. Heparan sulfate is one of the most common proteoglycans on the EC surface and importantly helps maintain the anti-inflammatory and anti-coagulant properties of healthy endothelium [39, 40]. In the present

study, loss of HS expression was observed in both contralateral and reperfused limbs following I/R, in line with previous data published by Rehm et al. [5]. Shedding of HS was prevented by pretreatment with both 50 IU/kg and 100 IU/kg, consistent with earlier reports in a xenograft rejection model [41] and our own data [25].

PAI-1 is a member of the serine protease inhibitor (serpin) family and a key regulator of the fibrinolytic system. Increased PAI-1 expression is a marker of EC activation, indicating decreased fibrinolytic activity of the endothelium [42]. Our data show that expression of PAI-1 was significantly increased in the vehicle group, confirming data from previous reperfusion injury studies in liver and kidney [43, 44]. Pretreatment with both doses of C1 INH reduced PAI-1 expression, mirrored by decreased fibrin deposition. Together, these data suggest that C1 INH administration preserved the endothelial heparan sulfate layer as well as the natural, anti-coagulant and pro-fibrinolytic phenotype of the endothelium as shown by reduced PAI-1 expression and fibrin deposition.

Expression of the adhesion molecules E-selectin and VCAM-1 was found in tissue from reperfused limbs in all groups. Pretreatment of the rats with C1 INH did not lead to significant down-regulation of E-selectin or VCAM-1 expression. Previous studies showed E-selectin binding via sialyl-Lewis^x tetrasaccharides on *N*-linked glycans of C1 INH, correlating with reduced leukocyte infiltration in a mouse peritonitis model [30, 45]. However, reduced expression of E-selectin by C1 INH has only been reported in xenotransplantation models, in which EC activation is completely dependent on complement [46]. Down-regulation of VCAM-1 mRNA in the liver was described in a rat LPS-induced sepsis model in which the animals were treated with 100 IU/kg C1 INH, but endothelial expression of VCAM-1 was not analyzed in this study [47].

In addition, higher levels of HMGB-1, as a potent mediator of inflammation, further supported severe I/R injury in the non-treated animals via toll like receptors (TLR) as shown in liver and kidney I/R injury model [9, 48]. Pretreatment with C1 INH significantly reduced plasma concentrations of HMGB-1, which has not been experimentally reported to date. Together with positive results of antibody, complement, coagulation, and EC activation from C1 INH-treated groups, suggesting that C1 INH is a multi-faceted anti-inflammation protein independent of its serine protease inhibition [49].

Interestingly in our study, treatment of the rats by vehicle alone also showed a significant reduction in edema formation, deposition of IgM and C4d, and release of HMGB-1. This might be explained by the presence of sodium citrate in the vehicle solution, which is an anti-coagulant, but also has an effect on complement activation in whole blood [50]. This may have been enough to reduce C4d binding to EC, leading to lower EC activation (lower expression of HMGB-1) and thus a reduced vascular leakage (reduced edema formation). However, vehicle-

only treatment had no effect on HS shedding and led to increased PAI-1 expression and therefore transition of the endothelium to a procoagulant and anti-fibrinolytic phenotype. Consequently, a (not significant) trend for increased fibrin deposition was seen in the vehicle-treated group and neither muscle viability nor histological injury scores were better in this group than no treatment.

Conclusions

In summary, pre-treatment of rats with both 50 IU/kg and 100 IU/kg C1 INH limited the extent of rat hind limb I/R injury. The mechanism by which C1 INH achieves this beneficial effect seems to be a reduction of EC activation, as reflected by prevention of HS shedding and reduced expression of PAI-1 and HMGB-1, which were particularly apparent at a dose of 100 IU/kg. Preoperative EC protection by C1 INH may therefore be a promising strategy to prevent I/R injury in the clinical setting of elective surgery on extremities, including replantation or transplantation.

Acknowledgements

We thank Claudia Dührkop for helpful discussions during rat experimentation. We also thank Marc Nolte and Sylvia Miescher for substance supply and helpful discussions. This work was support by the Microscopy Imaging Center (MIC), University of Bern, as well as the Translational Research Unit (TRU), Institute of Pathology, University of Bern.

Funding

Swiss National Science Foundation, grants no. 32003B_135272 and 320030_156193 to RR. The China Scholarship Council (CSC) awarded grant support to SZ in Switzerland.

Disclosure

The authors declare the following interests. Rolf Spirig is an employee of CSL Behring AG. Shengye Zhang was paid in part by a grant of CSL Behring AG. Robert Rieben received grant support from CSL Behring AG. C1 esterase inhibitor (Berinert) as well as the vehicle (10 mg/ml glycine, 2.9 mg/ml sodium citrate, 8.5 mg/ml sodium chloride, pH 7.0) were provided by CSL Behring (CSL Behring GmbH, Marburg, Germany for C1 INH and vehicle from CSL Behring AG, Bern, Switzerland). There are no further patents, products in development or marketed products to declare. All authors adhere to common scientific policy on sharing data and materials.

References

1. M. Zhang, E. M. Alicot, and M. C. Carroll, "Human natural IgM can induce ischemia/reperfusion injury in a murine intestinal model," *Molecular Immunology,* vol. 45, no. 15, pp. 4036-9, 2008.
2. V. I. Pavlov, Y. S. Tan, E. E. McClure, L. R. La Bonte, C. Zou, W. B. Gorsuch, and G. L. Stahl, "Human mannose-binding lectin inhibitor prevents myocardial injury and arterial thrombogenesis in a novel animal model," *American Journal of Pathology,* vol. 185, no. 2, pp. 347-55, 2015.
3. J. Kotimaa, P. van der Pol, S. Leijtens, N. Klar-Mohammad, G. Schilders, M. R. Daha, H. Rutjes, and C. van Kooten, "Functional assessment of rat complement pathway activities and quantification of soluble C5b-9 in an experimental model of renal ischemia/reperfusion injury," *Journal of Immunological Methods,* vol. 412, pp. 14-23, 2014.
4. E. Czarnowska, and E. Karwatowska-Prokopczuk, "Ultrastructural demonstration of endothelial glycocalyx disruption in the reperfused rat heart. Involvement of oxygen free radicals," *Basic Research in Cardiology,* vol. 90, no. 5, pp. 357-64, 1995.
5. M. Rehm, D. Bruegger, F. Christ, P. Conzen, M. Thiel, M. Jacob, D. Chappell, M. Stoeckelhuber, U. Welsch, B. Reichart, K. Peter, and B. F. Becker, "Shedding of the endothelial glycocalyx in patients undergoing major vascular surgery with global and regional ischemia," *Circulation,* vol. 116, no. 17, pp. 1896-906, 2007.
6. E. Rahbar, J. C. Cardenas, G. Baimukanova, B. Usadi, R. Bruhn, S. Pati, S. R. Ostrowski, P. I. Johansson, J. B. Holcomb, and C. E. Wade, "Endothelial glycocalyx shedding and vascular permeability in severely injured trauma patients," *Journal of Translational Medicine,* vol. 13, pp. 117, 2015.
7. D. Chappell, M. Jacob, K. Hofmann-Kiefer, M. Rehm, U. Welsch, P. Conzen, and B. F. Becker, "Antithrombin reduces shedding of the endothelial glycocalyx following ischaemia/reperfusion," *Cardiovascular Research,* vol. 83, no. 2, pp. 388-96, 2009.
8. H. H. Lipowsky, L. Gao, and A. Lescanic, "Shedding of the endothelial glycocalyx in arterioles, capillaries, and venules and its effect on capillary hemodynamics during inflammation," *American Journal of Physiology: Heart and Circulatory Physiology,* vol. 301, no. 6, pp. H2235-45, 2011.
9. H. Wu, J. Ma, P. Wang, T. M. Corpuz, U. Panchapakesan, K. R. Wyburn, and S. J. Chadban, "HMGB1 contributes to kidney ischemia reperfusion injury," *Journal of the American Society of Nephrology,* vol. 21, no. 11, pp. 1878-90, 2010.
10. S. Oozawa, S. Mori, T. Kanke, H. Takahashi, K. Liu, Y. Tomono, M. Asanuma, I. Miyazaki, M. Nishibori, and S. Sano, "Effects of HMGB1 on ischemia-reperfusion injury in the rat heart," *Circulation Journal,* vol. 72, no. 7, pp. 1178-84, 2008.
11. M. Andrassy, H. C. Volz, J. C. Igwe, B. Funke, S. N. Eichberger, Z. Kaya, S. Buss, F. Autschbach, S. T. Pleger, I. K. Lukic, F. Bea, S. E. Hardt, P. M. Humpert, M. E. Bianchi, H. Mairbaurl, P. P. Nawroth, A. Remppis, H. A. Katus, and A. Bierhaus, "High-mobility group box-1 in ischemia-reperfusion injury of the heart," *Circulation,* vol. 117, no. 25, pp. 3216-26, 2008.
12. Y. Lin, L. Chen, W. Li, and J. Fang, "Role of high-mobility group box-1 in myocardial ischemia/reperfusion injury and the effect of ethyl pyruvate," *Experimental and Therapeutic Medicine,* vol. 9, no. 4, pp. 1537-1541, 2015.
13. M. Zhang, E. M. Alicot, I. Chiu, J. Li, N. Verna, T. Vorup-Jensen, B. Kessler, M. Shimaoka, R. Chan, D. Friend, U. Mahmood, R. Weissleder, F. D. Moore, and M. C. Carroll, "Identification of the target self-antigens in reperfusion injury," *Journal of Experimental Medicine,* vol. 203, no. 1, pp. 141-52, 2006.
14. L. Kulik, S. D. Fleming, C. Moratz, J. W. Reuter, A. Novikov, K. Chen, K. A. Andrews, A. Markaryan, R. J. Quigg, G. J. Silverman, G. C. Tsokos, and V. M. Holers, "Pathogenic natural antibodies recognizing annexin IV are required to develop intestinal ischemia-reperfusion injury," *Journal of Immunology,* vol. 182, no. 9, pp. 5363-73, 2009.
15. I. U. Schraufstatter, K. Trieu, L. Sikora, P. Sriramarao, and R. DiScipio, "Complement c3a and c5a induce different signal transduction cascades in endothelial cells," *Journal of Immunology,* vol. 169, no. 4, pp. 2102-10, 2002.
16. J. Hughes, M. Nangaku, C. E. Alpers, S. J. Shankland, W. G. Couser, and R. J. Johnson, "C5b-9 membrane attack complex mediates endothelial cell apoptosis in experimental glomerulonephritis," *American Journal of Physiology: Renal Physiology,* vol. 278, no. 5, pp. F747-57, 2000.
17. T. V. Arumugam, I. A. Shiels, A. J. Strachan, G. Abbenante, D. P. Fairlie, and S. M. Taylor, "A small molecule C5a receptor antagonist protects kidneys from ischemia/reperfusion injury in rats," *Kidney International,* vol. 63, no. 1, pp. 134-42, 2003.
18. W. Zhou, C. A. Farrar, K. Abe, J. R. Pratt, J. E. Marsh, Y. Wang, G. L. Stahl, and S. H. Sacks, "Predominant role for C5b-9 in renal ischemia/reperfusion injury," *Journal of Clinical Investigation,* vol. 105, no. 10, pp. 1363-71, 2000.

19. F. Gao, T. L. Yue, D. W. Shi, T. A. Christopher, B. L. Lopez, E. H. Ohlstein, F. C. Barone, and X. L. Ma, "p38 MAPK inhibition reduces myocardial reperfusion injury via inhibition of endothelial adhesion molecule expression and blockade of PMN accumulation," *Cardiovascular Research,* vol. 53, no. 2, pp. 414-22, 2002.
20. S. P. Jones, S. D. Trocha, M. B. Strange, D. N. Granger, C. G. Kevil, D. C. Bullard, and D. J. Lefer, "Leukocyte and endothelial cell adhesion molecules in a chronic murine model of myocardial reperfusion injury," *American Journal of Physiology: Heart and Circulatory Physiology,* vol. 279, no. 5, pp. H2196-201, 2000.
21. T. Zhou, G. Z. Sun, M. J. Zhang, J. L. Chen, D. Q. Zhang, Q. S. Hu, Y. Y. Chen, and N. Chen, "Role of adhesion molecules and dendritic cells in rat hepatic/renal ischemia-reperfusion injury and anti-adhesive intervention with anti-P-selectin lectin-EGF domain monoclonal antibody," *World Journal of Gastroenterology,* vol. 11, no. 7, pp. 1005-10, 2005.
22. M. Ilic, N. Majkic-Singh, and K. Lalic, "Plasminogen activator inhibitor in patients with acute myocardial infarction and re-infarction in syndrome X," *Clínica y Laboratorio,* vol. 48, no. 3-4, pp. 125-8, 2002.
23. J. P. Collet, G. Montalescot, E. Vicaut, A. Ankri, F. Walylo, C. Lesty, R. Choussat, F. Beygui, M. Borentain, N. Vignolles, and D. Thomas, "Acute release of plasminogen activator inhibitor-1 in ST-segment elevation myocardial infarction predicts mortality," *Circulation,* vol. 108, no. 4, pp. 391-4, 2003.
24. B. H. Heijnen, I. H. Straatsburg, N. D. Padilla, G. J. Van Mierlo, C. E. Hack, and T. M. Van Gulik, "Inhibition of classical complement activation attenuates liver ischaemia and reperfusion injury in a rat model," *Clinical and Experimental Immunology,* vol. 143, no. 1, pp. 15-23, 2006.
25. C. Duehrkop, Y. Banz, R. Spirig, S. Miescher, M. W. Nolte, M. Spycher, R. A. Smith, S. H. Sacks, and R. Rieben, "C1 esterase inhibitor reduces lower extremity ischemia/reperfusion injury and associated lung damage," *PloS One,* vol. 8, no. 8, pp. e72059, 2013.
26. G. Castellano, R. Melchiorre, A. Loverre, P. Ditonno, V. Montinaro, M. Rossini, C. Divella, M. Battaglia, G. Lucarelli, G. Annunziata, S. Palazzo, F. P. Selvaggi, F. Staffieri, A. Crovace, M. R. Daha, M. Mannesse, S. van Wetering, F. Paolo Schena, and G. Grandaliano, "Therapeutic targeting of classical and lectin pathways of complement protects from ischemia-reperfusion-induced renal damage," *American Journal of Pathology,* vol. 176, no. 4, pp. 1648-59, 2010.
27. N. Heydenreich, M. W. Nolte, E. Gob, F. Langhauser, M. Hofmeister, P. Kraft, C. Albert-Weissenberger, M. Brede, C. Varallyay, K. Gobel, S. G. Meuth, B. Nieswandt, G. Dickneite, G. Stoll, and C. Kleinschnitz, "C1-inhibitor protects from brain ischemia-reperfusion injury by combined antiinflammatory and antithrombotic mechanisms," *Stroke,* vol. 43, no. 9, pp. 2457-67, 2012.
28. F. Lu, S. M. Fernandes, and A. E. Davis, 3rd, "The effect of C1 inhibitor on myocardial ischemia and reperfusion injury," *Cardiovascular Pathology,* vol. 22, no. 1, pp. 75-80, 2013.
29. F. Lu, A. K. Chauhan, S. M. Fernandes, M. T. Walsh, D. D. Wagner, and A. E. Davis, 3rd, "The effect of C1 inhibitor on intestinal ischemia and reperfusion injury," *American Journal of Physiology: Gastrointestinal and Liver Physiology,* vol. 295, no. 5, pp. G1042-9, 2008.
30. S. Cai, and A. E. Davis, 3rd, "Complement regulatory protein C1 inhibitor binds to selectins and interferes with endothelial-leukocyte adhesion," *Journal of Immunology,* vol. 171, no. 9, pp. 4786-91, 2003.
31. M. R. Weiser, J. P. Williams, F. D. Moore, Jr., L. Kobzik, M. Ma, H. B. Hechtman, and M. C. Carroll, "Reperfusion injury of ischemic skeletal muscle is mediated by natural antibody and complement," *Journal of Experimental Medicine,* vol. 183, no. 5, pp. 2343-8, 1996.
32. C. Atkinson, S. He, K. Morris, F. Qiao, S. Casey, M. Goddard, and S. Tomlinson, "Targeted complement inhibitors protect against posttransplant cardiac ischemia and reperfusion injury and reveal an important role for the alternative pathway of complement activation," *Journal of Immunology,* vol. 185, no. 11, pp. 7007-13, 2010.
33. E. W. Nielsen, C. Waage, H. Fure, O. L. Brekke, G. Sfyroera, J. D. Lambris, and T. E. Mollnes, "Effect of supraphysiologic levels of C1-inhibitor on the classical, lectin and alternative pathways of complement," *Molecular Immunology,* vol. 44, no. 8, pp. 1819-26, 2007.
34. A. E. Fiane, V. Videm, H. T. Johansen, O. J. Mellbye, E. W. Nielsen, and T. E. Mollnes, "C1-inhibitor attenuates hyperacute rejection and inhibits complement, leukocyte and platelet activation in an ex vivo pig-to-human perfusion model," *Immunopharmacology,* vol. 42, no. 1-3, pp. 231-43, 1999.
35. C. de Zwaan, A. H. Kleine, J. H. Diris, J. F. Glatz, H. J. Wellens, P. F. Strengers, M. Tissing, C. E. Hack, M. P. van Dieijen-Visser, and W. T. Hermens, "Continuous 48-h C1-inhibitor treatment, following reperfusion therapy, in patients with acute myocardial infarction," *European Heart Journal,* vol. 23, no. 21, pp. 1670-7, 2002.
36. M. G. De Simoni, E. Rossi, C. Storini, S. Pizzimenti, C. Echart, and L. Bergamaschini, "The powerful neuroprotective action of C1-inhibitor on brain ischemia-reperfusion injury does not require C1q," *American Journal of Pathology,* vol. 164, no. 5, pp. 1857-63, 2004.

37. Y. Okada, B. R. Copeland, R. Fitridge, J. A. Koziol, and G. J. del Zoppo, "Fibrin contributes to microvascular obstructions and parenchymal changes during early focal cerebral ischemia and reperfusion," *Stroke,* vol. 25, no. 9, pp. 1847-53; discussion 1853-4, 1994.
38. K. Okajima, N. Harada, S. Kushimoto, and M. Uchiba, "Role of microthrombus formation in the development of ischemia/reperfusion-induced liver injury in rats," *Thrombosis and Haemostasis,* vol. 88, no. 3, pp. 473-80, 2002.
39. C. R. Parish, "Heparan sulfate and inflammation," *Nature Immunology,* vol. 6, no. 9, pp. 861-2, 2005.
40. J. Liu, and L. C. Pedersen, "Anticoagulant heparan sulfate: structural specificity and biosynthesis," *Applied Microbiology and Biotechnology,* vol. 74, no. 2, pp. 263-72, 2007.
41. A. P. Dalmasso, and J. L. Platt, "Prevention of complement-mediated activation of xenogeneic endothelial cells in an in vitro model of xenograft hyperacute rejection by C1 inhibitor," *Transplantation,* vol. 56, no. 5, pp. 1171-6, 1993.
42. S. Handt, W. G. Jerome, L. Tietze, and R. R. Hantgan, "Plasminogen activator inhibitor-1 secretion of endothelial cells increases fibrinolytic resistance of an in vitro fibrin clot: evidence for a key role of endothelial cells in thrombolytic resistance," *Blood,* vol. 87, no. 10, pp. 4204-13, 1996.
43. K. Inoue, Y. Sugawara, K. Kubota, T. Takayama, and M. Makuuchi, "Induction of type 1 plasminogen activator inhibitor in human liver ischemia and reperfusion," *Journal of Hepatology,* vol. 33, no. 3, pp. 407-14, 2000.
44. X. S. Zhou, Y. F. Qiao, R. P. Wu, and R. S. Li, "Study on related indexes of the coagulation and fibrinolytic system after renal ischemia reperfusion injury in Wistar rats," *Saudi Medical Journal,* vol. 34, no. 6, pp. 579-83, 2013.
45. S. Cai, V. S. Dole, W. Bergmeier, J. Scafidi, H. Feng, D. D. Wagner, and A. E. Davis, 3rd, "A direct role for C1 inhibitor in regulation of leukocyte adhesion," *Journal of Immunology,* vol. 174, no. 10, pp. 6462-6, 2005.
46. U. O. Solvik, G. Haraldsen, A. E. Fiane, E. Boretti, J. D. Lambris, M. Fung, E. Thorsby, and T. E. Mollnes, "Human serum-induced expression of E-selectin on porcine aortic endothelial cells in vitro is totally complement mediated," *Transplantation,* vol. 72, no. 12, pp. 1967-73, 2001.
47. R. S. Croner, T. G. Lehmann, C. Fallsehr, C. Herfarth, E. Klar, and M. Kirschfink, "C1-inhibitor reduces hepatic leukocyte-endothelial interaction and the expression of VCAM-1 in LPS-induced sepsis in the rat," *Microvascular Research,* vol. 67, no. 2, pp. 182-91, 2004.
48. A. Tsung, R. Sahai, H. Tanaka, A. Nakao, M. P. Fink, M. T. Lotze, H. Yang, J. Li, K. J. Tracey, D. A. Geller, and T. R. Billiar, "The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion," *Journal of Experimental Medicine,* vol. 201, no. 7, pp. 1135-43, 2005.
49. F. Lu, S. M. Fernandes, and A. E. Davis, 3rd, "The role of the complement and contact systems in the dextran sulfate sodium-induced colitis model: the effect of C1 inhibitor in inflammatory bowel disease," *American Journal of Physiology: Gastrointestinal and Liver Physiology,* vol. 298, no. 6, pp. G878-83, 2010.
50. S. Huang, K. Sandholm, N. Jonsson, A. Nilsson, A. Wieslander, G. Grundstrom, V. Hancock, and K. N. Ekdahl, "Low concentrations of citrate reduce complement and granulocyte activation in vitro in human blood," *Clin Kidney J,* vol. 8, no. 1, pp. 31-7, 2015.

Figures and Legends

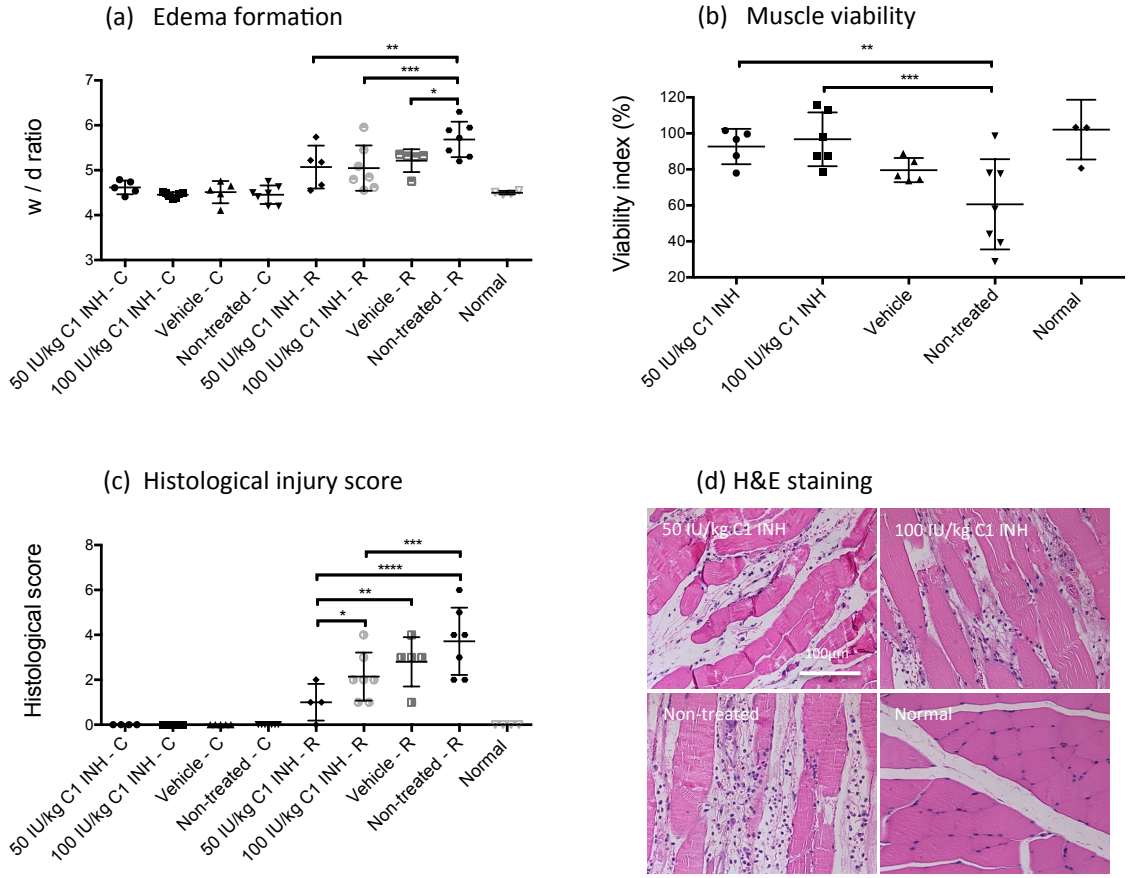


FIGURE 1**:** Effect of C1 INH on edema formation, muscle viability and histological injury in muscle tissue. (a) Analysis of edema formation in gastrocnemius muscle in both the contralateral and reperfused limbs. (b) Viability of gastrocnemius muscle assessed by MTT assay. (c) Histological assessment by H&E staining. (d) Representative images of H&E-stained tissue in each group. One-way ANOVA followed by Fisher's LSD post hoc test was used. Data of individual experiments are shown by dots with indication of mean ± SD by lines. *P<0.05; **P<0.01; ****P<0.0001. C - contralateral limb; R - reperfused limb. Scale bar represents 100 µm.

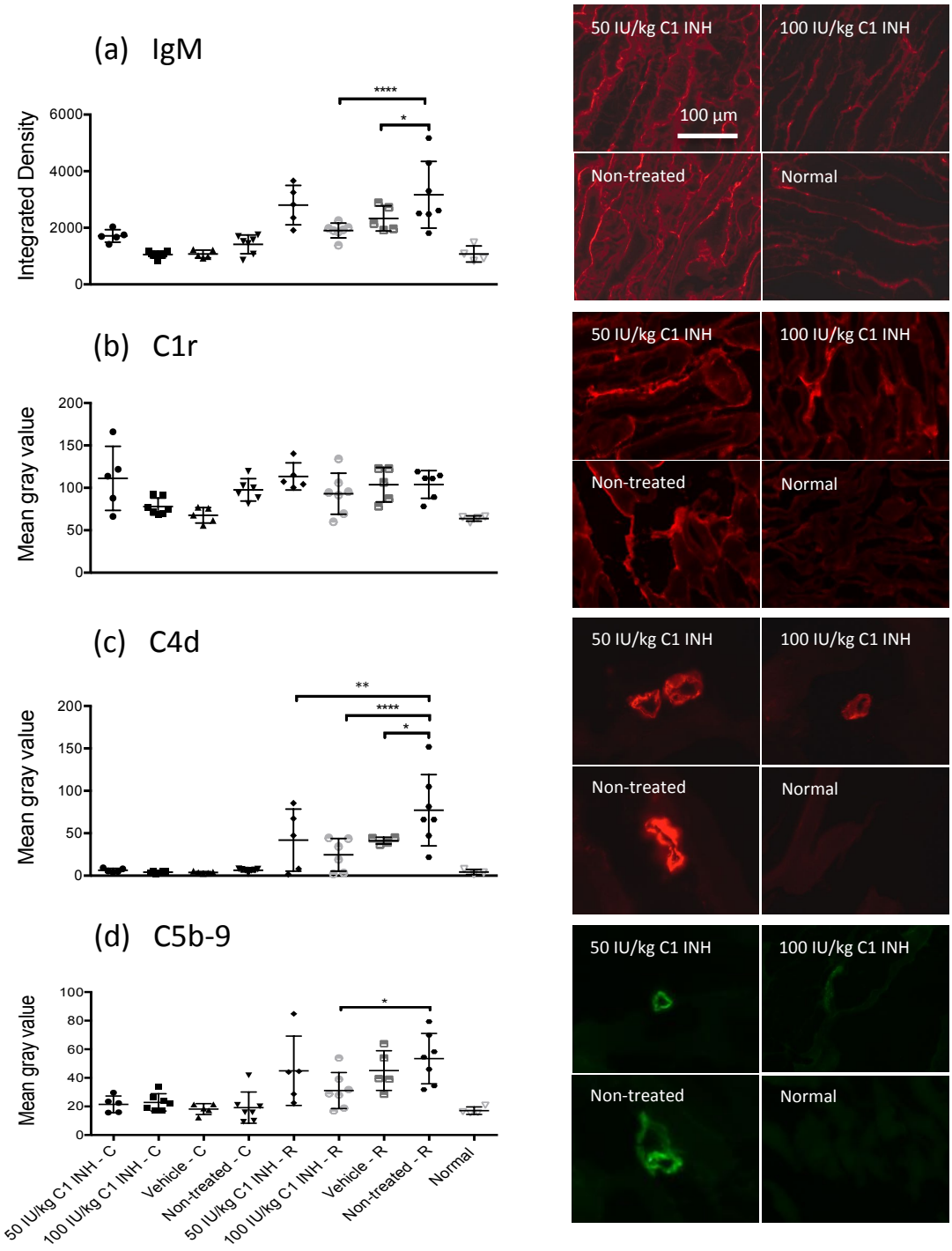


FIGURE 2: Analysis of deposition of antibody and complement fragments in muscle tissue. (a) Deposition of IgM. (b) Deposition of C1r. (c) Deposition of C4d. (d) Deposition of C5b-9. Statistical analyses are shown on the left and corresponding representative images on the right. One-way ANOVA followed by Fisher's LSD post hoc test was used. Data of individual experiments are shown by dots with indication of mean ± SD by lines. *P<0.05; **P<0.01; ****P<0.0001. C – contralateral limb; R – reperfused limb. Scale bar represents 100 µm.

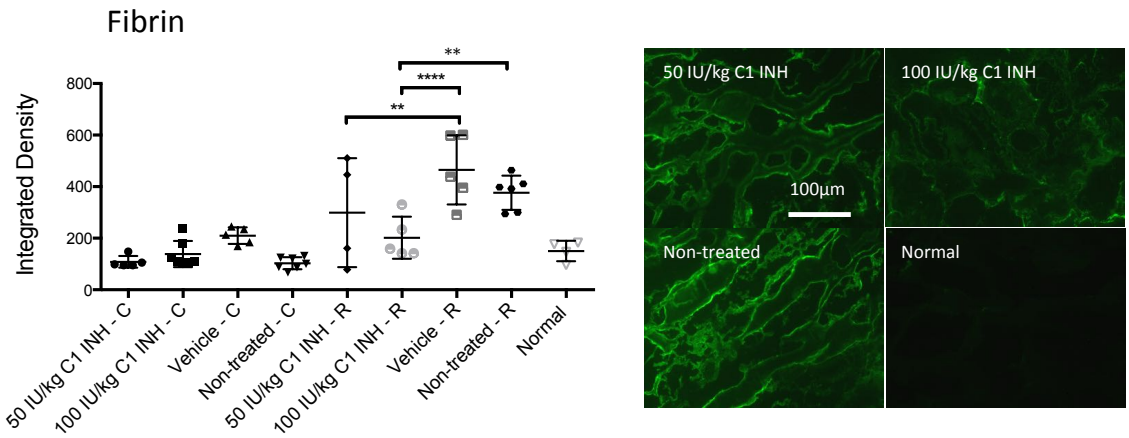


FIGURE 3: Deposition of fibrin formation in muscle tissue. Statistical analyses are shown on the left and the corresponding representative images on the right. One-way ANOVA followed by Fisher's LSD post hoc test was used. Data of individual experiments are shown by dots with indication of mean ± SD by lines. **P<0.01; ****P<0.0001. C – contralateral limb; R – reperfused limb. Scale bar represents 100 µm.

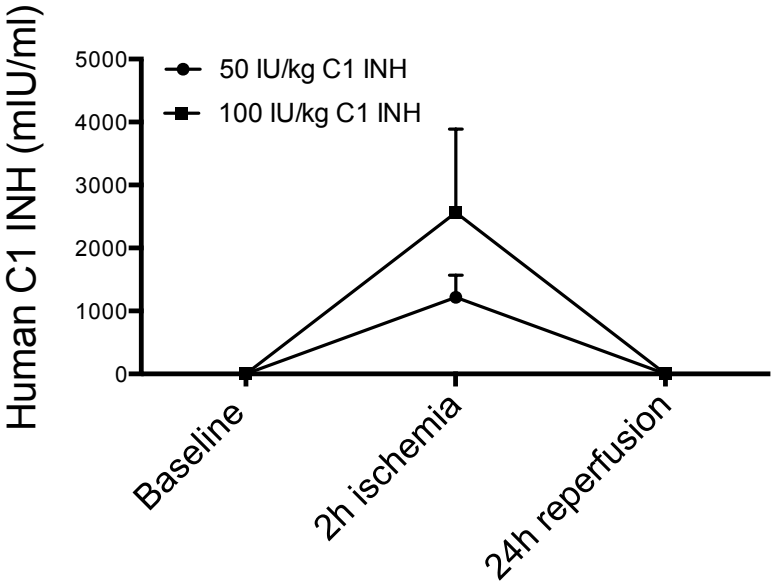


FIGURE 4: Plasma levels of human C1 INH. The concentration of human C1 INH in rat plasma was measured by species-specific ELISA. Statistical analysis was performed by non-paired t-test. Data show mean ± SD for C1 INH plasma levels over time.

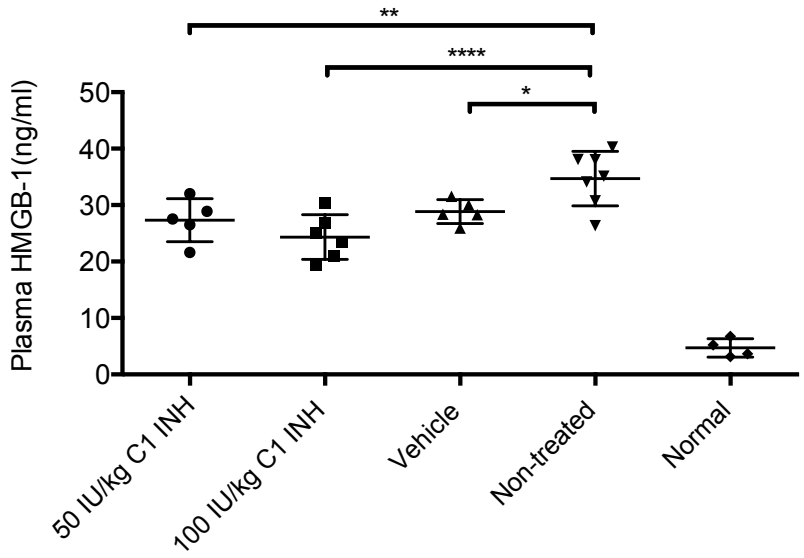


FIGURE 5: Plasma level of HMGB-1. The concentration of HMGB-1 in rat plasma was measured by species-specific ELISA. One-way ANOVA followed by Fisher's LSD post hoc test was used. Data of individual experiments are shown by dots with indication of mean ± SD by lines. *P<0.05; **P<0.01; ****P<0.0001. C – contralateral limb; R – reperfused limb. Scale bar represents 100 μm.

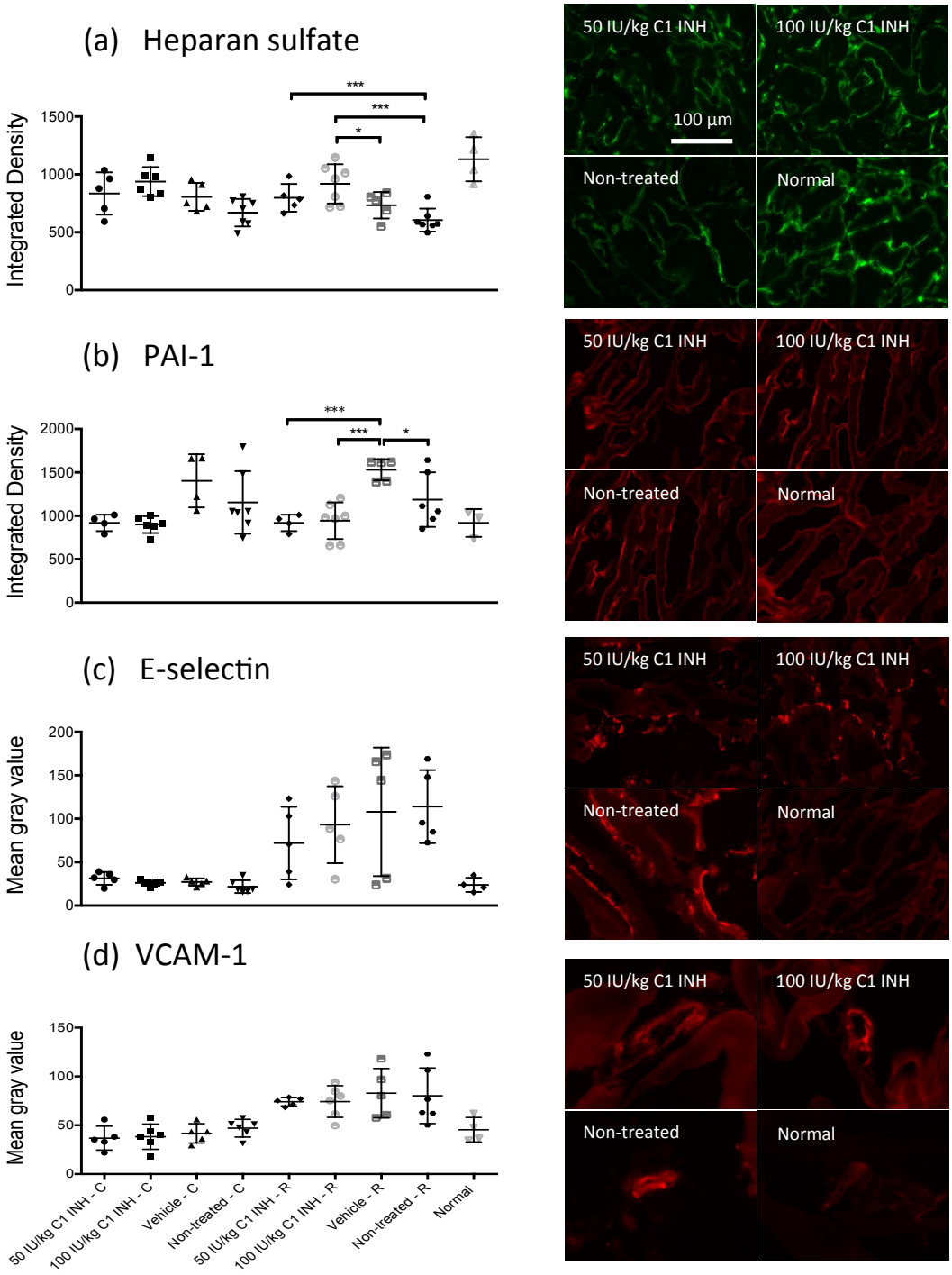


FIGURE 6: Analysis of endothelial cell markers in muscle tissue. (a) Expression of heparan sulfate. (b) Expression of PAI-1. (c) Expression of E-selectin. (d) Expression of VCAM-1. Statistical analyses are shown on the left, and the corresponding representative images are on the right. One-way ANOVA followed by Fisher's LSD post hoc test was used. Data of individual experiments are shown by dots with indication of mean ± SD by lines. *P<0.05; ***P<0.001. C - contralateral limb; R - reperfused limb. Scale bar represents 100 µm.

Paper III

Role of the plasma cascade systems in ischemia/reperfusion injury of bone

Shengye Zhang[1,2], Carlos Wotzkow[2], Anjan K. Bongoni[3], Jane Shaw-Boden[2], Mark Siegrist[2], Adriano Taddeo[2,4], Fabian Blank[2,5], Willy Hofstetter[2], Robert Rieben[2]

[1] Graduate School for Cellular and Biomedical Sciences, University of Bern, Bern, Switzerland; [2] Department of Clinical Research, University of Bern, Bern, Switzerland; [3] Immunology Research Centre, St. Vincent's Hospital, Melbourne, Australia; [4] Division of Plastic and Hand Surgery, Inselspital, Bern, Switzerland; [5] Pulmonary Medicine, Bern University Hospital, Bern, Switzerland.

Status: In submission for publication to Bone.

Aim: The aim was to see whether we can find signs of bone IRI and to study the involved pathophysiological mechanisms.

Conclusion: Ischemia and reperfusion of bone leads to activation of the complement and coagulation systems and a downregulation of the fibrinolytic cascade. In the acute phase, a vascular inflammation induced by activation of the plasma cascade systems therefore occurs in the bone. This is similar to I/R injury of other vascularized organs and tissues.

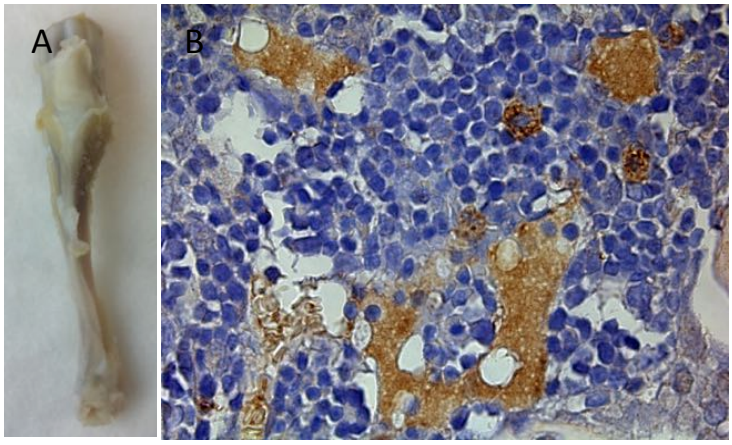


Figure: (A) The tibia bone. (B) Expression of tissue factor in normal bone marrow.

Role of the plasma cascade systems in ischemia/reperfusion injury of bone

Shengye Zhang[1,2], Carlos Wotzkow[2], Anjan K. Bongoni[3], Jane Shaw-Boden[2], Mark Siegrist[2], Adriano Taddeo[2,4], Fabian Blank[2,5], Willy Hofstetter[2], Robert Rieben[2]

[1] Graduate School for Cellular and Biomedical Sciences, University of Bern, Bern, Switzerland

[2] Department of Clinical Research, University of Bern, Bern, Switzerland

[3] Immunology Research Centre, St. Vincent's Hospital, Melbourne, Australia

[4] Division of Plastic and Hand Surgery, Inselspital, Bern, Switzerland

[5] Pulmonary Medicine, Bern University Hospital, Bern, Switzerland

Corresponding author

Robert Rieben
Department of Clinical Research, University of Bern
Murtenstrasse 50
CH-3008 Bern, Switzerland
Phone: +41 31 632 96 69
Fax: +41 31 632 75 94
Email: robert.rieben@dkf.unibe.ch

Highlights

- Ischemia/reperfusion (I/R) injury of bone was assessed in a rat hind limb model
- Complement and coagulation were up regulated in I/R injury of bone.
- Fibrinolysis was down regulated in I/R of bone.

	Cortical bone		**Bone marrow**	
	Reperfused	Contralateral	Reperfused	Contralateral
Complement C3b/c	↑	↑	↑	–
Tissue factor	–	–	–	↑
Fibrin	↑	↑	↑	↑
Tissue plasminogen activator (tPA)	↓	↓	↓	↓
Plasminogen activator inhibitor1 (PAI-1)	↑	–	**Not detectable**	**Not detectable**
E-selectin	–	–	**Not detectable**	**Not detectable**

Abstract

Ischemia/reperfusion (I/R) injury has been extensively studied in organs such as heart, brain, liver, kidney, and lung. As a vascularized organ, bone is known to be susceptible to I/R injury too, but the respective mechanisms are not well understood to date. We therefore asked the question whether plasma cascade-induced inflammation plays a role in bone I/R injury similar to other organs. Reperfusion injury in rat tibia was induced by unilateral clamping of the femoral artery and additional use of a tourniquet, while keeping the femoral vein patent to prevent venous congestion. Rats were subjected to 4h ischemia and 24h reperfusion. Deposition of complement fragment C3b/c and fibrin as well as expression of tissue factor (TF), tissue plasminogen activator (tPA), plasminogen activator inhibitor-1 (PAI-1), and E-selectin was detected by immunohistochemistry. In plasma, the levels of high mobility group box-1 (HMGB-1) were measured by ELISA. The total level of complement in serum was assessed by the CH50 test. Our results show that deposition of C3b/c was significantly increased with respect to healthy controls in cortical bone as well as in marrow of reperfused limbs. C3b/c deposition was also increased in cortical bone, but not in bone marrow of contralateral limbs. Deposition of fibrin, as well as expression of PAI-1, were significantly increased in bone after ischemia and reperfusion, whereas expression of tPA was reduced. These differences were most prominent in vessels of bone, both in marrow and cortical bone, and both in reperfused and contralateral limbs. However, PAI-1, was only increased in vessels of reperfused cortical bone and there were no significant changes in expression of E-selectin. CH50 values in serum decreased over time and HMGB-1 was significantly increased in plasma of animals at the end of reperfusion. We conclude that ischemia and reperfusion of bone leads to activation of the complement and coagulation systems and a downregulation of the fibrinolytic cascade. In the acute phase, a vascular inflammation induced by activation of the plasma cascade systems therefore occurs in the bone. This is similar to I/R injury of other vascularized organs and tissues.

Keywords: Bone, ischemia/reperfusion injury, complement, coagulation, fibrinolysis

1. Introduction

As a vascularized organ, bone is known to be susceptible to ischemia/reperfusion (I/R) injury [1, 2]. This can be the case in traumatic or non-traumatic conditions, for example in limb replantation, transplantation, but also in surgery on the extremities in general. All cells including osteoblasts, osteocytes, osteoclasts, chondrocytes, and bone marrow cells can be affected and involved in sterile inflammation during reperfusion injury, leading to disruption of bone homeostasis or cell death [3, 4]. We therefore wanted to study the mechanisms involved in bone I/R injury, focusing on the activation of the plasma cascade systems and involvement of bone vasculature.

Accumulating studies have already documented the role of the complement cascade in reperfusion injury [5-7]. During ischemia, damage associated molecule patterns (DAMPs) such as HMGB-1 are induced and expressed on the cell surface, which can be recognized by circulating natural antibodies and trigger complement activation [8-10]. Anaphylatoxins C3a and C5a as well as the membrane attack complex C5b-9 are subsequently formed. It was reported that mice with C3 deficiency were protected from skeletal muscle I/R injury, which was restored after reconstitution with serum from normal mice [11]. Also C5-deficient mice showed 48% less reperfusion injury than wild-type mice in a hind limb I/R injury model [12]. However, reperfusion injury was reestablished after reconstitution with wild-type serum, suggesting an important role of C5b-9 in the development of skeletal muscle I/R injury [12].

Also the coagulation cascade plays an important role in I/R injury. This was for example demonstrated in hind limb I/R injury, in which fibrin deposition and plasma levels of thrombotic marker were increased [13, 14]. Under quiescent conditions, endothelium maintains its anti-coagulant and pro-fibrinolytic state by regulation of the fibrinolytic system. Among others, tissue-type plasminogen activator (tPA) is a major fibrinolytic marker. Fibrinolysis was shown to be impaired in a clinical limb I/R injury study as confirmed by the reduced levels of tPA in blood [15]. In addition to its effect in the fibrinolytic cascade, tPA modulates reperfusion injury-induced inflammation via neutrophil influx in lung and kidney in mice [16, 17]. The principle inhibitor of tPA, plasminogen activator inhibitor 1 (PAI-1), is mainly synthesized by activated endothelial cells (ECs). Increased concentrations of PAI-1 are associated with hypercoagulant disorders in thrombotic diseases and I/R injury [18, 19]. Higher PAI-1 and/or lower tPA levels shift healthy endothelium into a pro-coagulant and anti-fibrinolytic phenotype.

Studies on reperfusion injury of bone are limited and the respective mechanisms of bone I/R injury are not well understood to date. In the present study we therefore asked the question whether plasma cascade systems-induced inflammation plays a role in bone I/R injury as occurs in other organs.

2. Materials and methods

2.1 Animals

Male Wistar rats (wild type, bred at the central animal facility, University of Bern), weighing between 280g and 320g, were used in this study. All animals were housed under standard conditions with water and food ad lib. All animal experiments were performed in accordance with the U.K. Animals Act (scientific procedures) and the health Guide for the Care and Use of Laboratory Animals, as well as the Swiss animal protection law. All animal studies complied with the ARRIVE guidelines. The animal experimentation committee of the cantonal veterinary service (Canton of Bern, Switzerland) approved all animal procedures, permission no. BE70/14. Experimental protocols were refined according to the 3R principles and state-of-the-art anesthesia and pain management were used to minimize the number of animals and reduce the exposure of the animals to stress and pain during the experiments.

2.2 Surgical procedure

Anesthesia was induced with 3% isoflurane in 50% oxygen / air and then maintained at 2% isoflurane using a standard rat nose mask (Provet, Lyssach, Switzerland). Analgesia was administered with 0.05 mg/kg of buprenorphine (Temgesic, Reckitt Benckiser, Switzerland AG), subcutaneously injected 30 min before induction of the surgical procedure. Rats were placed on a heating pad and the rectal temperature kept at 37 ± 1°C which was monitored by a Mouse-Ox plus System (Starr Life Sciences, Oakmont, PA, USA) throughout the anesthesia. Following groin incision in the left hind limb, femoral artery, vein, and nerve were isolated from the surrounding tissue. Then the femoral artery was clamped with two microvascular clamps (B1-V, S&T, Neuhausen, Switzerland) proximal to the deep femoral artery branch. Meanwhile, a tourniquet, connected to a fixed weight of 450 g, was placed underneath the femoral vessels around the thigh as high as possible to block collateral blood supply, while keeping the femoral vein open. The animals were subjected to 4h ischemia and 24h reperfusion (by releasing the clamps and the tourniquet). Upon reperfusion, the rats were allowed to wake up and another dose of buprenorphine was given to provide adequate analgesia. At the end of the 24h reperfusion period, the rats were anesthetized again and sacrificed by exsanguination during organ removal in deep anesthesia. Blood and tibia bone samples were taken for further analysis. Two groups were included in this study, the I/R injury group (n=6) and normal, healthy rats as control group (n=4).

2.3 Sample preparation

All reperfused and contralateral tibia bone samples from rat hind limbs were dissected, freed from the surrounding soft tissue, rinsed with phosphate-buffered saline (PBS), blotted dry, and immediately

fixed in 4% paraformaldehyde for 24h at room temperature, then washed under tap water overnight and finally transferred into 70% ethanol for further use. When all bones were available, decalcification was performed in 15% EDTA/0.5% paraformaldehyde and checked by X-ray. After complete decalcification, bone tissue was rinsed under tap water overnight then dehydrated. Samples including transverse sections from the middle of the shaft were then embedded in paraffin and 4µm sections were cut. Blood was drawn at baseline, end of ischemia and end of reperfusion to collect plasma and serum.

2.4 Immunohistochemistry

To assess the involvement of the complement, coagulation, and fibrinolytic cascades after 24h reperfusion, immunohistochemistry (IHC) staining was performed. Briefly, sections were deparaffinized in xylene, rehydrated through graded alcohols, and washed in distilled H_2O. Then antigen retrieval was performed in 15 µg/ml of Proteinase K (Ref. 03115 879001, Roche Diagnostics, Indianapolis, IN, USA) in water bath at 37°C for 5 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 min. Primary antibodies were then applied to the sections in a humid chamber at 4°C overnight. The following primary antibodies were used: rabbit anti-human C3b/c (A0062, Dako, Glostrup, Denmark), goat anti-rat tissue factor (TF) (sc-23596, Santa Cruz Biotechnology, Inc., Texas, USA), rabbit anti-human fibrinogen (A0080, Dako), rabbit anti-rat tissue plasminogen activator (tPA) (ab14198, Abcam, Cambridge, UK), goat anti-rat PAI-1 (SC-6644, Santa Cruz), and rabbit anti-human E-selectin (bs-1273, Bioss Inc., Woburn, Massachusetts, USA). Then affinity-purified, biotin-conjugated rabbit anti-goat (E0466, Dako) and goat anti-rabbit (E0432, Dako) secondary antibodies were applied to all sections for 90 minutes at room temperature. Afterwards, sections were incubated with avidin-biotin-peroxidase complex (PK6100, Vectastain, Burlingame CA, USA) for 45 min. Bound peroxidase was detected by 3,3'-diaminobenzidine (DAB) substrate for 1-3 min in the dark. Finally, sections were counterstained with hematoxylin (GHS232, Sigma-Aldrich, St. Louis, MO, USA) and mounted in Aquatex (108562, Darmstadt, Germany). For visualization and acquisition, images were taken with a bright field microscope (Leica DMI 4000B, Leica Microsystems Schweiz, Heerbrugg, Switzerland) equipped with an HCX PL APO 63x 1.40-0.6 lens and a color camera (DFC425). For each marker, 3-5 representative images were taken per slide and the mean was calculated and used for comparison.

The intensity of chromogen stain in IHC was quantified within the vessels by Fiji software (ImageJ) (http://fiji.sc/Fiji). Vessels were identified visually and delineated by hand. The intensity of the staining was then calculated by Fiji software using the color deconvolution feature for H DAB staining. The stronger the staining visually, the weaker the intensity. This leads to an inverse correlation between the staining signal and the measured intensity. We followed the protocol

published by Nguyen et al. using reciprocal intensity (subtract the measured intensity from the maximum intensity value of 255), which is proportional to the actual staining intensity [20].

2.5 Hemolytic complement (CH50) assay

Activity of the classical pathway in serum was analyzed using a standard hemolytic complement (CH50) assay. Basically, sheep erythrocytes were washed in veronal buffered saline (VBS^{++}) at 1:30 until the supernatant was clear. The number of cells was determined at 10^9/ml, which were then incubated with rabbit anti-sheep erythrocyte antibody (S1389, Sigma-Aldrich) for 20 min at 37°C. After centrifugation, antibody-sensitized erythrocytes were resuspended in Alsever's solution (A3551, Sigma-Aldrich) at 4°C overnight. On the second day, the cells were washed again and adjusted to 10^8/ml. Serum samples were then added to a transparent 96 well microplate (Nunc, Roskilde, Denmark) and incubated with the above adjusted cells at 37°C for 60 min, then stopped with PBS. For the controls, erythrocytes were incubated in a veronal buffer ($DGVB^{++}$) stopped with water (T100, 100% lysis) or PBS (T0, 0% lysis, background). At the end, optical density was measured at 412nm (Infinite M1000 spectrophotometer, Tecan, Männedorf, Switzerland). A lysis percentage was calculated as: $\frac{OD\ sample - OD\ T0}{OD\ T100 - OD\ T0} * 100$.

2.6 Measurement of plasma HMGB-1 by ELISA

To determine plasma HMGB-1, a homemade sandwich-ELISA was performed. In brief, a capture antibody, rabbit anti-rat HMGB-1 (H9539; Sigma) and HRP-conjugated detection antibody, HRP conjugated rabbit anti-rat HMGB-1 (ab128129; Abcam) were added, followed by SureBlue™ TMB Microwell Peroxidase Substrate (52-00-01, KPL, Gaithersburg, MD, USA) for color development and stopped by 1 mol/L H_2SO_4. At the end, optical density was measured at 450/540 nm using a FLUOstar Omega microplate reader (BMG LABTECH GmbH, Ortenberg, Germany).

2.7 Statistical analysis

All data are presented as mean ± standard deviation (SD). Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Fisher's LSD post hoc test with GraphPad Prism 6 software (GraphPad, San Diego, CA, USA). P values <0.05 were considered statistically significant.

3. Results

3.1 Deposition of C3b/c

To assess activation of the complement cascade in reperfusion injury of bone, deposition of the central component fragment C3b/c was analyzed. In cortical bone, C3b/c deposition was significantly increased in both reperfused and contralateral limbs compared with normal, healthy controls ($p=0.006$ and $p=0.023$, respectively). In bone marrow, a significant increase of C3b/c was shown in the reperfused, but not in the contralateral marrow ($p=0.005$ reperfused vs. contralateral and $p=0.008$ reperfused vs. normal, Fig. 1).

3.2 Expression of tissue factor and deposition of fibrin

To assess the role of the coagulation cascade in bone I/R injury, IHC staining was performed for expression of TF and deposition of fibrin. TF expression was significantly raised in the contralateral bone marrow compared with normal controls without any forms of intervention ($p=0.046$), but the increase in staining intensity was not statistically significant in reperfused bones. In the cortical bone, no significant differences were detected for expression of TF between groups (Fig. 2).

Deposition of fibrin was significantly increased in both reperfused and contralateral cortical bones compared to normal ones without manipulation (both $p<0.0001$), as well as in the respective bone marrow sections ($p=0.002$ and $p=0.005$, respectively, Fig. 3).

3.3 Expression of tPA and PAI-1

To assess the involvement of the fibrinolytic system in bone I/R injury, expression of tPA and PAI-1 was evaluated by IHC. Expression of tPA was significantly down regulated in both reperfused and contralateral cortical bones as compared to healthy controls without intervention ($p=0.007$ and $p=0.015$, respectively). Also in the bone marrow, a significant downregulation of tPA expression was found both in the reperfused and contralateral limbs (both $p<0.0001$, respectively, Fig. 4).

PAI-1 expression was only visible in the vessels of cortical bone, not in bone marrow. Expression of PAI-1 was significantly up regulated in the reperfused cortical bones rather than the contralateral and normal, healthy controls ($p=0.016$ and $p=0.006$, respectively, Fig. 5).

3.4 Expression of E-selectin

To evaluate expression of the adhesion molecule E-selectin on ECs, IHC staining was performed. In a similar manner to PAI-1, E-selectin expression was only found in vessels of cortical bones, but not in bone marrow. A trend, however not significant, was observed to increased E-selectin expression in

the reperfused cortical bones as compared to cortical bones from unmanipulated, healthy animals (p=0.073). (Fig. 6).

3.5 Serum complement activity

In animals undergoing 4h ischemia and 24h reperfusion, serum complement activity was reduced over time. At baseline, the percentage of erythrocyte lysis was 96 ±3.6%. After 2h ischemia, values dropped to 89.3 ± 2.8%, which was significantly lower than baseline (p=0.0183). At the end of reperfusion, 85.9 ± 6.0% erythrocyte lysis was measured (p=0.0012 vs. baseline, Fig. 7).

3.6 HMGB-1 in plasma

As an inflammatory marker, concentrations of HMGB-1 in plasma were measured by ELISA. A significantly higher level of HMGB-1 in plasma was found in animals subjected to bone I/R injury (24.1 ± 6.0 ng/ml) compared with normal, healthy animals (4.7 ± 1.6 ng/ml, p<0.001, Fig. 8).

Discussion

Our results show that activation of the plasma cascades, including complement, coagulation and fibrinolysis, may play a role in I/R injury of bone. This was supported by increased deposition of C3b/c and fibrin and increased expression of PAI-1, as well as reduced expression of tPA in bones from ischemic and reperfused rat hind limbs.

In the present study with 4h ischemia and 24h reperfusion, the coagulation cascade was activated as reflected by a significantly increased deposition of fibrin – a final product of coagulation activation – which was present in vessels of both reperfused and contralateral bones, including cortical bone and bone marrow. Deposition of fibrin in the contralateral limb was also found in an earlier study by our group, in which I/R injury of the gastrocnemius muscle was investigated in the same rat model [5]. The presence of fibrin deposits in the vasculature of contralateral bones suggests activation of the respective ECs due to the systemic presence of pro-inflammatory mediators. In fact, remote organ damage caused by limb I/R injury has been shown by us [5] and others [21-23] in experimental models, and clinical cases [24, 25] have been reported too.

Transition of bone vascular ECs to a pro-coagulant and anti-fibrinolytic phenotype was further evidenced by downregulation of tPA, which activates plasmin to break down emerging fibrin clots. Downregulation of tPA expression, measured as impaired release of tPA into the circulation, was also reported in an extremity I/R injury study in humans [15]. This upregulation of PAI-1 plays a role in I/R injury as reported previously that PAI-1 deficient mice showed reduced fibrin deposition and a lesser degree of inflammation in a lung I/R injury model [26]. Also data from human studies in I/R

injury of the liver as well as trauma support the importance of PAI-1 expression in the pathophysiological development of reperfusion injury [27, 28].

EC activation is often associated with expression of adhesion molecules like E-selectin. In our study E-selectin expression was not significantly upregulated after I/R injury. This finding is in line with earlier data from a cerebral I/R injury model with 3h ischemia and 24h reperfusion, in which tissue injury occurred in a P- and E-selectin-independent manner, possibly due to the presence of a low shear stress in the affected vasculature [29, 30]. In contrast, other studies reported upregulation of E-selectin in I/R injury models [31, 32].

As an initiator of the coagulation pathway, TF is normally located in the subendothelium but can be induced on the surface of ECs due to vascular injury. There were no significant differences for TF expression except a slight increase in the contralateral bone marrow. This could mean two things: Either fibrin formation may occur independently of TF expression, mainly via contact activation, or we miss a peak of TF expression that might occur earlier than 24h after reperfusion. Previous data showed an increased expression of TF on ECs at 12h of reperfusion in an I/R injury of kidney, but TF staining was not shown at 24h of reperfusion [33].

In addition to coagulation, the complement system was also activated in our rat hind limb I/R injury model. This was shown by a significant increase of C3b/c deposits in cortical bone and bone marrow of reperfused limbs, as well as cortical bone of the contralateral limbs. In circulation, we found a significant reduction of functional complement as reflected by CH50 values. However, the damage to skeletal muscle tissue occurring in our model (supplemental Fig. 1 and 2) also contributes to this complement consumption.

In some models of I/R injury, complement activation was shown to be linked to recognition of ischemia-induced neoepitopes by natural antibodies [34, 35]. However, in contrast to myocardial I/R injury [36], skeletal muscle reperfusion injury was not attenuated in the rat hind limb model by complement inhibition alone [5]. It remains to be determined whether in case of bone I/R injury EC activation relies on complement activation or not. In fact, ECs can be activated by HMGB-1, independent of complement activation, as shown recently in a xenotransplantation model [37]. We found highly increased levels of HMGB-1 in circulation after 24h of reperfusion, which are probably caused by extensive injury to the skeletal muscle tissue in our model (supplemental Fig. 1-2). We might therefore speculate that EC activation in the bone vasculature might be mediated – at least in part – by HMGB-1.

In conclusion, our results show that ischemia and reperfusion of bone leads to activation of the coagulation and complement cascades and a downregulation of the fibrinolytic system. In the acute phase, a vascular inflammation induced by activation of the plasma cascade systems therefore occurs in the bone. This is similar to I/R injury of other vascularized organs and tissues.

Funding

Swiss National Science Foundation (SNSF), grants no. 32003B_135272 and 320030_156193 to RR and the SNSF Fellowship: P2BEP3_155459 to AKB. The China Scholarship Council (CSC) awarded grant support to SZ in Switzerland.

Disclosure statement

The authors declare no conflicts of interest.

Acknowledgements

We thank Prof. Anne Angelillo Scherrer, University of Bern, for helpful discussion and critical comments. This study was support by the Microscopy Imaging Center (MIC), University of Bern.

References

1. A. Sckell, T. J. Demhartner, C. R. Fraitzl, and M. Leunig, "Reperfusion injury in free bone grafts after tourniquet-induced ischemia," *Clinical Orthopaedics and Related Research,* no. 409, pp. 306-16, 2003.
2. Y. Tamura, G. Inoue, T. Miura, N. Ishiguro, and T. Shimizu, "Reperfusion injury in bone: effects of CV-3611, a free radical scavenger, on ischemic revascularized bone grafts in rats," *Journal of Reconstructive Microsurgery,* vol. 8, no. 6, pp. 471-9, 1992.
3. A. S. Hsieh, H. Winet, J. Y. Bao, H. Glas, and H. Plenk, "Evidence for reperfusion injury in cortical bone as a function of crush injury ischemia duration: a rabbit bone chamber study," *Bone,* vol. 28, no. 1, pp. 94-103, 2001.
4. M. Sato, N. Sugano, K. Ohzono, S. Nomura, Y. Kitamura, Y. Tsukamoto, and S. Ogawa, "Apoptosis and expression of stress protein (ORP150, HO1) during development of ischaemic osteonecrosis in the rat," *Journal of Bone and Joint Surgery (British Volume),* vol. 83, no. 5, pp. 751-9, 2001.
5. C. Duehrkop, Y. Banz, R. Spirig, S. Miescher, M. W. Nolte, M. Spycher, R. A. Smith, S. H. Sacks, and R. Rieben, "C1 esterase inhibitor reduces lower extremity ischemia/reperfusion injury and associated lung damage," *PLoS One,* vol. 8, no. 8, pp. e72059, 2013.
6. B. H. Heijnen, I. H. Straatsburg, N. D. Padilla, G. J. Van Mierlo, C. E. Hack, and T. M. Van Gulik, "Inhibition of classical complement activation attenuates liver ischaemia and reperfusion injury in a rat model," *Clinical and Experimental Immunology,* vol. 143, no. 1, pp. 15-23, 2006.
7. C. Costa, L. Zhao, Y. Shen, X. Su, L. Hao, S. P. Colgan, G. L. Stahl, T. Zhou, and Y. Wang, "Role of complement component C5 in cerebral ischemia/reperfusion injury," *Brain Research,* vol. 1100, no. 1, pp. 142-51, 2006.
8. S. Oozawa, S. Mori, T. Kanke, H. Takahashi, K. Liu, Y. Tomono, M. Asanuma, I. Miyazaki, M. Nishibori, and S. Sano, "Effects of HMGB1 on ischemia-reperfusion injury in the rat heart," *Circulation Journal,* vol. 72, no. 7, pp. 1178-84, 2008.
9. M. Andrassy, H. C. Volz, J. C. Igwe, B. Funke, S. N. Eichberger, Z. Kaya, S. Buss, F. Autschbach, S. T. Pleger, I. K. Lukic, F. Bea, S. E. Hardt, P. M. Humpert, M. E. Bianchi, H. Mairbaurl, P. P. Nawroth, A. Remppis, H. A. Katus, and A. Bierhaus, "High-mobility group box-1 in ischemia-reperfusion injury of the heart," *Circulation,* vol. 117, no. 25, pp. 3216-26, 2008.
10. Y. Lin, L. Chen, W. Li, and J. Fang, "Role of high-mobility group box-1 in myocardial ischemia/reperfusion injury and the effect of ethyl pyruvate," *Experimental and Therapeutic Medicine,* vol. 9, no. 4, pp. 1537-1541, 2015.
11. M. R. Weiser, J. P. Williams, F. D. Moore, Jr., L. Kobzik, M. Ma, H. B. Hechtman, and M. C. Carroll, "Reperfusion injury of ischemic skeletal muscle is mediated by natural antibody and complement," *Journal of Experimental Medicine,* vol. 183, no. 5, pp. 2343-8, 1996.
12. C. Kyriakides, W. Austen, Jr., Y. Wang, J. Favuzza, L. Kobzik, F. D. Moore, Jr., and H. B. Hechtman, "Skeletal muscle reperfusion injury is mediated by neutrophils and the complement membrane attack complex," *American Journal of Physiology,* vol. 277, no. 6 Pt 1, pp. C1263-8, 1999.
13. C. Duehrkop, J. Denoyelle, S. Shaw, and R. Rieben, "Use of dextran sulfate in tourniquet-induced skeletal muscle reperfusion injury," *Journal of Surgical Research,* vol. 187, no. 1, pp. 150-61, 2014.
14. M. F. Conrad, D. H. Stone, H. Albadawi, H. T. Hua, F. Entabi, M. C. Stoner, and M. T. Watkins, "Local inflammatory and thrombotic responses differ in a murine model of partial and complete hindlimb ischemia/reperfusion," *Surgery,* vol. 138, no. 2, pp. 375-81, 2005.
15. C. M. Pedersen, G. Barnes, M. R. Schmidt, H. E. Botker, R. K. Kharbanda, D. E. Newby, and N. L. Cruden, "Ischaemia-reperfusion injury impairs tissue plasminogen activator release in man," *European Heart Journal,* vol. 33, no. 15, pp. 1920-7, 2012.
16. Y. Zhao, A. K. Sharma, D. J. LaPar, I. L. Kron, G. Ailawadi, Y. Liu, D. R. Jones, V. E. Laubach, and C. L. Lau, "Depletion of tissue plasminogen activator attenuates lung ischemia-reperfusion injury via inhibition of neutrophil extravasation," *American Journal of Physiology: Lung Cellular and Molecular Physiology,* vol. 300, no. 5, pp. L718-29, 2011.
17. J. J. Roelofs, K. M. Rouschop, J. C. Leemans, N. Claessen, A. M. de Boer, W. M. Frederiks, H. R. Lijnen, J. J. Weening, and S. Florquin, "Tissue-type plasminogen activator modulates inflammatory responses and renal function in ischemia reperfusion injury," *Journal of the American Society of Nephrology,* vol. 17, no. 1, pp. 131-40, 2006.
18. D. E. Vaughan, "PAI-1 and atherothrombosis," *Journal of Thrombosis and Haemostasis,* vol. 3, no. 8, pp. 1879-83, 2005.
19. J. I. Kim, K. J. Jung, H. S. Jang, and K. M. Park, "Gender-specific role of HDAC11 in kidney ischemia- and reperfusion-induced PAI-1 expression and injury," *American Journal of Physiology: Renal Physiology,* vol. 305, no. 1, pp. F61-70, 2013.
20. D. H. Nguyen, T. Zhou, J. Shu, and J. Mao, "Quantifying chromogen intensity in immunohistochemistry via reciprocal intensity " *Protocol exchange -Nature,* 2013.

21. M. M. Yassin, D. W. Harkin, A. A. Barros D'Sa, M. I. Halliday, and B. J. Rowlands, "Lower limb ischemia-reperfusion injury triggers a systemic inflammatory response and multiple organ dysfunction," *World Journal of Surgery,* vol. 26, no. 1, pp. 115-21, 2002.
22. D. W. Harkin, A. Barros D'sa A, K. McCallion, M. Hoper, M. I. Halliday, and F. C. Campbell, "Circulating neutrophil priming and systemic inflammation in limb ischaemia-reperfusion injury," *International Angiology,* vol. 20, no. 1, pp. 78-89, 2001.
23. M. D. Bianco-Batlles, A. Sosunov, R. A. Polin, and V. S. Ten, "Systemic inflammation following hind-limb ischemia-reperfusion affects brain in neonatal mice," *Developmental Neuroscience,* vol. 30, no. 6, pp. 367-73, 2008.
24. L. N. Lin, L. R. Wang, W. T. Wang, L. L. Jin, X. Y. Zhao, L. P. Zheng, L. D. Jin, L. M. Jiang, and X. Q. Xiong, "Ischemic preconditioning attenuates pulmonary dysfunction after unilateral thigh tourniquet-induced ischemia-reperfusion," *Anesthesia and Analgesia,* vol. 111, no. 2, pp. 539-43, 2010.
25. W. K. Edrees, L. L. Lau, I. S. Young, M. G. Smye, K. R. Gardiner, B. Lee, R. J. Hannon, and C. V. Soong, "The effect of lower limb ischaemia-reperfusion on intestinal permeability and the systemic inflammatory response," *European Journal of Vascular and Endovascular Surgery,* vol. 25, no. 4, pp. 330-5, 2003.
26. C. L. Lau, Y. Zhao, J. Kim, I. L. Kron, A. Sharma, Z. Yang, V. E. Laubach, J. Linden, G. Ailawadi, and D. J. Pinsky, "Enhanced fibrinolysis protects against lung ischemia-reperfusion injury," *Journal of Thoracic and Cardiovascular Surgery,* vol. 137, no. 5, pp. 1241-8, 2009.
27. K. Inoue, Y. Sugawara, K. Kubota, T. Takayama, and M. Makuuchi, "Induction of type 1 plasminogen activator inhibitor in human liver ischemia and reperfusion," *Journal of Hepatology,* vol. 33, no. 3, pp. 407-14, 2000.
28. C. Kluft, J. H. Verheijen, A. F. Jie, D. C. Rijken, F. E. Preston, H. M. Sue-Ling, J. Jespersen, and A. O. Aasen, "The postoperative fibrinolytic shutdown: a rapidly reverting acute phase pattern for the fast-acting inhibitor of tissue-type plasminogen activator after trauma," *Scandinavian Journal of Clinical and Laboratory Investigation,* vol. 45, no. 7, pp. 605-10, 1985.
29. S. G. Soriano, Y. F. Wang, D. D. Wagner, and P. S. Frenette, "P- and E-selectin-deficient mice are susceptible to cerebral ischemia-reperfusion injury," *Brain Research,* vol. 835, no. 2, pp. 360-4, 1999.
30. L. Birk-Sorensen, C. L. Kerrigan, and G. S. Jensen, "E-selectin and L-selectin blockade in pure skin flaps exposed to ischaemia and reperfusion injury," *Scandinavian Journal of Plastic and Reconstructive Surgery and Hand Surgery,* vol. 32, no. 4, pp. 365-71, 1998.
31. R. L. Zhang, M. Chopp, Z. G. Zhang, M. L. Phillips, C. L. Rosenbloom, R. Cruz, and A. Manning, "E-selectin in focal cerebral ischemia and reperfusion in the rat," *Journal of Cerebral Blood Flow and Metabolism,* vol. 16, no. 6, pp. 1126-36, 1996.
32. J. Russell, C. J. Epstein, M. B. Grisham, J. S. Alexander, K. Y. Yeh, and D. N. Granger, "Regulation of E-selectin expression in postischemic intestinal microvasculature," *American Journal of Physiology: Gastrointestinal and Liver Physiology,* vol. 278, no. 6, pp. G878-85, 2000.
33. M. Matsuyama, R. Yoshimura, K. Akioka, M. Okamoto, H. Ushigome, Y. Kadotani, T. Nakatani, and N. Yoshimura, "Tissue factor antisense oligonucleotides prevent renal ischemia-reperfusion injury," *Transplantation,* vol. 76, no. 5, pp. 786-91, 2003.
34. M. Zhang, E. M. Alicot, I. Chiu, J. Li, N. Verna, T. Vorup-Jensen, B. Kessler, M. Shimaoka, R. Chan, D. Friend, U. Mahmood, R. Weissleder, F. D. Moore, and M. C. Carroll, "Identification of the target self-antigens in reperfusion injury," *Journal of Experimental Medicine,* vol. 203, no. 1, pp. 141-52, 2006.
35. L. Kulik, S. D. Fleming, C. Moratz, J. W. Reuter, A. Novikov, K. Chen, K. A. Andrews, A. Markaryan, R. J. Quigg, G. J. Silverman, G. C. Tsokos, and V. M. Holers, "Pathogenic natural antibodies recognizing annexin IV are required to develop intestinal ischemia-reperfusion injury," *Journal of Immunology,* vol. 182, no. 9, pp. 5363-73, 2009.
36. Y. Banz, O. M. Hess, S. C. Robson, E. Csizmadia, D. Mettler, P. Meier, A. Haeberli, S. Shaw, R. A. Smith, and R. Rieben, "Attenuation of myocardial reperfusion injury in pigs by Mirococept, a membrane-targeted complement inhibitor derived from human CR1," *Cardiovascular Research,* vol. 76, no. 3, pp. 482-93, 2007.
37. W. E. Bongoni A.K., Ayares D, Rieben R, Cowan P.J. , "Transgenic expression of human thrombomodulin inhibits HMGB1-induced porcine aortic endothelial cell activation," *Xenotransplantation,* 2016.

Figures and Legends

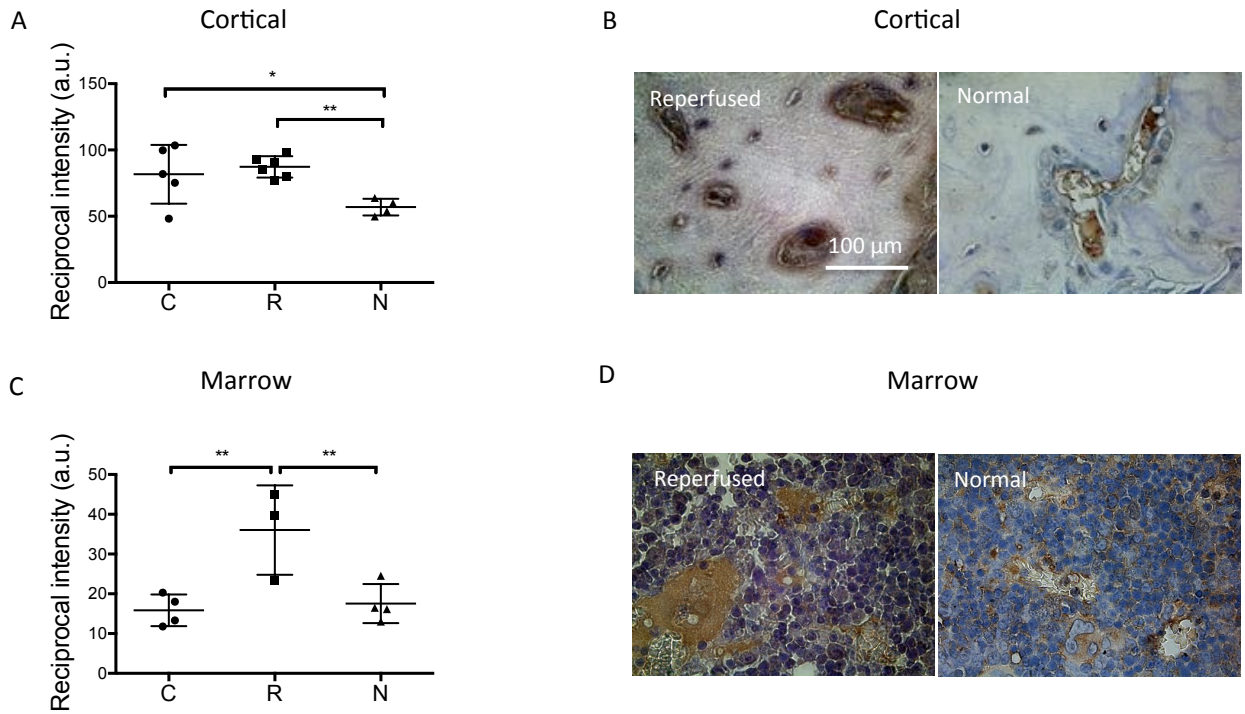


Fig. 1. Deposition of C3b/c in bone tissue. A, quantitative comparison of C3b/c deposition in cortical bone. B, representative images for C3b/c staining in reperfused and normal cortical bones from healthy, unmanipulated animals. C, quantitative comparison of C3b/c deposition in marrow. D, representative images for C3b/c staining in reperfused and normal marrow from healthy, unmanipulated animals. Data of individual experiments are represented by dots with indication of mean ± SD by lines. Scale bar represents 100 µm. C - contralateral; R - reperfused; N - normal. *P<0.05; **P<0.01.

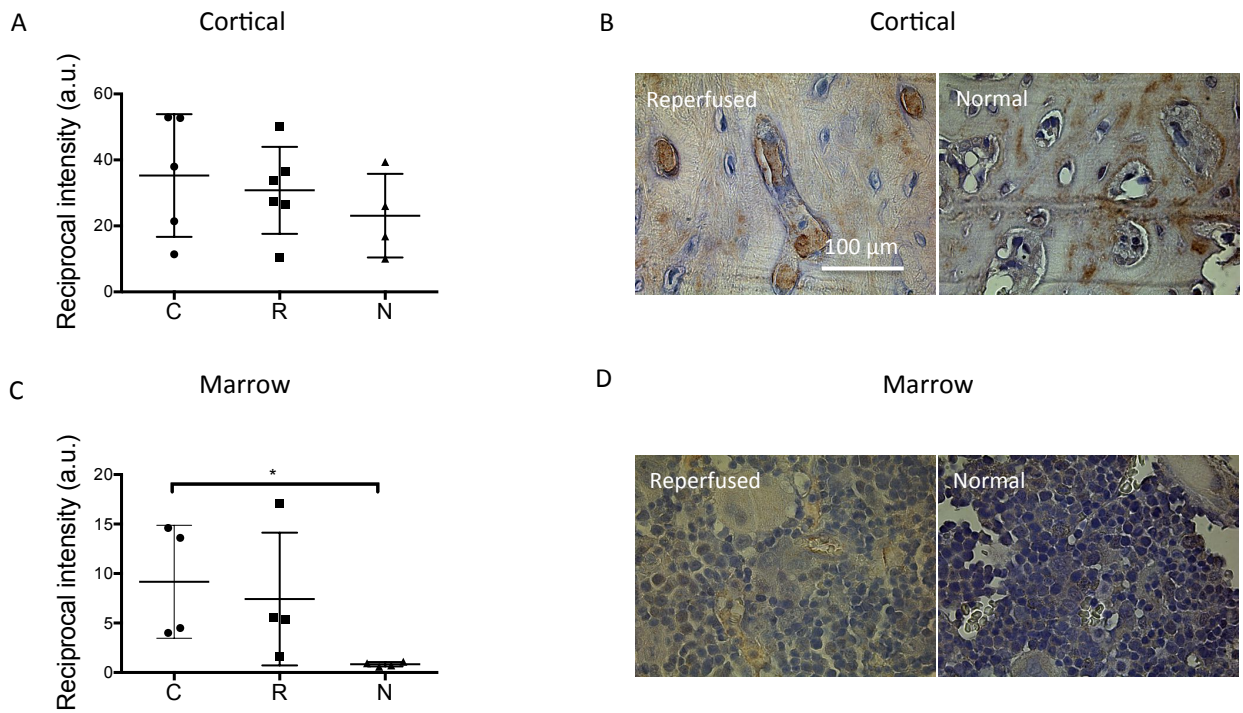


Fig. 2. Expression of tissue factor in bone tissue. A, quantitative comparison of TF expression in cortical bone. B, representative images for TF staining in reperfused and normal cortical bones from healthy, unmanipulated animals. C, quantitative comparison of TF expression in marrow. D, representative images for TF staining in reperfused and normal marrow from healthy, unmanipulated animals. Data of individual experiments are represented by dots with indication of mean ± SD by lines. Scale bar represents 100 µm. C - contralateral; R - reperfused; N - normal. *P<0.05.

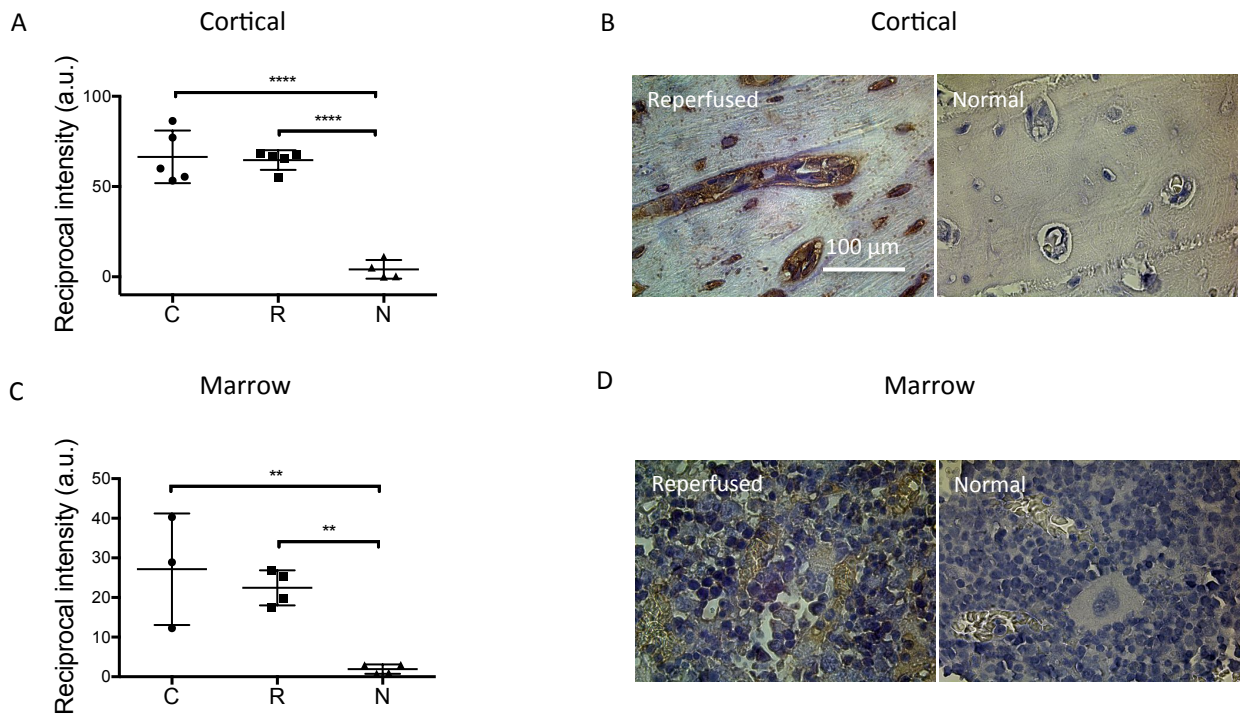


Fig. 3. Deposition of fibrin in bone tissue. A, quantitative comparison of fibrin deposition in cortical bone. B, representative images for fibrin staining in reperfused and normal cortical bones from healthy, unmanipulated animals. C, quantitative comparison of fibrin deposition in marrow. D, representative images for fibrin staining in reperfused and normal marrow from healthy, unmanipulated animals. Data of individual experiments are shown by dots with indication of mean ± SD by lines. Scale bar represents 100 µm. C - contralateral; R - reperfused; N - normal. **P<0.01; ****p<0.0001.

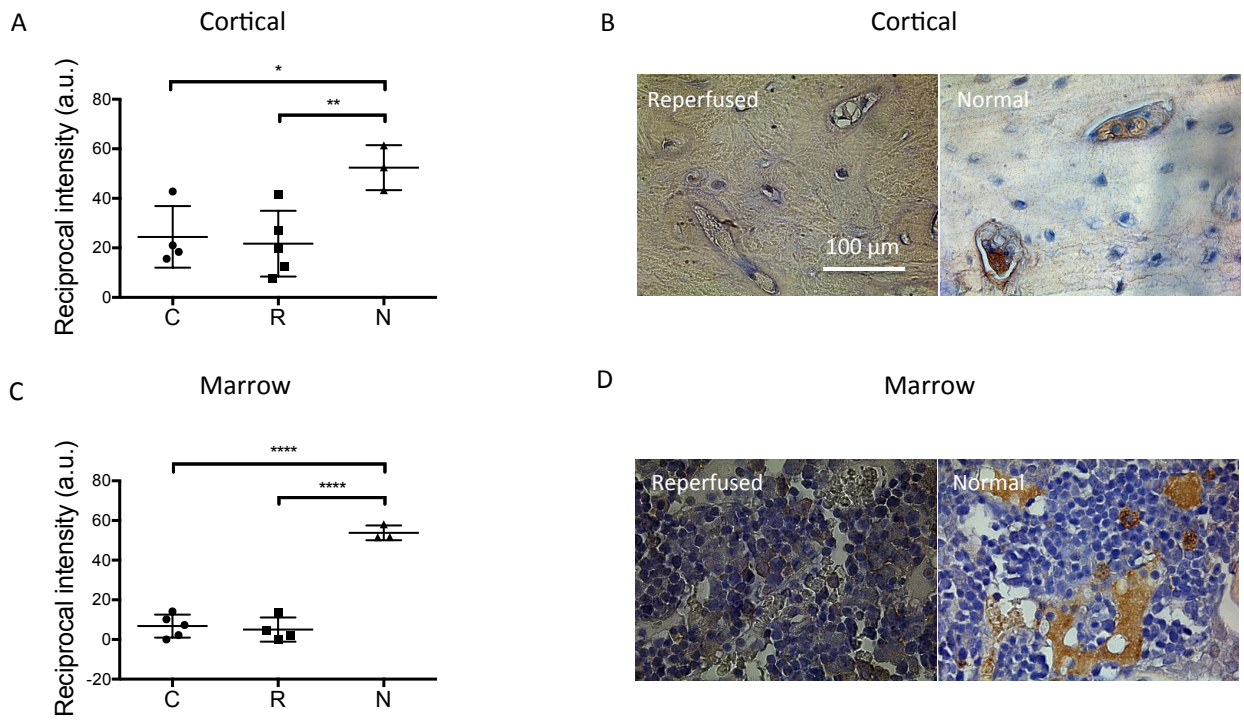


Fig. 4. Expression of tPA in bone tissue. A, quantitative comparison of tPA expression in cortical bone. B, representative images for tPA staining in reperfused and normal cortical bones from healthy, unmanipulated animals. C, quantitative comparison of tPA expression in marrow. D, representative images for tPA staining in reperfused and normal marrow from healthy, unmanipulated animals. Data of individual experiments are represented by dots with indication of mean ± SD by lines. Scale bar represents 100 µm. C - contralateral; R - reperfused; N - normal. *P<0.05; **p<0.01; ***p<0.001.

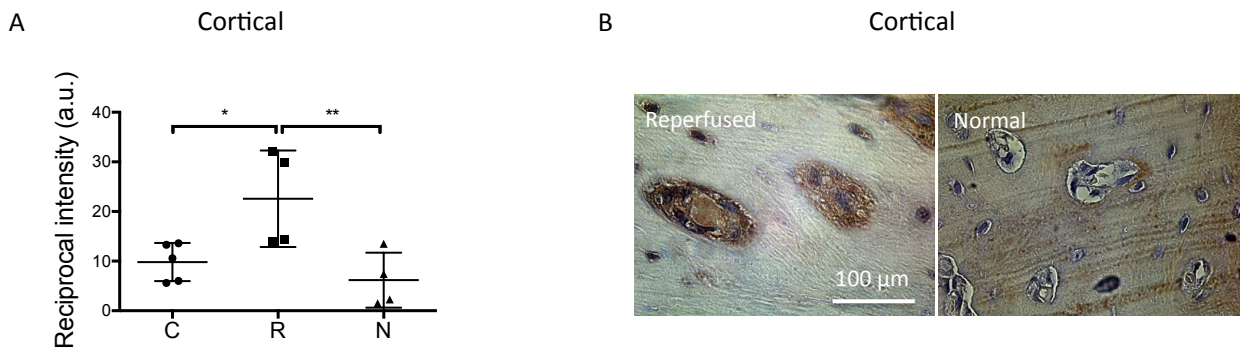


Fig. 5. Expression of PAI-1 in bone tissue. A, quantitative comparison of PAI-1 expression in cortical bone. B, representative images for PAI-1 staining in reperfused and normal cortical bones from healthy, unmanipulated animals. Data of individual experiments are represented by dots with indication of mean ± SD by lines. Scale bar represents 100 µm. C - contralateral; R - reperfused; N - normal. *P<0.05; **p<0.01.

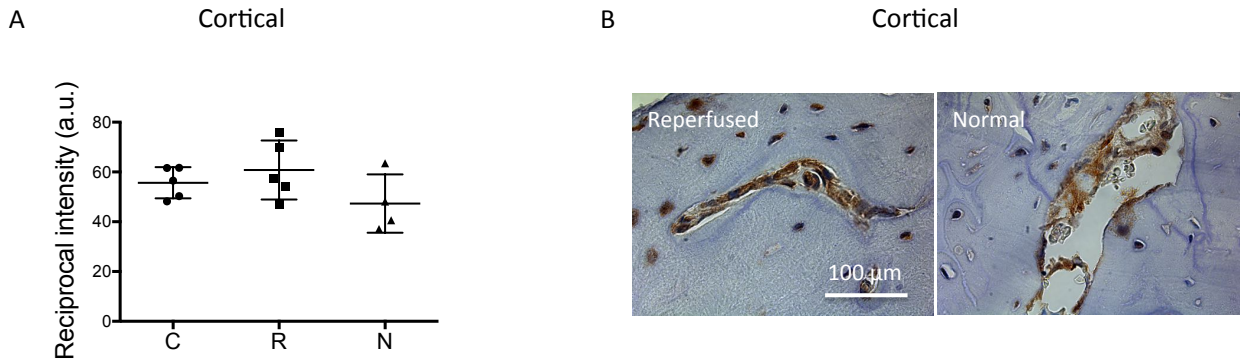


Fig. 6. Expression of E-selectin in bone tissue. A, quantitative comparison of E-selectin expression in cortical bone. B, representative images for E-selectin staining in reperfused and normal cortical bones from healthy, unmanipulated animals. Data of individual experiments are represented by dots with indication of mean ± SD by lines. Scale bar represents 100 µm. C - contralateral; R – reperfused; N - normal.

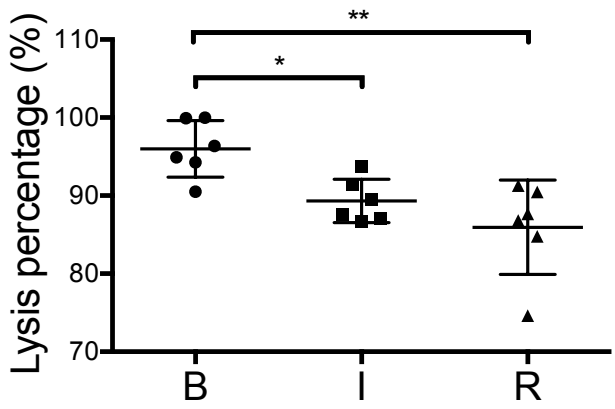


Fig. 7. Hemolytic complement activity by CH50 assay in serum at baseline, end of ischemia, and end of reperfusion. Data of individual experiments are represented by dots with indication of mean ± SD by lines. B - baseline; I - end of ischemia; R - end of reperfusion. *P<0.05; **p<0.01.

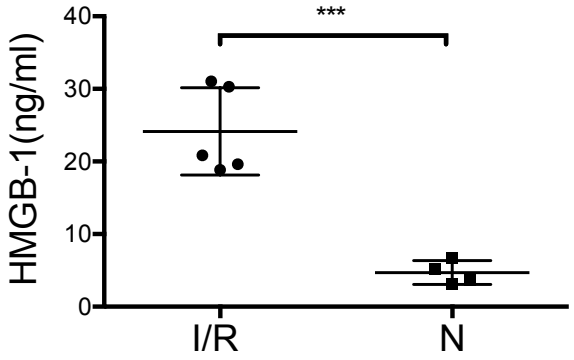
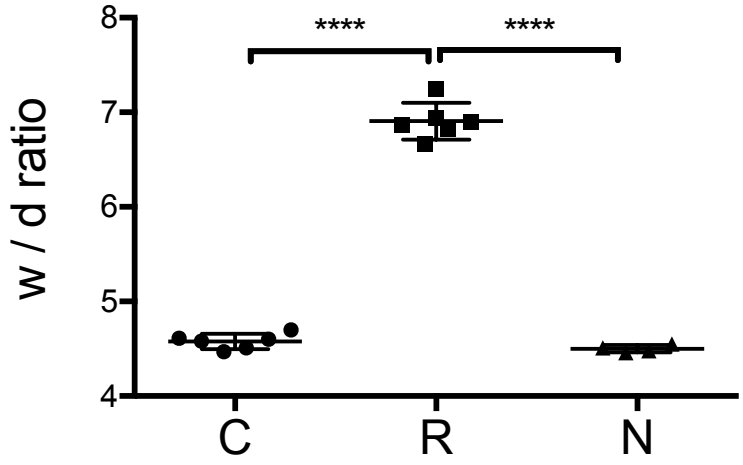
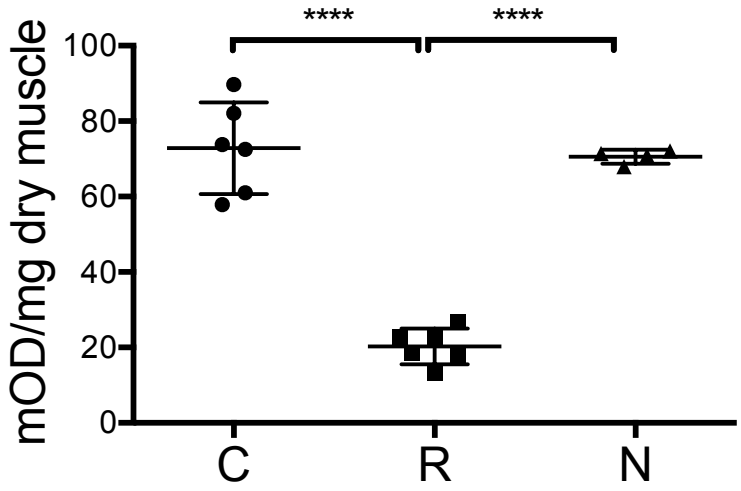


Fig. 8. Plasma levels of HMGB-1 measured by ELISA. Data of individual experiments are represented by dots with indication of mean ± SD by lines. I/R – ischemia/reperfusion injury group; N – normal control group. ***p<0.001.

Supplemental Figures and Legends



Supplemental Fig. 1. Edema formation in gastrocnemius muscle tissue after 4h ischemia and 24h reperfusion in rat hind limbs. The ratio of wet weight to dry weight ratio (w/d ratio) was calculated as an indicator for edema formation within the muscle. Edema formation was significantly increased in muscle tissue of reperfused limbs compared with the contralateral and normal controls (both $p<0.0001$). One-way analysis of variance (ANOVA) followed by Fisher's LSD post hoc test. Data of individual experiments are represented by dots with indication of mean ± SD by lines. Scale bar represents 100 µm. C – contralateral limbs; R – reperfused limbs; N – normal controls. ****$p<0.0001$.



Supplemental Fig. 2. Viability in gastrocnemius muscle tissue after 4h ischemia and 24h reperfusion in rat hind limbs. Muscle viability was assessed by MTT assay. OD per mg dry muscle weight was used to quantitate muscle viability. Muscle viability was significantly reduced in muscle tissue of reperfused limbs compared with contralateral and normal controls (both $p<0.0001$). One-way analysis of variance (ANOVA) followed by Fisher's LSD post hoc test. Data of individual experiments are represented by dots with indication of mean ± SD by lines. Scale bar represents 100 μm. C – contralateral limbs; R – reperfused limbs; N – normal controls. ****$p<0.0001$.

Paper IV

3D culture and analysis of endothelial cells under physiological flow conditions in a xenotransplantation setting

Shengye Zhang[1,2], Colette Andrea Bichsel[1,3], Rahel Klossner[2], Oliver Steck[2], Alain Despont[2], Oliver Thierry Guenat[3], Robert Rieben[2]

[1]Graduate School for Cellular and Biomedical Sciences, University of Bern, Switzerland; [2]Department of Clinical Research, University of Bern, Switzerland; [3]ARTORG Center, University of Bern, Switzerland

Status: In submission for publication to ALTEX (Alternative to Animal Experiments)

Aim: The aim of this study was to develop an in vitro system to grow and investigate EC under physiological flow conditions, simulating shear stress as occurs in medium sized arteries.

Conclusion: We have developed an in vitro system for 3-dimensional growth of EC in microfluidic channels with circular cross sections under physiological flow conditions, mimicking small to medium sized arteries. The system is able to reproduce key findings of EC activation and complement activation in a xenotransplantation model. Compared to conventional 96-well plate assays the system provides a >350 times larger ratio of fluid volume exposure to EC surface, equaling in vivo conditions.

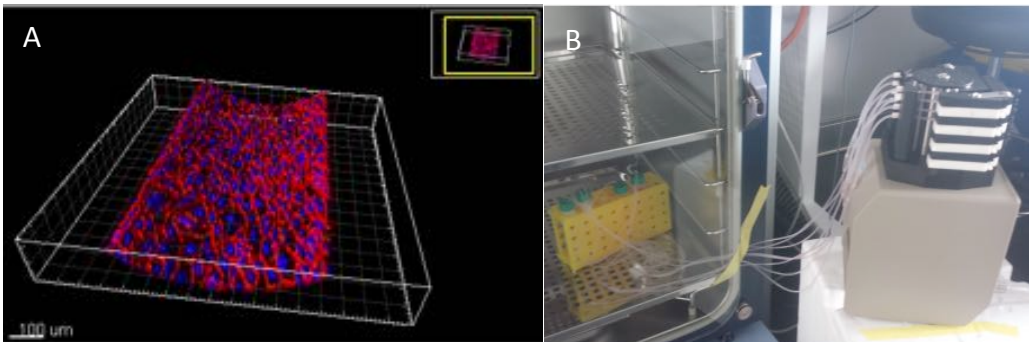


Figure: (A) Expression of CD31 in microfluidic channels in 3-dimentional rendering view. (B) The whole setting of circulating loop for culturing our endothelial cells under physiological flow conditions.

3D culture and analysis of endothelial cells under physiological flow conditions in a xenotransplantation setting

Shengye Zhang[1,2], Colette Andrea Bichsel[1,3], Rahel Klossner[2], Oliver Steck[2], Alain Despont[2], Oliver Thierry Guenat[3], Robert Rieben[2]

[1]Graduate School for Cellular and Biomedical Sciences, University of Bern, Switzerland.

[2]Department of Clinical Research, University of Bern, Switzerland

[3]ARTORG Center, University of Bern, Switzerland

Corresponding author

Robert Rieben, PhD

University of Bern

Department of Clinical Research

Murtenstrasse 50

CH-3008 Bern, Switzerland

Phone: +41 31 632 96 69

Fax: +41 31 632 75 94

Email: robert.rieben@dkf.unibe.ch

Summary

Based on the 3R principles and microfluidic technology, we developed an in vitro system to grow and investigate endothelial cells (EC) under physiological flow conditions. Microfluidic channels with circular cross-sections of 330 µm diameter were produced in polydimethylsiloxane. Porcine aortic endothelial cells (PAEC) were loaded in microfluidic channels, and a physiological shear stress of 10 dyn/cm^2 was applied for 2-7 days. After exposure to shear stress, PAEC were elongated and aligned in the direction of flow, while maintaining their endothelial properties as shown by expression of CD31, VE-cadherin and von Willebrand factor. To test EC activation a pig-to-primate xenotransplantation was simulated by perfusing the porcine cells with normal human serum (NHS) with or without complement inhibitors for 1h. Complement was deposited when PAEC were perfused with NHS alone and EC became activated as shown by positive staining for C4b/c, C3b/c and C6, expression of E-selectin, deposition of thrombin, and formation of soluble C5b-9. A significant reduction of these parameters was observed when complement inhibitors were used together with NHS. In conclusion, we describe the establishment of a simple in vitro model mimicking small to medium sized arteries under physiological flow conditions. The system is able to reproduce key findings of EC activation and complement activation in a xenotransplantation model. Compared to conventional 96-well plate assays the system provides a >350 times larger ratio of fluid volume exposure to EC surface, equaling in vivo conditions.

Keywords: endothelial cells, antibody, complement, physiological flow conditions, xenotransplantation

1. Introduction

Endothelial cell (EC) activation plays an important role in the pathophysiology of ischemia reperfusion injury, sepsis, vascular rejection of transplanted organs, and other diseases linked to the vascular system. In transplantation, the vascular endothelium of the donor organ is the first tissue to come in contact with the blood of the recipient. If pre-formed anti-donor antibodies are present in the recipient's blood, an immediate activation of the donor endothelium occurs due to antibody binding followed by activation of the complement system. This is for example the case in blood group ABO-incompatible transplantations, recipients sensitized to donor HLA antigens, and in experimental pig-to-primate xenotransplantation [1]. EC activation in turn triggers the coagulation cascade and leads to the clinical picture of hyperacute or acute vascular rejection [2, 3]. Xenotransplantation experiments in animal models have been carried out extensively to investigate mechanisms of EC activation [4-6], but also ex vivo perfusions of porcine organs with human blood, plasma or serum have been used for this purpose [7-9]. In order to reduce – in accordance with the 3R principles – the number of animals used for investigation of EC activation in hyperacute and acute vascular rejection, we developed an in vitro system to grow and investigate EC under physiological flow conditions, simulating shear stress as occurs in medium sized arteries.

In standard 2D cell culture the amount of serum or plasma in contact with EC grown on the bottom of the wells is small and may often be the limiting factor for activation or cytotoxicity of EC in vitro: In a typical experiment using 96-well microtiter plates, the ratio of fluid volume to EC surface is only 0.2 ml/cm^2 (100 µl per well with a bottom surface of 0.5 cm^2). This ratio is far away from a physiological situation in which blood circulates through vessels. Using in vitro systems based on 3D culture of EC on the inner surface of 'artificial blood vessels' and perfusion with a physiological flow the in vivo ratio of fluid volume to EC surface can be reached.

Over the last decade, microfluidics technologies have been developed, and commercial systems have been made available in which cells can be cultured under flow using convenient slide– or microtiter-based setups [10, 11]. Usually, these systems are used for growing the EC two-dimensionally, on the bottom of a rectangular shaped micro channel. Also 3D growth of EC has been reported on the inner surface of rectangular channels [12, 13]. However, the flow pattern in circular microchannels better approaches the physiological situation than in rectangular channels. In order to fabricate circular microchannels, different technologies have been reported such as a combination of mechanical micromilling and soft lithography, or introducing a pressurized air stream into liquid PDMS filled microchannels [14, 15]. Most often, however, those "circular cross-sections" were not equal.

Based on the use of needles as molds published by Chrobak et al [16], we therefore produced microfluidics chips with circular microchannels. In the present study porcine EC grown under physiological shear stress were perfused with normal human serum (NHS) as a source of xenoreactive natural antibody and complement under physiological flow conditions in the context of xenotransplantation.

2. Material and methods

2.1 Isolation and culture of porcine aortic endothelial cells

Porcine aortic endothelial cells (PAEC) were isolated from aortas by using a mechanical procedure. In brief, aortas were cut open and PAEC isolated by gently rubbing the inner surface with a cotton wool bud. The cells were transferred to fibronectin-coated tissue culture flasks (Nalge Nunc International, Kamstrup, DK) at 37°C in a 5% CO_2 incubator until confluence. DMEM cell culture medium (Thermo Fisher Scientific, Waltham, MA, USA) was used, supplemented with 10% heat-inactivated fetal bovine medium (FBS, Biochrom, Berlin, Germany), 100 IU/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific), and 0.4% endothelial cell growth medium 2 (ECGM2, PromoCell, Heidelberg, Germany). Cells were used between passage 3 and 6 for this study.

No animals were used specifically for the present study. Porcine aortas used for PAEC isolation were from animal experiments with pigs in the context of evaluation of surgical techniques and devices, as well as studies on xenotransplantation. All animal experiments were approved by the Veterinary Service of the Canton of Bern, Switzerland, and performed in accordance with national and international 3R and ARRIVE guidelines [17].

2.2 Construction of microfluidics channels with round cross section

Polydimethylsiloxane (PDMS, Sylgard, Dow Corning, Wiesbaden, Germany) was prepared by mixing 10 parts of elastomer silicone and 1 part of curing agent, and casted in a petri dish (Thermo Fisher Scientific). Sterile and pyrogen free needles with a diameter of 120 µm and a length of 3 cm (Seirin, Hamburg, MA, USA) were laid in parallel in the liquid PDMS, at the bottom of the petri dish. Four needles of 330 µm diameter and 2.5 cm length (BD Biosciences, New Jersey, USA) were placed at a 90° angle on top of the thinner needles as shown in Fig. 1. The Luer connectors of the needles were cut off with a diagonal cutter before using the needles as molds. The PDMS with the needle-molds was cured at 60°C overnight. Rectangular microfluidic chips containing 4 round channels of 330 µm were cut out from the petri dish, while needles were extracted horizontally. Inlet and outlet connectors to the microchannels were made with 2 mm biopsy punches (Shoney Scientific, Waukesha, USA). The hole, left from extraction of needles, between the edge of the PDMS gel and the inlet and outlet, respectively, was sealed with liquid PDMS and cured at 60°C overnight. The final microfluidics chips contained microchannels, mimicking medium sized arteries, with a diameter of 330 µm and a length of 1 cm. The schematic for microchannel fabrication is shown in Fig.1.

2.3 Modification of PDMS surface in microchannels

Before seeding cells in the microfluidic channels, the inner surface of PDMS was modified to covalently bond extracellular matrix molecules [18]. Briefly, PDMS chips and standard glass slides were cleaned, activated in an oxygen plasma cleaner (Harrick Plasma, New York, USA) at 650 mTorr for 3 min, and bonded together. Immediately after bonding, 5% 3-Triethoxysilylpropylamine (APTES) (Sigma-Aldrich, Buchs, Switzerland) was added to fill the microchannels and incubated for 20 min at room temperature, then treated with 0.1% glutaraldehyde (Sigma-Aldrich) for 30 min for PDMS surface salinization. To immobilize extracellular protein onto the PDMS surface, microchannels were coated with 50 µg/ml fibronectin (Millipore, Schaffhausen, Switzerland) at room temperature overnight and 100 mg/ml collagen I bovine protein (Gibco, Thermo Fisher Scientific) at room temperature for 1.5 h, respectively. Cell culture medium was rinsed through the microfluidic channels to adsorb unspecific protein sites before cell loading.

2.4 Cell loading and pulsatile flow

PAEC grown to confluence in T75 flasks were trypsinized with 0.05% EDTA-trypsin (Gibco, Thermo Fisher Scientific) and suspended in normal cell culture medium with 4% dextran from Leuconostoc spp. (Mr ~70,000 Da, Sigma-Aldrich), to increase viscosity and promote cell adhesion. Cells at a density of $1x10^6$/ml were loaded into the microfluidic channels. The whole device was then fixed on an intelli-mixer rotator with tapes (ELMI ltd, Riga, Latvia) and placed in the 37°C / 5% CO_2 incubator at 1 rpm for 45 min to permute uniform cell attachment. Cells were then cultured under static conditions for one day and medium was changed three times per day by pipetting. Afterwards, a peristaltic pump – Minipuls 3 with 8 channels (Gilson, Villiers le bel, France) – was connected to the microfluidic channels via sterile silicon tubing with stoppers (Gilson) and extension silicon tubing (Gobatec, Bern, Switzerland). These tubes were extensively flushed with PBS and distilled water, followed by cell culture medium with 4% dextran. A medium reservoir in a 15 ml sterile tube (Corning, Berlin, Germany) was attached to each microchannel and put in the 37°C incubator together with the microfluidic device. After 1 day of static incubation the PAEC in the microfluidic channels were incubated in cell culture medium under pulsatile flow, starting with a low shear stress of 0.04 dyn/cm^2, corresponding to 5 pulses per minute (bpm), overnight. Thereafter the shear stress and pulse rate was gradually increased by 10 bpm per hours, until the desired shear stress of 10 dyn/cm^2 at 70 bpm was reached (shear stress = $4\mu Q/pi*R^3$, Q = 0.0898x - 0.0026, µ: medium viscosity; Q: flow rate; R: radius; x: rpm; 1rpm = 10bpm). This shear flow of 10 dyn/cm^2 was maintained for two days in the present study. But the system can be maintained for 7 days with exchange of the medium every 2-3 days. Cell morphology was assessed under a bright field microscope (Leica DMI 4000B, Microsystems Schweiz, Heerbrugg, Switzerland).

2.5 Perfusion of normal human serum and interventions

After two days of pulsatile flow, cell culture medium was replaced with normal human serum diluted 1:10 in PBS supplemented with 0.15 mM Ca^{++} and 0.5 mM Mg^{++} (NHS) with or without complement inhibitors for 1h under the same flow condition. Four groups were made: Group 1: NHS alone, Group 2: NHS + 10 IU/ml C1 INH (Berinert, provided by CSL Behring, Marburg, Germany), Group 3: NHS + 0.25 mg/ml APT070 (provided by Richard Smith, King's College, London, UK), Group 4: NHS + 0.3 mg/ml DXS (M_r 5KDa, provided by Tikomed, Viken, Sweden). For each group, experiments with 3-5 channels were performed. Finally, perfusate was collected and stored at -80°C. EC in the microchannels were used for immunofluorescence staining.

2.6 Immunofluorescence staining

To characterize endothelial cells in the microchannels and to assess deposition of complement and thrombin as well as EC activation in our model, immunofluorescence staining was performed. In brief, cells in the microfluidic channels were washed with PBS, fixed with 4% formaldehyde for 15 min, and blocked with PBS-3%BSA for 45 min. Incubation with primary antibodies was at 4°C overnight, followed by secondary antibodies and 4',6-diamidino-2-phenylindole (DAPI). The primary antibodies used were: rat anti-porcine CD31 (mAB33871, R&D, Minneapolis, USA), goat anti-human VE-cadherin (sc-6458, Santa Cruz, Texas, USA), rabbit anti-human von Willebrand factor (vWF, A0082, Dako, Glostrup, Denmark), rabbit anti-human C3b/c-FITC (F0201, Dako), rabbit anti-human C4b/c-FITC (F0169, Dako), Goat anti-human C6 (A307, Quidel, San Diego, USA), mouse anti-heparan sulfate IgM (370255-1, Amsbio, Abingdon, UK), mouse anti-human E-selectin IgG (S-9555, Sigma-Aldrich), rabbit anti-thrombin (ab92621, Abcam). The secondary antibodies were goat anti-rat IgG Cy3 (112-166-003, Jackson ImmunoResearch, West Grove, PA, USA), donkey anti-goat alexa488 (A21082, Thermo Fisher Scientific), sheep anti-rabbit IgG Cy3 (C2306, Sigma-Aldrich), donkey anti-goat IgG alexa488 (A11055, Thermo Fisher Scientific, MA, USA), goat anti-mouse IgM FITC (115-097-020; Jackson ImmunoResearch), goat anti-mouse IgG alexa488 (A21121, Thermo Fisher Scientific). Nuclei were stained with DAPI (Boehringer, Roche Diagnostics, Indianapolis, IN, USA). In addition, cytoskeleton filamentous actin (F-actin) was stained with Rhodamine Phalloidin (PHDR1, LuBioScience, Luzern, Switzerland). Images were taken at 63x with a confocal laser-scanning microscope (Zeiss LSM 710, Feldbach, Switzerland) and analyzed by ImageJ (National Institutes of Health, Bethesda, MD, USA). In addition, z-stack images were processed by Imaris 8.2 software (Bitplane, Zurich, Switzerland).

2.7 Quantification of cellular alignment

To quantify cellular alignment with the direction of flow, cell orientation was analyzed and quantified using the FibrilTool plugin function in Fiji (http://fiji.sc./Fiji) following the published protocol [19] both under static and flow conditions. Fluorescent signals from CD31 and F-actin staining were used.

2.8 Detection of sC5b-9

To determine the concentrations of human soluble C5b-9 (sC5b-9) in perfusate, a single Bio-Plex assay was performed according to a protocol published by our group [20]. In brief, microbeads (Luminex) were coupled with mouse anti-human sC5b-9 antibody (Dia011-01, Dianova, Hamburg, Germany) using Bio-Plex amine coupling kit (Bio-Rad). Coupled beads were then incubated with samples, followed by biotinylated anti-human C6 detection antibody (A706, Quidel, San Diego, USA) and Streptavidin-R-PE (922721, Qiagen, Hilden, Germany). Measurement and data analysis were performed with a Bio-Plex 100 array reader and the Bio-Plax Manager software version 6.1 (Bio-Rad).

2.9 Statistical analysis

All data are presented as mean ± standard deviation (SD). Statistical analyses were performed by GraphPad Prism 6 software (GraphPad, San Diego, CA, USA) using one-way analysis of variance (ANOVA) followed by Fisher's LSD post hoc test to compare means of all groups. For comparison of cell orientation, Mann-Whitney U test was used. P values <0.05 were considered statistically significant.

3. Results

3.1 Cell morphology and alignment with direction of pulsatile flow

Cells started to attach to the inner surface of the microchannels 3-4h after seeding. Afterwards, cells became elongated and a confluent monolayer formed in one day. When a pulsatile flow was applied, cells started to align with the flow over time. After 2 days of peristaltic flow, cells were aligned with the direction of flow (Fig. 2A).

Cell alignment in the direction of flow was assessed by staining of the cytoskeleton protein F-actin as well as CD31. For F-actin, after 2 days of pulsatile flow, the average angle of the cells with respect to the flow direction of the microchannels was 9.6 ± 8.1°, which was significantly smaller than under static conditions (70.7 ± 32.1°, p=0.007). For CD31 the respective values were 21.8 ± 26.3° and 74.2 ± 13.7°, respectively, p=0.047 (Fig. 2B-C).

3.2 Endothelial cell characterization and overview of PAEC distribution in the microfluidic channels

To confirm that EC from porcine aortas still expressed typical endothelial markers when cultured in microfluidic channels, staining for CD31, VE-cadherin, and vWF was performed by immunofluorescence. All of these markers were expressed on PAEC after culturing in a 3D microfluidic system under both static and flow conditions (Fig. 3A). Confocal 3D imaging also revealed that the PAEC do indeed cover the complete inner surface of the circular microfluidic channels, mimicking arteries with a diameter of 330 µm. This is shown in 3D rendering and orthogonal views, in Fig. 3B-C.

3.3 Use of PAEC in microfluidic channels in a xenotransplantation model

PAEC grown under pulsatile flow in PDMS microchannels were perfused with normal human serum, with or without addition of complement inhibitors. Activation of the complement cascade was confirmed by positive staining for C3b/c, C4b/c, and C6. Deposition of C3b/c, C4b/c, and C6 was significantly reduced by all of the three used complement inhibitors compared to perfusion by NHS alone. The respective data are shown in Fig. 4, both quantitated as column graphs and as representative immunofluorescence images.

3.4 Expression of heparan sulfate and E-selectin

To evaluate expression of HS and E-selectin on PAEC after perfusion with NHS with or without complement inhibitors, immunofluorescence staining was performed. DXS effectively reduced the shedding of HS compared with the NHS alone perfusion group ($p=0.002$). However, this was not the case with C1 INH and APT070 (Fig. 5A).

Expression of E-selectin was upregulated on cells perfused with NHS alone. Addition of the complement inhibitors C1 INH, APT070 and DXS led to a significant downregulation of E-selectin expression as compared to the cells perfused with NHS alone (all $p<0.01$, Fig. 5B).

3.5 Binding of thrombin to PAEC

To investigate the transformation of EC to a pro-coagulant phenotype in our system, thrombin binding to PAEC was assessed by immunofluorescence staining. Thrombin was found to be present on PAEC that were perfused with NHS. As compared with NHS alone, perfusion of the PAEC microchannels with NHS + DXS led to a significant reduction of thrombin staining ($p=0.010$), whereas addition of C1 INH or APT070 to NHS did not have a significant effect on thrombin binding in our model (Fig.6).

3.6 Soluble C5b-9 in perfusate

Levels of sC5b-9 in perfusate were measured as a marker of terminal complement pathway activation in our experiments. High levels of sC5b-9 in perfusate were found when cells were perfused with NHS alone

(30547 ± 2932 ng/ml), while addition of complement inhibitors significantly reduced sC5b-9 generation (C1 INH: 19019 ± 10501 ng/ml, p=0.004; APT070: 725 ± 585 ng/ml, p<0.0001; DXS: 18605 ± 4181 ng/ml, p=0.005 Fig.7).

4. Discussion

We established a simple in vitro system to grow endothelial cells on the inner surface of round PDMS microchannels under pulsatile flow conditions. The diameter of the channels (330 µm) and the used shear stress (10 dyn/cm^2) mimic the conditions of small to medium sized arteries in vivo [21]. This microfluidic system was used to investigate endothelial cell activation in the context of a xenotransplantation setting.

Endothelial cells seeded into the microfluidic channels and grown under static conditions for the first two days aligned in the direction of flow as soon as exposure to shear stress was induced by pulsatile perfusion with cell culture medium. This cell alignment was described earlier in microfluidic studies and is supposed to be based on mechanically affected distribution of cytoskeleton proteins [22, 23]. In our model we could show complete coverage of the inner surface of the round microchannels by a confluent monolayer of EC, creating the impression of artificial small to medium sized arteries in a 3-dimensional rending view. Maintenance of the endothelial phenotype of the cells in the microchannels was confirmed by expression of CD31, VE-cadherin, and vWF, both under static and flow conditions.

After establishment of our in vitro model, we wanted to know whether this system is able to reproduce complement activation as occurs in hyperacute or acute vascular rejection in a xenotransplantation setting [1]. We therefore perfused PAEC-microchannels with diluted normal human serum with or without complement inhibitors. Deposition of the complement components C3b/c, C4b/c, and C6 was observed and complement activation further confirmed by an increased level of sC5b-9 in NHS perfused through PAEC-microchannels.

One application of our microfluidics system could be the screening of inhibitors of complement and/or EC activation. Three known complement inhibitors were therefore tested in our model: C1 INH, APT070, and DXS. C1 INH is a physiological, fluid phase inhibitor of complement and coagulation, acting mainly on the C1 complex, which initiates classical pathway complement activation [24-26]. APT070 is a recombinant derivative of soluble complement receptor 1, regulating complement activation at the level of C4 / C3 [27]. DXS, finally, is a highly sulfated polyglucose and a member of the glycosaminoglycan family. It acts as an EC protectant and a complement inhibitor [28, 29]. As expected, all inhibitors blocked complement activation on the C4 / C3 level and further downstream.

In addition to direct, antibody- and complement-mediated cytotoxicity, EC activation also contributes to the pathophysiology of hyperacute or acute vascular rejection. One of the earliest events in EC activation is the shedding of HS from the endothelial surface, transforming the endothelium from an anti-coagulant and anti-inflammatory to a pro-coagulant and pro-inflammatory phenotype [30]. In line with data published

before [31] we found a low expression of HS when PAEC-microchannels were perfused with NHS alone. An increased HS staining was observed when DXS was added to the serum, but not with the other complement inhibitors. This finding supports earlier data from our group, showing that the proteoglycan analog DXS acts as an EC protectant both in vitro and in vivo [28, 32]. However, anti-HS antibodies also detect DXS bound to EC, which may lead to overestimation of the prevention of HS shedding by DXS.

To detect EC activation expression of E-selectin was investigated. We found an upregulation of E-selectin expression in the PAEC-microchannels perfused by NHS alone, as reported previously in an in vitro model of xenotransplantation [33]. Also in our model upregulation of E-selectin corresponded to complement activation on cells perfused with NHS, which is in line with in vitro data showing that endothelial E-selectin expression in xenotransplantation is fully dependent on complement [34]. This is further supported by the fact that all of the three used complement inhibitors in our study also down regulated the expression of E-selectin.

Thrombin, formed from cleavage of prothrombin, is present in serum after blood coagulation [35]. Deposition of thrombin was observed on PAEC perfused with NHS, suggesting binding of thrombin to receptors expressed on activated EC such as protease-activated receptors [36, 37]. Interestingly, only DXS treatment significantly reduced thrombin binding in our model, whereas C1 INH and APT070 had no effect.

A limitation of our model is the use of 1:10 diluted serum to study activation of the EC growing on the inner surface of the microfluidic channels. In vivo, EC activation in transplantation, ischemia/reperfusion injury and other clinical settings occurs in the whole blood environment. Our study only includes the effect of complement and omits possible effects of the other plasma cascade systems, namely coagulation, fibrinolysis and kallikrein/kinin, as well as blood cells. Coating of the silicon tubings and connectors with heparin might allow the use of whole, non-anticoagulated blood for perfusion of the EC-microchannels and further improve the model. Within the microchannels the ratio of EC-surface to blood volume is 120 cm^2/ml, which would allow the exploitation of the natural, anticoagulant properties of EC when working with non-anticoagulated whole blood [38].

In conclusion, we have established an in vitro system for 3-dimensional growth of EC in microfluidic channels with circular cross sections under physiological flow conditions, mimicking small to medium sized arteries. The system is able to reproduce key findings of EC activation and complement activation in a xenotransplantation model. Compared to conventional 96-well plate assays the system provides a >350 times larger ratio of fluid volume exposure to EC surface, equaling in vivo conditions.

References

1. U. Galili, "Interaction of the natural anti-Gal antibody with alpha-galactosyl epitopes: a major obstacle for xenotransplantation in humans," *Immunology Today,* vol. 14, no. 10, pp. 480-2, 1993.
2. B. Gollackner, S. K. Goh, I. Qawi, L. Buhler, C. Knosalla, S. Daniel, E. Kaczmarek, M. Awwad, D. K. Cooper, and S. C. Robson, "Acute vascular rejection of xenografts: roles of natural and elicited xenoreactive antibodies in activation of vascular endothelial cells and induction of procoagulant activity," *Transplantation,* vol. 77, no. 11, pp. 1735-41, 2004.
3. R. Oriol, Y. Ye, E. Koren, and D. K. Cooper, "Carbohydrate antigens of pig tissues reacting with human natural antibodies as potential targets for hyperacute vascular rejection in pig-to-man organ xenotransplantation," *Transplantation,* vol. 56, no. 6, pp. 1433-42, 1993.
4. T. Laumonier, A. J. Walpen, K. M. Matozan, E. Y. Korchagina, N. V. Bovin, A. Haeberli, P. J. Mohacsi, and R. Rieben, "Multimeric tyrosine sulfate acts as an endothelial cell protectant and prevents complement activation in xenotransplantation models," *Xenotransplantation,* vol. 11, no. 3, pp. 262-8, 2004.
5. N. Domenech, T. Diaz, I. Moscoso, A. Centeno, E. Lopez-Pelaez, and R. Manez, "Porcine endothelial cell activation in hDAF pig hearts transplanted in baboons with prolonged survival and lack of rejection," *Transplantation Proceedings,* vol. 35, no. 5, pp. 2045-6, 2003.
6. H. Xu, D. Yin, B. Naziruddin, L. Chen, A. Stark, Y. Wei, Y. Lei, J. Shen, J. S. Logan, G. W. Byrne, and A. S. Chong, "The in vitro and in vivo effects of anti-galactose antibodies on endothelial cell activation and xenograft rejection," *Journal of Immunology,* vol. 170, no. 3, pp. 1531-9, 2003.
7. A. Baquerizo, A. Mhoyan, M. Kearns-Jonker, W. S. Arnaout, C. Shackleton, R. W. Busuttil, A. A. Demetriou, and D. V. Cramer, "Characterization of human xenoreactive antibodies in liver failure patients exposed to pig hepatocytes after bioartificial liver treatment: an ex vivo model of pig to human xenotransplantation," *Transplantation,* vol. 67, no. 1, pp. 5-18, 1999.
8. T. J. Kroshus, R. M. Bolman, 3rd, and A. P. Dalmasso, "Selective IgM depletion prolongs organ survival in an ex vivo model of pig-to-human xenotransplantation," *Transplantation,* vol. 62, no. 1, pp. 5-12, 1996.
9. J. V. Guarrera, J. Stone, J. Tulipan, J. Jhang, B. Arrington, J. Boykin, G. Markowitz, and L. E. Ratner, "A novel ex-vivo porcine renal xenotransplantation model using a pulsatile machine preservation system," *Annals of Transplantation,* vol. 16, no. 1, pp. 80-2, 2011.
10. D. Wilson, and B. Hube, "Hgc1 mediates dynamic Candida albicans-endothelium adhesion events during circulation," *Eukaryot Cell,* vol. 9, no. 2, pp. 278-87, 2010.
11. C. G. Conant, M. A. Schwartz, and C. Ionescu-Zanetti, "Well plate-coupled microfluidic devices designed for facile image-based cell adhesion and transmigration assays," *J Biomol Screen,* vol. 15, no. 1, pp. 102-6, 2010.
12. M. B. Esch, D. J. Post, M. L. Shuler, and T. Stokol, "Characterization of in vitro endothelial linings grown within microfluidic channels," *Tissue Eng Part A,* vol. 17, no. 23-24, pp. 2965-71, 2011.
13. K. Hattori, Y. Munehira, H. Kobayashi, T. Satoh, S. Sugiura, and T. Kanamori, "Microfluidic perfusion culture chip providing different strengths of shear stress for analysis of vascular endothelial function," *J Biosci Bioeng,* vol. 118, no. 3, pp. 327-32, 2014.
14. M. E. Wilson, N. Kota, Y. Kim, Y. Wang, D. B. Stolz, P. R. LeDuc, and O. B. Ozdoganlar, "Fabrication of circular microfluidic channels by combining mechanical micromilling and soft lithography," *Lab Chip,* vol. 11, no. 8, pp. 1550-5, 2011.
15. M. Abdelgawad, C. Wu, W. Y. Chien, W. R. Geddie, M. A. Jewett, and Y. Sun, "A fast and simple method to fabricate circular microchannels in polydimethylsiloxane (PDMS)," *Lab Chip,* vol. 11, no. 3, pp. 545-51, 2011.
16. K. M. Chrobak, D. R. Potter, and J. Tien, "Formation of perfused, functional microvascular tubes in vitro," *Microvascular Research,* vol. 71, no. 3, pp. 185-96, 2006.
17. C. Kilkenny, W. J. Browne, I. C. Cuthill, M. Emerson, and D. G. Altman, "Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research," *PLoS Biology,* vol. 8, no. 6, pp. e1000412, 2010.
18. S. Kuddannaya, Y. J. Chuah, M. H. Lee, N. V. Menon, Y. Kang, and Y. Zhang, "Surface chemical modification of poly(dimethylsiloxane) for the enhanced adhesion and proliferation of mesenchymal stem cells," *ACS Appl Mater Interfaces,* vol. 5, no. 19, pp. 9777-84, 2013.
19. A. Boudaoud, A. Burian, D. Borowska-Wykret, M. Uyttewaal, R. Wrzalik, D. Kwiatkowska, and O. Hamant, "FibrilTool, an ImageJ plug-in to quantify fibrillar structures in raw microscopy images," *Nature Protocols,* vol. 9, no. 2, pp. 457-63, 2014.
20. A. K. Bongoni, J. Lanz, R. Rieben, and Y. Banz, "Development of a bead-based multiplex assay for the simultaneous detection of porcine inflammation markers using xMAP technology," *Cytometry A,* vol. 83, no. 7, pp. 636-47, 2013.
21. T. G. Papaioannou, and C. Stefanadis, "Vascular wall shear stress: basic principles and methods," *Hellenic Journal of Cardiology. Hellenike Kardiologike Epitheorese,* vol. 46, no. 1, pp. 9-15, 2005.

22. P. L. Voyvodic, D. Min, and A. B. Baker, "A multichannel dampened flow system for studies on shear stress-mediated mechanotransduction," *Lab Chip,* vol. 12, no. 18, pp. 3322-30, 2012.
23. C. L. Gouget, Y. Hwang, and A. I. Barakat, "Model of cellular mechanotransduction via actin stress fibers," *Biomech Model Mechanobiol,* 2015.
24. G. J. Arlaud, A. Reboul, R. B. Sim, and M. G. Colomb, "Interaction of C1-inhibitor with the C1r and C1s subcomponents in human C1," *Biochimica et Biophysica Acta,* vol. 576, no. 1, pp. 151-62, 1979.
25. H. Jiang, E. Wagner, H. Zhang, and M. M. Frank, "Complement 1 inhibitor is a regulator of the alternative complement pathway," *Journal of Experimental Medicine,* vol. 194, no. 11, pp. 1609-16, 2001.
26. A. E. Fiane, V. Videm, H. T. Johansen, O. J. Mellbye, E. W. Nielsen, and T. E. Mollnes, "C1-inhibitor attenuates hyperacute rejection and inhibits complement, leukocyte and platelet activation in an ex vivo pig-to-human perfusion model," *Immunopharmacology,* vol. 42, no. 1-3, pp. 231-43, 1999.
27. F. Xiao, L. Ma, M. Zhao, R. A. Smith, G. Huang, P. M. Jones, S. Persaud, A. Pingitore, A. Dorling, R. Lechler, and G. Lombardi, "APT070 (mirococept), a membrane-localizing C3 convertase inhibitor, attenuates early human islet allograft damage in vitro and in vivo in a humanized mouse model," *British Journal of Pharmacology,* vol. 173, no. 3, pp. 575-87, 2016.
28. T. Laumonier, A. J. Walpen, C. F. Maurus, P. J. Mohacsi, K. M. Matozan, E. Y. Korchagina, N. V. Bovin, B. Vanhove, J. D. Seebach, and R. Rieben, "Dextran sulfate acts as an endothelial cell protectant and inhibits human complement and natural killer cell-mediated cytotoxicity against porcine cells," *Transplantation,* vol. 76, no. 5, pp. 838-43, 2003.
29. R. Spirig, T. Gajanayake, O. Korsgren, B. Nilsson, and R. Rieben, "Low molecular weight dextran sulfate as complement inhibitor and cytoprotectant in solid organ and islet transplantation," *Molecular Immunology,* vol. 45, no. 16, pp. 4084-94, 2008.
30. N. S. Ihrcke, and J. L. Platt, "Shedding of heparan sulfate proteoglycan by stimulated endothelial cells: evidence for proteolysis of cell-surface molecules," *Journal of Cellular Physiology,* vol. 168, no. 3, pp. 625-37, 1996.
31. J. L. Platt, G. M. Vercellotti, B. J. Lindman, T. R. Oegema, Jr., F. H. Bach, and A. P. Dalmasso, "Release of heparan sulfate from endothelial cells. Implications for pathogenesis of hyperacute rejection," *Journal of Experimental Medicine,* vol. 171, no. 4, pp. 1363-8, 1990.
32. Y. Banz, T. Gajanayake, K. Matozan, Z. Yang, and R. Rieben, "Dextran sulfate modulates MAP kinase signaling and reduces endothelial injury in a rat aortic clamping model," *Journal of Vascular Surgery,* vol. 50, no. 1, pp. 161-70, 2009.
33. I. Kourtzelis, A. Ferreira, I. Mitroulis, D. Ricklin, S. R. Bornstein, C. Waskow, J. D. Lambris, and T. Chavakis, "Complement inhibition in a xenogeneic model of interactions between human whole blood and porcine endothelium," *Hormone and Metabolic Research,* vol. 47, no. 1, pp. 36-42, 2015.
34. U. O. Solvik, G. Haraldsen, A. E. Fiane, E. Boretti, J. D. Lambris, M. Fung, E. Thorsby, and T. E. Mollnes, "Human serum-induced expression of E-selectin on porcine aortic endothelial cells in vitro is totally complement mediated," *Transplantation,* vol. 72, no. 12, pp. 1967-73, 2001.
35. P. O'Mullan, D. Craft, J. Yi, and C. A. Gelfand, "Thrombin induces broad spectrum proteolysis in human serum samples," *Clinical Chemistry and Laboratory Medicine,* vol. 47, no. 6, pp. 685-93, 2009.
36. T. Minami, A. Sugiyama, S. Q. Wu, R. Abid, T. Kodama, and W. C. Aird, "Thrombin and phenotypic modulation of the endothelium," *Arteriosclerosis, Thrombosis, and Vascular Biology,* vol. 24, no. 1, pp. 41-53, 2004.
37. K. Bartha, T. Kovacs, I. Lerant, B. Papp, E. Csonka, K. Kolev, and R. Machovich, "Interaction of antithrombin III and thrombin-antithrombin III complex with cultured aortic endothelial cells," *Thrombosis Research,* vol. 47, no. 5, pp. 541-52, 1987.
38. H. P. Kohler, M. Muller, T. Bombeli, P. W. Straub, and A. Haeberli, "The suppression of the coagulation of nonanticoagulated whole blood in vitro by human umbilical endothelial cells cultivated on microcarriers is not dependent on protein C activation," *Thrombosis and Haemostasis,* vol. 73, no. 4, pp. 719-24, 1995.

Figures and Legends

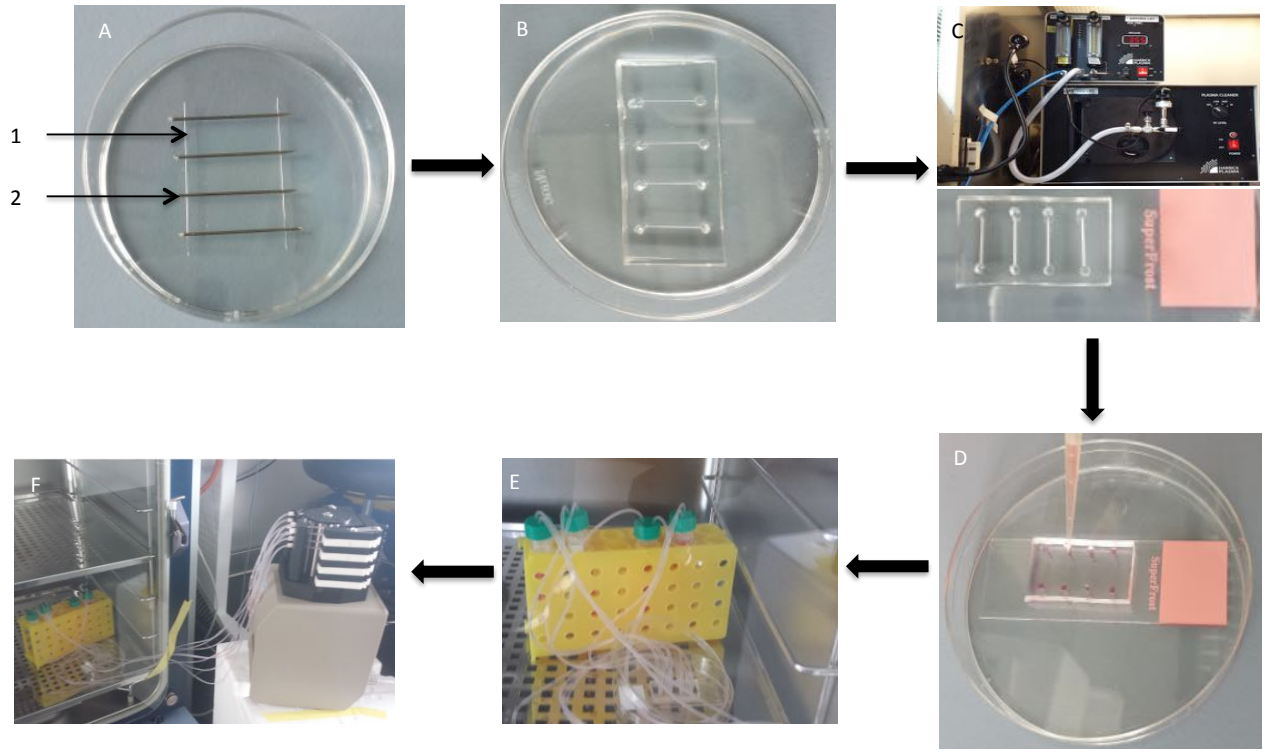


Fig. 1: Schematic of microchannel fabrication, modification of PDMS surface, cell loading, and pulsatile pump connection. A: Needle placement in liquid PDMS in a petri dish. (1) support needles of 120µm diameter; (2) mold needles with a diameter of 330 µm. B: PDMS chips after removing support and mold needles, as well as punching inlets and outlets and sealing holes. C: Oxygen plasma bonding of PDMS chips and glass slides. D: Cell loading after PDMS inner surface modification with APTES, glutaraldehyde, fibronectin, and collagen I. E: Silicon tubing connecting to microfluidic channels in the 37°C incubator with 5% CO_2. F: Overview of peristaltic pump connected to microfluidic channels.

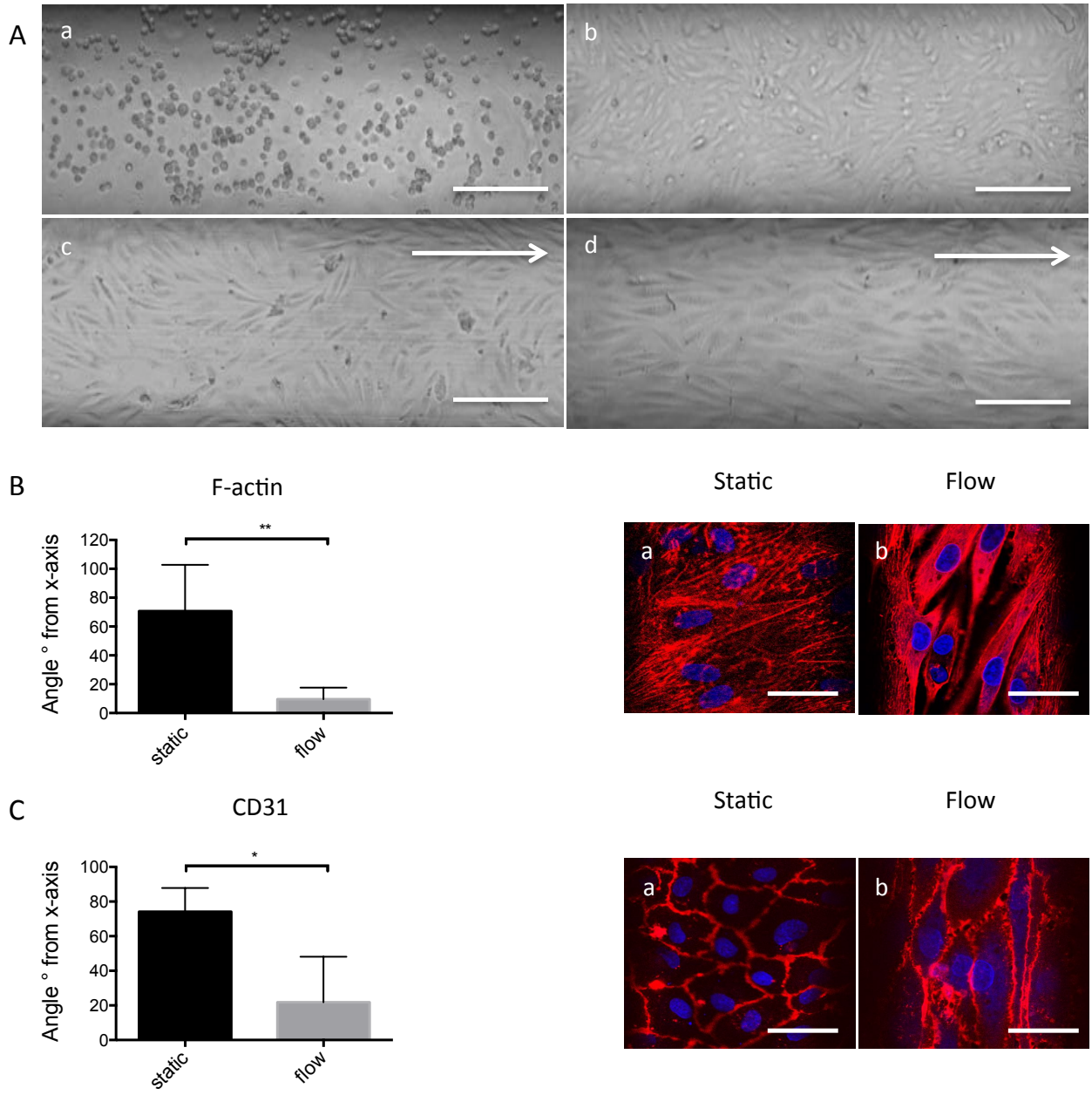


Fig. 2: Cell morphology and quantification of cell alignment. A: Cell morphology over time. (a) day 0, cells randomly distributed immediately after seeding; (b) day 1, cells attach and elongate under static conditions; (c) day 3, cells start to become aligned under flow for one day; (d) day 4, most of the cells are aligned under flow for two days. Arrows indicate the direction of pulsatile flow in the microfluidic channels. Scale bar represents 100 µm. B-C: Quantification of cell alignment to the x-axis of the microfluidic channels by immunofluorescence staining for the cytoskeleton protein F-actin and CD31, respectively. On the left panel, column graphs of the average cell angle in degrees to the x-axis are shown under static and pulsatile flow conditions (mean values ± SD, p-value: *<0.05, **<0.01). Representative immunofluorescence images are shown on the right panel (a-b). Scale bar represents 50 µm.

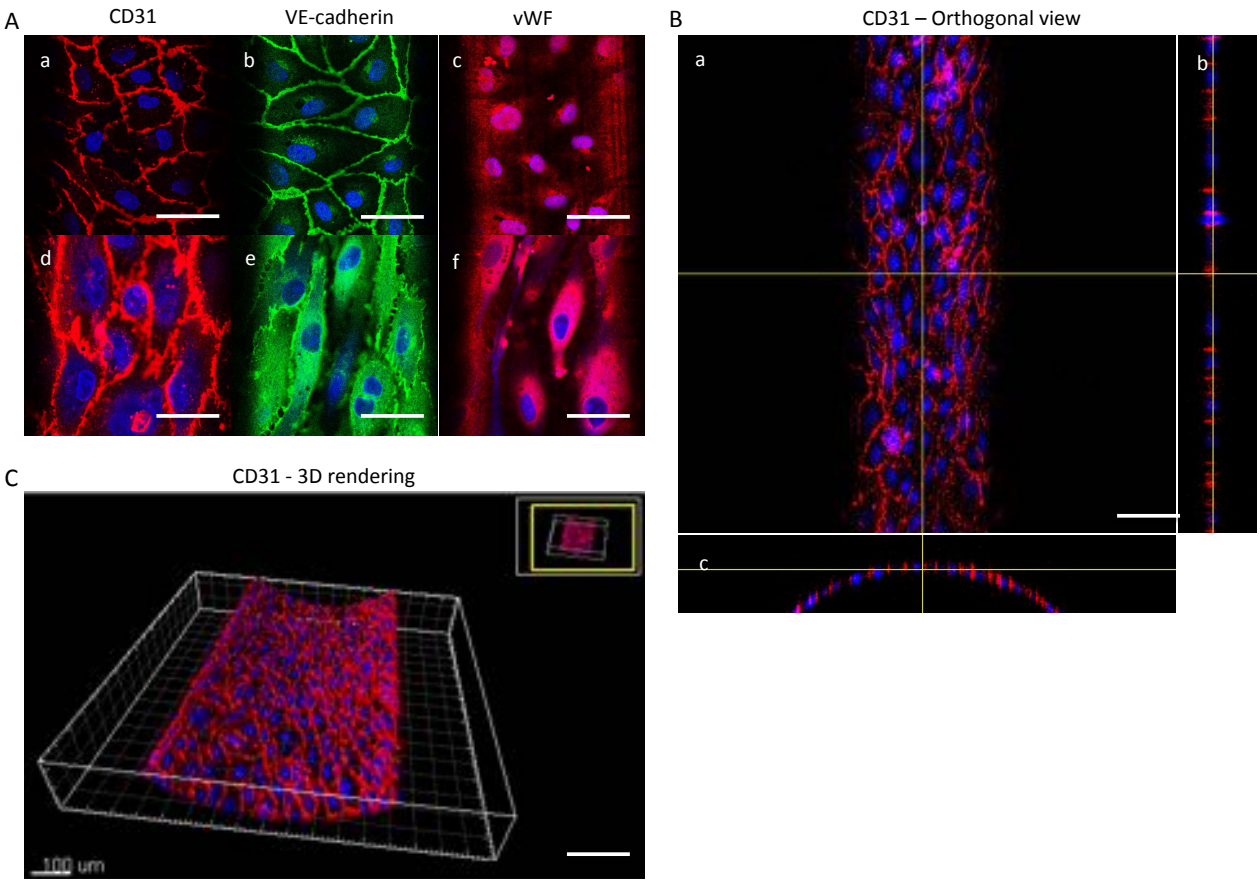


Fig. 3: EC characterization and overview of cell distribution in microfluidic channels both under static and flow conditions. A: EC characterization by expression of CD31, VE-cadherin, and vWF. (a-c) representative images for CD31, VE-cadherin, and vWF expression under static conditions; (d-f) representative images for CD31, VE-cadherin, and vWF expression under flow conditions. B: A representative orthogonal view of CD31 expression in the microfluidic channel. (a) 3D expression of CD31 in the circular microfluidic channel; (b) CD31 expression in the horizontal cross-section of the microfluidic channel; (c) CD31 expression in the vertical cross-section of the microfluidic channel. Scale bar represents 50 µm. C: A representative overview of CD31 expression in the microfluidic channel in 3D rendering. Scale bar represents 50 µm.

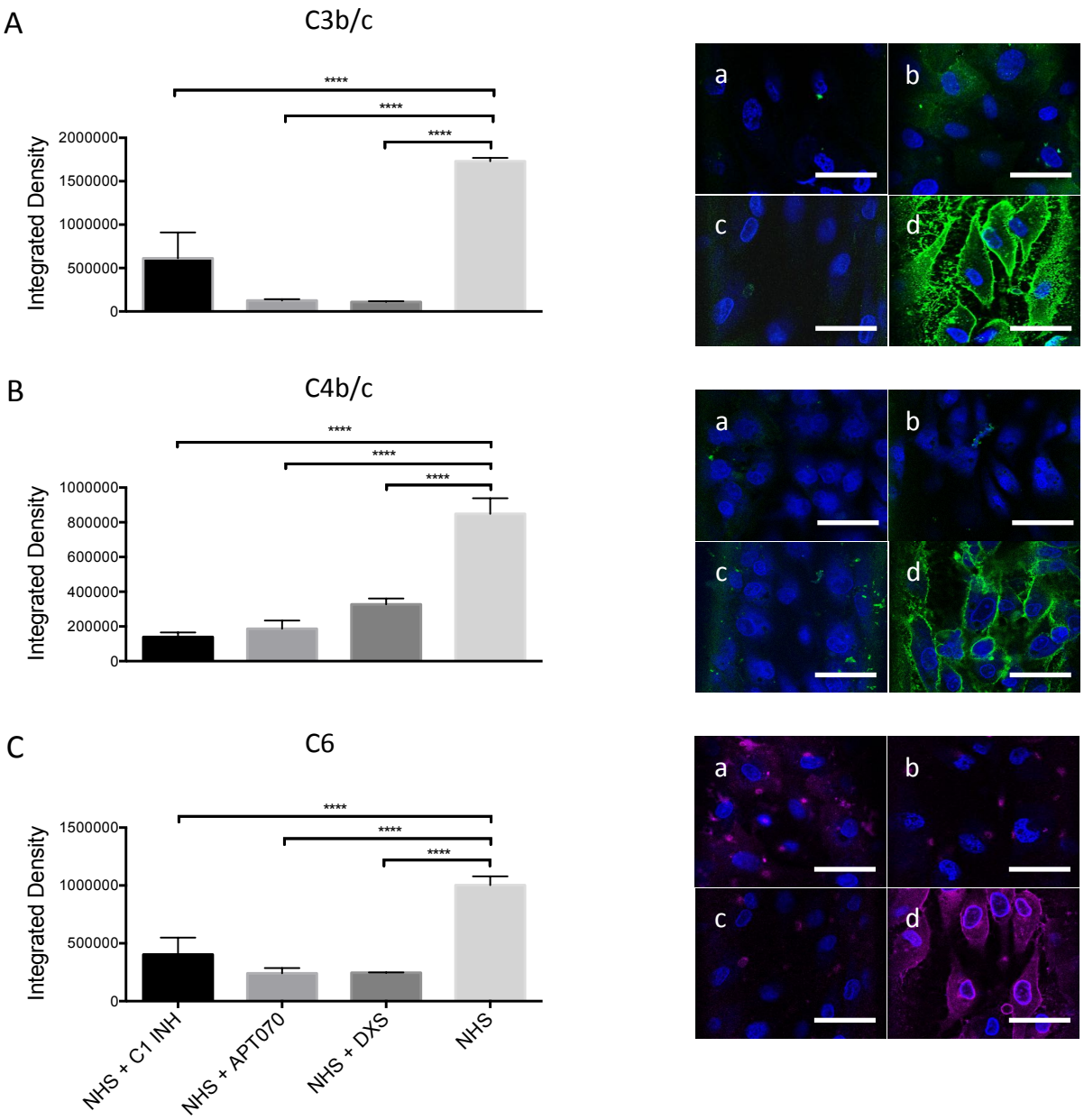


Fig. 4: Deposition of C3b/c, C4b/c, and C6 in PAEC assessed by immunofluorescence after perfusion with NHS with or without complement inhibitors. Column graphs on the left panels show quantification of immunofluorescence staining for deposition of C3b/c (A), C4b/c (B) and C6 (C). Shown are mean values ± SD with indication of statistically significant differences between complement inhibitor groups and NHS alone. ****$p<0.0001$. Representative images are shown on the right panels. PAEC in microfluidic channels were perfused with NHS + C1 INH (a), NHS + APT070 (b), NHS + DXS (c), and NHS alone (d). Scale bar represents 50 µm.

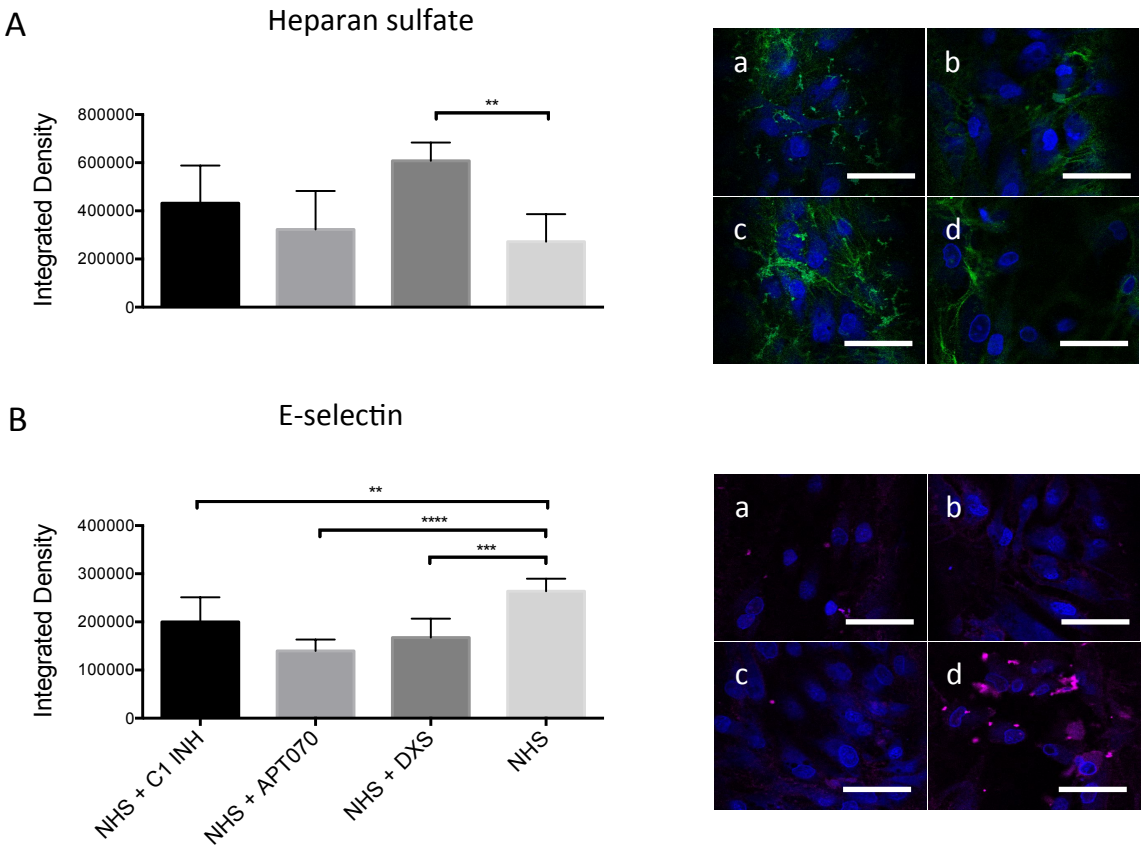


Fig. 5: Expression of heparan sulfate and E-selectin in PAEC assessed by immunofluorescence after perfusion with NHS with or without complement inhibitors. A: On the left panel, quantification for expression of heparan sulfate is shown (mean values ± SD, p-value: *<0.05, **p<0.01). On the right panel, (a-d) representative images for heparan sulfate expression in four groups were shown. Scale bar represents 50 µm. B: On the left panel, quantification for expression of E-selectin is shown (mean values ± SD, p-value: **<0.01, ***p<0.001, ****p<0.0001). On the right panel, (a-d) representative images for E-selectin expression in four groups were shown. Scale bar represents 50 µm.

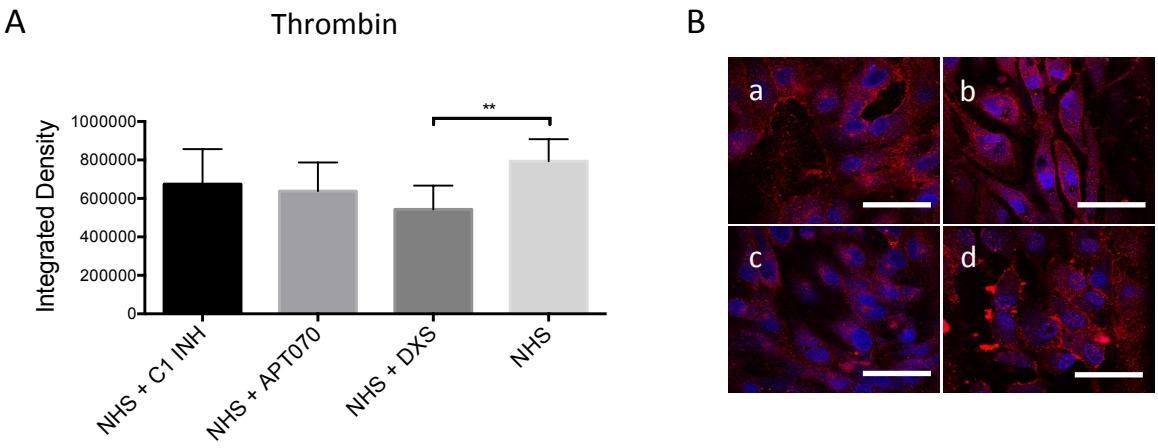


Fig. 6: Binding of thrombin to PAEC after perfusion with NHS with or without complement inhibitors. A: Quantification of thrombin deposition by immunofluorescence (mean values ± SD, p-value: **p<0.01). B: (a-d) Representative images for thrombin deposition in the four treatment groups. Scale bar represents 50 µm.

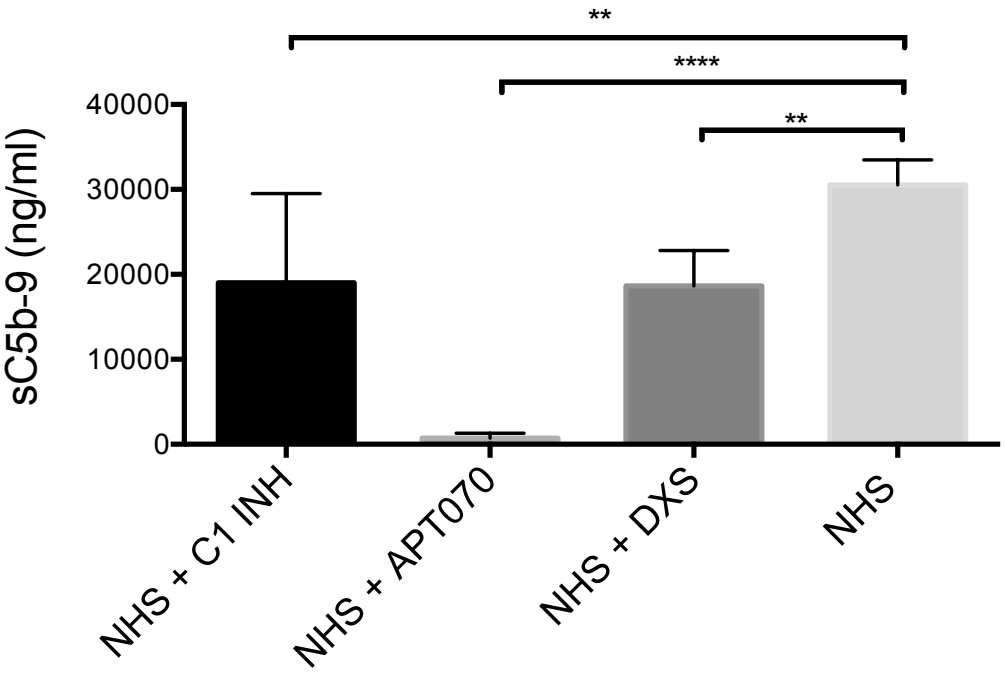


Fig. 7: Concentrations of soluble C5b-9 after perfusion with NHS with or without complement inhibitors. Soluble C5b-9 levels were measured by Bio-Plex analysis. Columns represent mean values, error bars indicate standard deviations, p-values: **p<0.01, ****p<0.0001.

Acknowledgements

We thank Drs. Rolf Spirig, CSL Behring AG, Bern, Switzerland; Marc Nolte, CSL Behring GmbH, Marburg, Germany; Anders Waas, Tikomed AB, Viken, Sweden; and Richard Smith, King's College, London, UK, for providing complement inhibitors used in this study. Help with text editing by Jane Shaw-Boden is gratefully acknowledged. This work was supported by the Microscopy Imaging Center (MIC), University of Bern. Funding was provided by the 3R Research Foundation Switzerland, project no. 133-12 and the Swiss National Science Foundation, project no. 320030_156193. SZ was supported by the China Scholarship Council (CSC).

Conflict of interest statement

None of the authors declare any conflicts of interest.

Paper V

Botulinum toxin A and B raise blood flow and increase survival of critically ischemic skin flaps

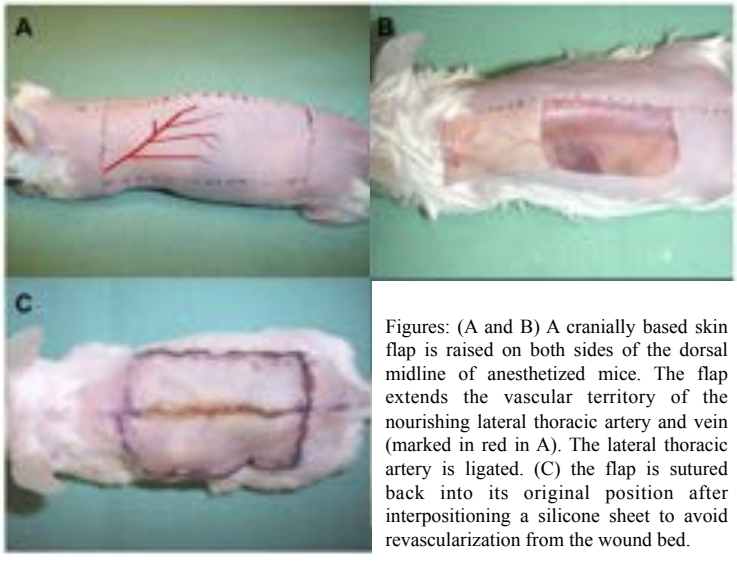
Dennis F. Schweizer, MD,[a] Riccardo Schweizer, MD,[a] Shengye Zhang, MSc,[a] Pranitha Kamat, PhD,[a,b] Claudio Contaldo, MD,[b] Robert Rieben, PhD,[a] Daniel Eberli, MD, PhD,[c] Pietro Giovanoli, MD,[b] Dominique Erni, MD,[a] and Jan A. Plock, MD [a,b,c],*

[a]Department of Clinical Research, University of Bern, Bern, Switzerland; [b]Division of Plastic and Hand Surgery, University of Zurich, Zurich, Switzerland; [c]Center of Clinical Research, University Hospital Zurich, Zurich, Switzerland.

Status: Published in J Surg Res. Doi: 10.1016/j.jss.2013.04.004.

Aim: Our aim was to evaluate BTX A and B in a mouse model of critical flap ischemia for preoperative and intraoperative application.

Conclusion: BTX application to the vascular pedicle of an axial pattern flap leads to significantly better flap perfusion, oxygenation, tissue metabolism, and tissue viability. In addition, we were able to demonstrate data supporting a mechanism for BTX A and B through the Rho/Rho kinase system and eNOS expression.



Figures: (A and B) A cranially based skin flap is raised on both sides of the dorsal midline of anesthetized mice. The flap extends the vascular territory of the nourishing lateral thoracic artery and vein (marked in red in A). The lateral thoracic artery is ligated. (C) the flap is sutured back into its original position after interpositioning a silicone sheet to avoid revascularization from the wound bed.

Available online at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: www.JournalofSurgicalResearch.com

Botulinum toxin A and B raise blood flow and increase survival of critically ischemic skin flaps

Dennis F. Schweizer, MD,[a] Riccardo Schweizer, MD,[a] Shengye Zhang, MSc,[a] Pranitha Kamat, PhD,[a,b] Claudio Contaldo, MD,[b] Robert Rieben, PhD,[a] Daniel Eberli, MD, PhD,[c] Pietro Giovanoli, MD,[b] Dominique Erni, MD,[a] and Jan A. Plock, MD[a,b,c,]*

[a] *Department of Clinical Research, University of Bern, Bern, Switzerland*
[b] *Division of Plastic and Hand Surgery, University of Zurich, Zurich, Switzerland*
[c] *Center of Clinical Research, University Hospital Zurich, Zurich, Switzerland*

ARTICLE INFO

Article history:
Received 20 November 2012
Received in revised form
23 February 2013
Accepted 4 April 2013
Available online 24 April 2013

Keywords:
Botox
Endothelium
eNOS
Microcirculation
Microhemodynamics
Preconditioning
Rho kinase
Smooth muscle cells
Vasodilation

ABSTRACT

Background: Botulinum toxin (BTX) A and B are commonly used for aesthetic indications and in neuromuscular disorders. New concepts seek to prove efficacy of BTX for critical tissue perfusion. Our aim was to evaluate BTX A and B in a mouse model of critical flap ischemia for preoperative and intraoperative application.

Methods: BTX A and B were applied on the vascular pedicle of an axial pattern flap in mice preoperatively or intraoperatively. Blood flow, tissue oxygenation, tissue metabolism, flap necrosis rate, apoptosis assay, and RhoA and eNOS expression were endpoints.

Results: Blood-flow measurements 1 d after the flap operation revealed a significant reduction to 53% in the control group, while flow was maintained or increased in all BTX groups (103%–129%). Over 5 d all BTX groups showed significant increase in blood flow to 166-187% ($P < 0.01$). Microdialysis revealed an increase of glucose and reduced lactate/pyruvate ratio and glycerol levels in the flap tissue of all BTX groups. This resulted in significantly improved tissue survival in all BTX groups compared with the control group (62% ± 10%; all $P < 0.01$): BTX A preconditioning (84% ± 5%), BTX A application intraoperatively (88% ± 4%), BTX B preconditioning (91% ± 4%), and intraoperative BTX B treatment (92% ± 5%). This was confirmed by TUNEL assay. Immunofluorescence demonstrated RhoA and eNOS expression in BTX groups. All BTX applications were similarly effective, despite pharmacologic dissimilarities and different timing.

Conclusions: In conclusion, we were able to show on a vascular, tissue, cell, and molecular level that BTX injection to the feeding arteries supports flap survival through ameliorated blood flow and oxygen delivery.

1. Introduction

Over the last decades the use of botulinum toxin (BTX) has seen an enormous increase. Although it was first used for aesthetic indications like facial wrinkles and hyperhidrosis, the medical use started 20 y ago with Food and Drug Administration approval for treating blepharospasm and facial spasms with BTX A and cervical dystonia with BTX B.

* *Corresponding author*. Division of Plastic and Hand Surgery, University Hospital Zurich, Raemistrasse 100, CH-8091 Zurich, Switzerland. Tel.: +41 44 255 11 11; fax: +41 44 255 44 25.
E-mail address: jan.plock@usz.ch (J.A. Plock).
0022-4804/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.jss.2013.04.004

Nowadays there is also a broad spectrum of indications for neuromuscular disorders like spasmodic torticollis, spasms of the extremities, and treatment of migraine, urinary bladder dysfunction, and anal fissures [1–5]. New concepts have evaluated its clinical efficacy for critical perfusion in Raynaud disease [6,7]. As a neurotoxin, BTX irreversibly blocks the neuromuscular junction, inducing a muscular relaxation, which accounts for its popular use in aesthetic applications. However, there is no direct mechanistic evidence for a positive effect to date on a vascular level.

BTX exists in seven (A–G) serotypes, each of which has additional subtypes. BTX is produced by the bacterium *Clostridium botulinum*. It is a polypeptide, with a protease being the active part in inhibiting the acetylcholine release at the neuromuscular junction. Pharmacologically only the former two serotypes are investigated more in detail. BTX A and BTX B belong to the same family of *Clostridium botulinum* toxins, yet they do have different pharmacologic characteristics and biological effects. BTX A is stabilized through vacuum drying, whereas BTX B undergoes pH reduction [8,9].

Here we present our results on using BTX A and B in a critically ischemic axial pattern skin flap model in mice. Preconditioning potential and intraoperative application were evaluated regarding flap perfusion and flap tissue viability.

2. Methods

2.1. Animals/protocol

All experiments were performed according to Swiss National Institutes of Health guidelines for the care and use of laboratory animals and with the approval of the local Animal Ethics Committee. Thirty-eight female BALB/C mice with 20–24 g body weight were used. Animals were assigned to five different groups, resulting in sample sizes of $n = 9$ for the control and $n = 6$ each for the BTX A preconditioning, the BTX B preconditioning, and the BTX A and B treatment groups. Five animals with unexpected death or total flap necrosis were excluded.

All animals underwent baseline measurements (BL) for laser Doppler flowmetry (LDF), microdialysis, and partial tissue oxygen tension (PtO_2) in the determined flap area of the skin. The preconditioned animals received either 0.1 IU BTX A or 0.5 IU BTX B by means of subcutaneous injection near the vascular pedicle after baseline measurements. After 24 h all animals underwent flap surgery. The treatment groups received intraoperatively a subcutaneous injection near to the vascular pedicle of either 0.1 IU BTX A or 0.5 IU BTX B. Controls received no injection. On postoperative days 1, 3, and 5 the measurements for tissue oxygenation and blood flow were repeated. Microdialysis was performed on postoperative day 1. The development of tissue necrosis was analyzed on day 5 by computer-based planimetry. All animals were sacrificed after day 5.

2.2. Anesthesia

For all manipulations, the animals were anesthetized with an intraperitoneal injection of medetomidine 500 μg/kg body weight (BW), climazolam 5 mg/kg BW, and fentanyl 50 μg/kg BW, as described previously [10,11]. Reversion was induced by injection of atipamezole 1.25 mg/kg BW (Antisedan; Pfizer, Zurich, Switzerland), sarmazenil 0.5 mg/kg BW (Sarmasol; Gräub, Bern, Switzerland), and naloxone 0.6 mg/kg BW (Naloxon; OrPha, Küsnacht, Switzerland) after all manipulations were completed. The anesthetized animals were placed on a heating pad in a prone position, and the room temperature was set at 28°C to keep their skin temperature constant at 32°C, which was verified with a microthermometer placed on the abdominal skin. Postoperatively, animals received analgesic treatment with metamizole 0.25 mg/g BW, (Novalgin; Sanofi-Aventis, Geneva, Switzerland) and 0.1 mL glucose 5% for volume replacement purposes due to the large wound size.

2.3. Surgery

A modified axially perfused dorsal skin flap model was used as described previously [12]. In brief, the back skin was shaved and epilated. In all the animals, the flap was overextended to achieve a defined necrotic area. A cranially based flap (40 × 15 mm) nourished by the lateral thoracic artery was dissected lateral from the midline of the back. The superficial circumflex iliac artery was secured when the flap was raised. The flap consisted of skin, a thin layer of panniculus carnosus muscle, and subcutaneous tissue. During surgery, the flap was regularly irrigated with 0.9% NaCl to prevent it from drying out. The flaps were sutured back into their original position after having placed a silicone sheet underneath to prevent vascular interference with the wound bed.

2.4. Blood flow

Blood flow in the flap was measured with a laser Doppler imager (moorLDI2-IR; Moor Instruments, Axminster, United Kingdom) on postoperative days 1, 3, and 5. The anesthetized animals were placed on a heating pad in a prone position. Measurements were taken in a room with no daylight and standard ambient room light. The LDF device was fixed at a distance of 31 cm above the animals and the scan parameters were set to a resolution of 97 × 153 pixels and a speed of 10 ms/pixel. The data were processed and analyzed using the Moor Instruments LDI Scan Software, Research Version 5.3 (Moore Instruments).

2.5. Tissue oxygenation

Tissue partial oxygen tension was monitored on postoperative days 1, 2, and 5 with Clark-type microprobes consisting of polarographic electrodes and an oxygen-sensitive microcell with a PtO_2-sensitive area of 1 mm^2 (Revoxode CC1; GMS, Kiel, Germany). The probe was inserted into the dermal layer in the center of the flap under microscopic magnification. Care was taken not to place the probes near arterioles or large venules. The mean value over an observation period of 15 min was used for further analysis.

2.6. Tissue metabolism

Microdialysis measurements were performed on postoperative day 1 to quantify the oxidative energy metabolism (glucose,

lactate/pyruvate [L/P] ratio) and lipid membrane integrity (glycerol) of the tissue, as previously described [10,13–15]. The microprobes (CMA20, 100 kDa, PES; CMA, Stockholm, Sweden) were inserted into the dermis and perfused with isotonic Ringer solution using a microinjection pump (CMA/100; CMA, Stockholm, Sweden). The perfusion rate was set to 0.75 μL/min. The probes were perfused for 30 min for equilibration before the sample was collected over 15 min. The dialysates were analyzed photometrically using the CMA600 computer-based system (CMA 600; CMA, Stockholm, Sweden).

2.7. Tissue necrosis

Flap necrosis was analyzed utilizing the built-in CCD camera of the laser Doppler imager (moorLDI2-IR; Moor Instruments) on day 5. The resolution was 132 × 212 pixels and the necrosis area was calculated with the Moor Instruments LDI Scan Software, Research Version 5.3.

2.8. Immunofluorescence

On day 5 after surgery, full-thickness transverse flap segments were harvested, fixed in buffered formalin 4%, and embedded in paraffin. The sections were deparaffinized with xylene and rehydrated in ethanol, followed by antigen retrieval in a citrate buffer solution (pH 6.0) for 15 min at 95°C. Sections were then incubated overnight at 4°C with the primary antibody eNOS (rabbit polyclonal to eNOS antibody; 1:50 dilution in PBS-TBS-1% BSA; ab66127; Abcam, Cambridge, United Kingdom) or RhoA (rabbit polyclonal to RhoA antibody; 1:100 dilution in PBS-TBS-1% BSA; ab68826; Abcam), followed by 1 h incubation at room temperature with the secondary antibody (sheep anti-rabbit IgG Cy3-conjugated antibody; 1:500 dilution in PBS-TBS-1% BSA; c-2306; Sigma-Aldrich, St. Louis, MO). All sections were then incubated for 5 min at room temperature with DAPI (1:1000 dilution; 4′, 6-diamidino-2-phenylindole). Before and after each step, the sections were washed three times in TBS.

The TUNEL assay was performed as instructed by the kit (Roche, Roth, Switzerland). In brief, sections were immersed for 30 min at 15°C–25°C in Tris-HCl serum (pH 7.5, containing 3% BSA and 20% normal bovine serum), incubated for 60 min at 37°C in a humidified atmosphere in the dark in a 1:1 TUNEL reaction-buffer mixture (TUNEL buffer: 30 mM Tris/HCl, 140 mM sodium cacodylate, and 1 mM $CoCl_2$), and finally stained with DAPI as described before.

For visualization, a confocal laser scanning microscope (LSM 510 and LSM exciter; Carl Zeiss MicroImaging GmbH, Jena, Germany) was utilized and a computerized semi-quantitative analysis of the fluorescent staining intensity of the arteriolar wall (eNOS and RhoA) was performed with ImageJ 64-Bit version 1.41o software (U.S. National Institutes of Health, Bethesda, MD) and Prism version 4.0 software (GraphPad Software, San Diego, CA). The dermal layer was selected as the region of interest for quantitative analysis of the TUNEL assay and from each sample three high-power fields were evaluated and averaged.

2.9. Statistical analysis

InStat version 3.0 software and Prism version 4.0 software (Graph Pad Software, La Jolla) were used for statistical analysis. The data were presented as mean ± standard deviation. Differences between time points were assessed by one-way analysis of variance and the Bonferroni post test. A value of $P < 0.05$ was taken to represent statistical significance. For the computed fluorescence analysis of eNOS, outliers were identified and eliminated by applying Peirce's criterion eliminated [16].

3. Results

3.1. Animals

Overall the animal experimentations were uneventful. Five animals were excluded due to total flap necrosis or unsuspected death in the first postoperative night according to the exclusion criteria (1 flap necrosis BTX B group; 4 deaths—1 belonged to the control group, 1 to the BTX B pretreatment group, and 2 to the BTX A pretreatment group).

3.2. Blood flow

The cumulative blood flow was measured by laser Doppler flowmetry and showed an average baseline value of 238 ± 53 U in healthy animals prior to experimentation (Fig. 1A).

On the first postoperative day after flap surgery, blood flow was significantly decreased in the control group to 126 ± 54 U. The BTX groups showed higher blood flow when compared with the control (BTX A preconditioning 243 ± 33 U and BTX A intraoperative 244 ± 53 U) and was significant for BTX B preconditioning 296 ± 39 U and BTX B intraoperative 307 ± 49 U ($P < 0.01$). On day 3, improved blood flow was observed with control (199 ± 61 U), BTX A preconditioning (315 ± 74 U), BTX B preconditioning (406 ± 67 U), BTX A intraoperative (330 ± 46 U), and BTX B intraoperative (463 ± 96 U). On day 5, the blood flow in the control group returned to baseline value of 268 ± 105 U. Until day 5 blood flow increased to 175% (468 ± 69 U in BTX A preconditioning), 166% (445 ± 104 U for BTX B preconditioning), 187% (502 ± 114 U in BTX A intraoperative), and 180% (482 ± 130 U in BTX B intraoperative). This increase in all BTX groups was significant with respect to the control ($P < 0.01$).

The vascular axis could be identified from day 1 onward as a strongly intensified signal axis in the BTX groups under LDF visualization (Fig. 2).

3.3. Tissue oxygenation

PtO_2 (Fig. 1B) was significantly reduced on postoperative day 1 in all the control group and BTX A groups compared with baseline measurements (22.2 ± 4.3 mm Hg). Intraoperative treatment with BTX B (18.8 ± 2.0 mm Hg; not significant *versus* baseline) and preoperative treatment with BTX B (15.6 ± 4.3 mm Hg; not significant *versus* baseline) did not lead to significant reduction of tissue oxygenation. Only intraoperative treatment with BTX B showed a significant difference from the control group on day 1 (9.5 ± 3.3 mm Hg; $P < 0.01$). On days 3 and 5 PtO_2 could not be measured in the control group reliably, because of development of a large zone of necrosis. In the BTX groups oxygenation increased stepwise, reaching baseline values on day 5.

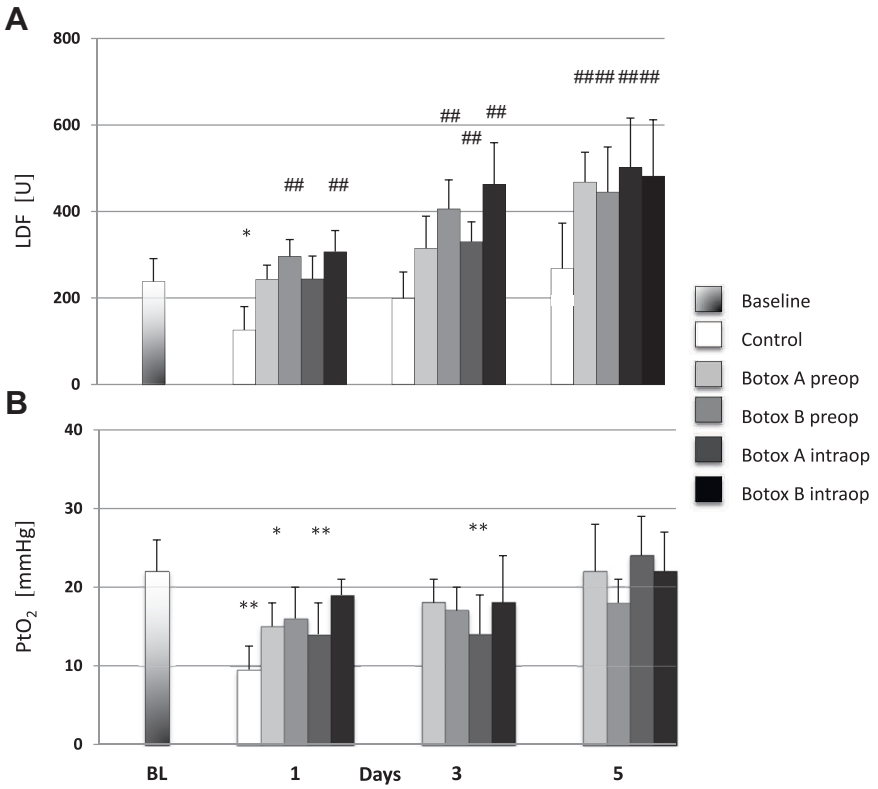


Fig. 1 – Blood flow and tissue oxygenation. Comparison of experimental groups over 5 d *versus* baseline for (A) laser Doppler flow and (B) partial tissue oxygen tension. Partial tissue oxygen tension was not measurable on days 3 and 5 in the control group, because of established tissue necrosis in the flap tissue. $^{*}P < 0.05$ *versus* BL, $^{**}P < 0.01$ *versus* BL, $^{\#\#}P < 0.01$ *versus* control.

3.4. *Tissue metabolism*

The *in vivo* cellular metabolism analyzed by microdialysis at day 1 revealed a significantly reduced level of glucose in the control group (2.3 ± 2.4 mmol/L) in comparison to baseline (19.0 ± 3.8 mmol/L; $P < 0.01$) and all BTX groups (20.2 ± 6.2 mmol/L in BTX A preconditioning, 19.4 ± 2.9 mmol/L in BTX B preconditioning, 19.4 ± 3.4 mmol/L in BTX A intraoperative, 19.7 ± 2.7 mmol/L in BTX B intraoperative; all $P < 0.01$). Glucose values were similar for all BTX groups when compared with baseline (Fig. 3A).

The decrease of glucose in the control group was associated with an accumulation of acid lactate. L/P ratio was elevated to 122 ± 45 in the control group on day 1 ($P < 0.01$ *versus* baseline L/P 25 ± 8), whereas the results for all BTX groups were significantly lower than in the control group ($P < 0.01$) and comparable to normal (38 ± 20 in BTX A preconditioning; 37 ± 23 in BTX A intraoperative, 26 ± 22 in BTX B preconditioning; 21 ± 9 in BTX B intraoperative) (Fig. 3B).

Baseline values of glycerol were measured to be 159 ± 23 μmol/L and were similar to BTX B groups (163 ± 26 μmol/L in preconditioning and 147 ± 44 μmol/L in intraoperative). Both BTX A groups exhibited slightly higher glycerol levels (BTX A preconditioning 232 ± 70 μmol/L and BTX A intraoperative 196 ± 66 μmol/L) when compared with the control group, which showed a significant increase to 517 ± 127 μmol/L ($P < 0.01$) (Fig. 3C).

Due to technical limitations we performed microdialysis only on day 1. Development of necrosis damaged the probes during insertion on the following days (Fig. 3A–C).

3.5. *Tissue necrosis*

After 5 d, 62% ± 10% of the flap area was vital by planimetric evaluation in the control group (Fig. 4). Flap viability was significantly increased in all BTX groups, reaching 84% ± 5% after BTX A preconditioning, 88% ± 4% after BTX A application intraoperatively, 91% ± 4% after BTX B preconditioning, and 92% ± 5% after intraoperative BTX B treatment (all $P < 0.01$) (Fig. 4).

3.6. *Histomorphometric evaluation*

Immunofluorescence revealed a significantly higher level of fluorescence when stained for eNOS in the arteriolar wall of flaps treated with BTX A and B preoperatively and intraoperatively compared with controls (BTX A preoperative *versus* control $P < 0.05$, all other groups *versus* control $P < 0.01$) (Fig. 5, Table). RhoA was also expressed significantly higher along the endothelium (BTX A preoperative *versus* control $P < 0.05$, all other groups *versus* control $P < 0.01$). Analysis of the TUNEL assay revealed a significant reduction of apoptotic cells in the dermis of flaps after BTX A and B preoperative treatment ($P < 0.01$) compared with controls. Intraoperative

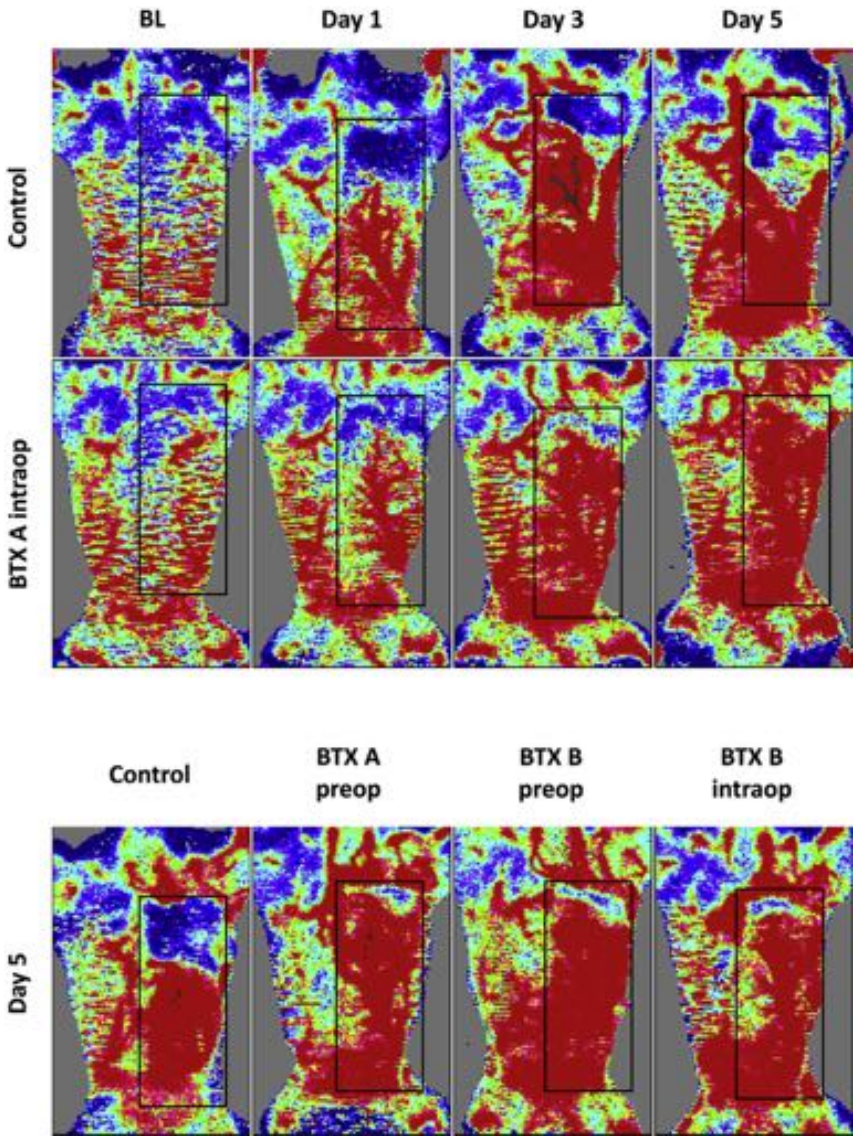


Fig. 2 – Laser Doppler image. Control and BTX A after intraoperative (intraop) application. BL, day 1, day 3, and day 5. The marked area represents the flap area. Control, BTX A preoperative (preop), BTX B preop, and BTX B intraop on day 5. (Color version of figure is available online.)

treatment was not significantly different from controls for BTX A, but differed significantly for BTX B at 5 d ($P < 0.05$).

4. Discussion

This study was dedicated to evaluate the effects of BTX A and B injections to the feeding pedicle in a critically ischemic skin flap. The principal finding was an early and compelling increase of tissue perfusion and tissue oxygenation, resulting in improved flap survival. BTX treatment was successful for increasing flap viability, with similar results for both BTX subtypes (A and B). Further, we found no advantage for BTX injection in the early preoperative setting.

Recent experimental studies focused mainly on the effects of the use of BTX A on vascularity in randomly perfused flaps [17–19]. However, the results presented so far could only show limited success and the mechanism of action remained unclear. We intended to investigate if there are any differences with regard to vascular effects, which is completely novel for BTX B. Botulinum toxin A has a higher potency than BTX B; however, there is no clear equivalent dosis according to the literature. Rates of equivalency in humans for BTX A and B range between 1:20 and more than 1:125 [20–23]. Based on the study by Aoki, in which he compared the efficacy of muscle weakening for BTX A and B, we chose a 1:5 ratio of BTX A to BTX B in mice [20].

Since early onset of vasodilation might be critical for early flap survival, we included BTX B, the subform with an earlier onset of action, into this research. BTX B has already been proven to be highly effective in the treatment of neuromuscular diseases [24,25]. From a pharmacologic point of view, BTX A is clinically effective in muscular disorders and aesthetic indications within 3 d and lasts for up to 6 mo. BTX B has a faster clinical onset of 1–2 d and lasts for up to 3 mo

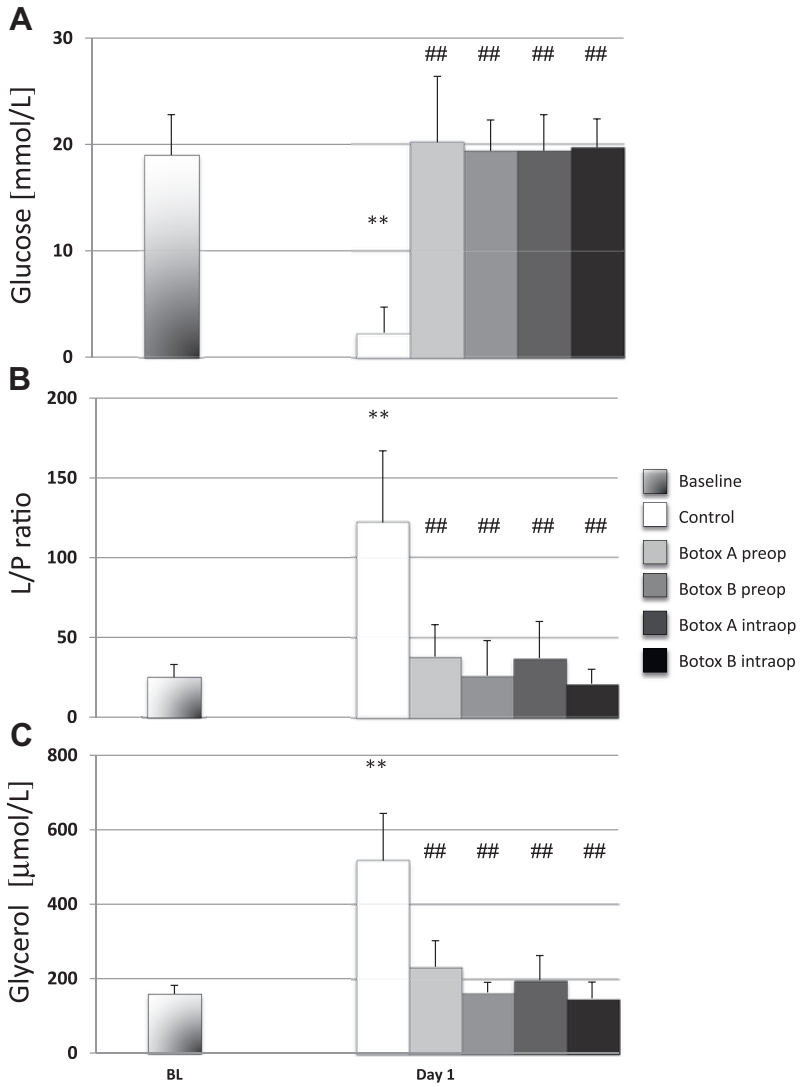


Fig. 3 – Microdialysis analysis. (A) Glucose, (B) lactate/pyruvate ratio, and (C) glycerol. Comparison of experimental groups on day 1 *versus* baseline. $^{**}P < 0.01$ *versus* BL, $^{\#\#}P < 0.01$ *versus* control.

[26,27]. Both agents irreversibly block the neuromuscular junction, leading to muscle relaxation. Theoretically, this should also work for smooth muscle cells along the vascular wall, which are innervated by the sympathetic nerve system. Our hypothesis was that sympathicolysis would lead to vasodilation and improved perfusion of the dependent tissue section. Theoretically this could also be true for pretreatment with BTX leading to chemical denervation of the vascularity and thus vasodilation in the tissue. We could not, however, observe superior effects of BTX pretreatment compared with intraoperative treatment.

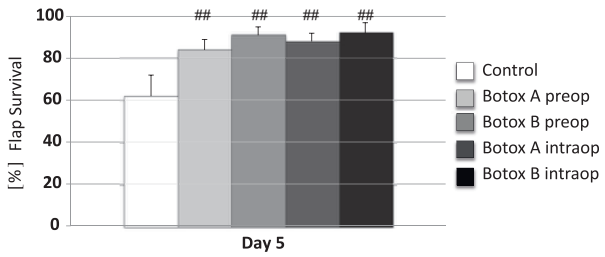


Fig. 4 – Flap survival. Comparison of percentages of vital flap tissue in experimental groups on day 5. $^{\#\#}P < 0.01$ *versus* control.

We could show a significant improvement of flap tissue oxygenation in all BTX groups. This was paralleled by glucose values equaling the baseline values on day 1 after surgery in the BTX groups, compared with significantly reduced glucose levels in the control group. According to a previous study [13], reduction of glucose is the most sensitive metabolic value for detecting hypoxia. The most specific value, however, is the L/P ratio [13], which was comparable to physiological levels in the presented BTX groups. Therefore the preoperative and intraoperative application of BTX A or B resulted in normalization of tissue oxygen levels. The impaired tissue oxygenation and cellular metabolic state in the control resulted in promoted disintegration of cellular membranes and cell death, as demonstrated by elevated glycerol levels.

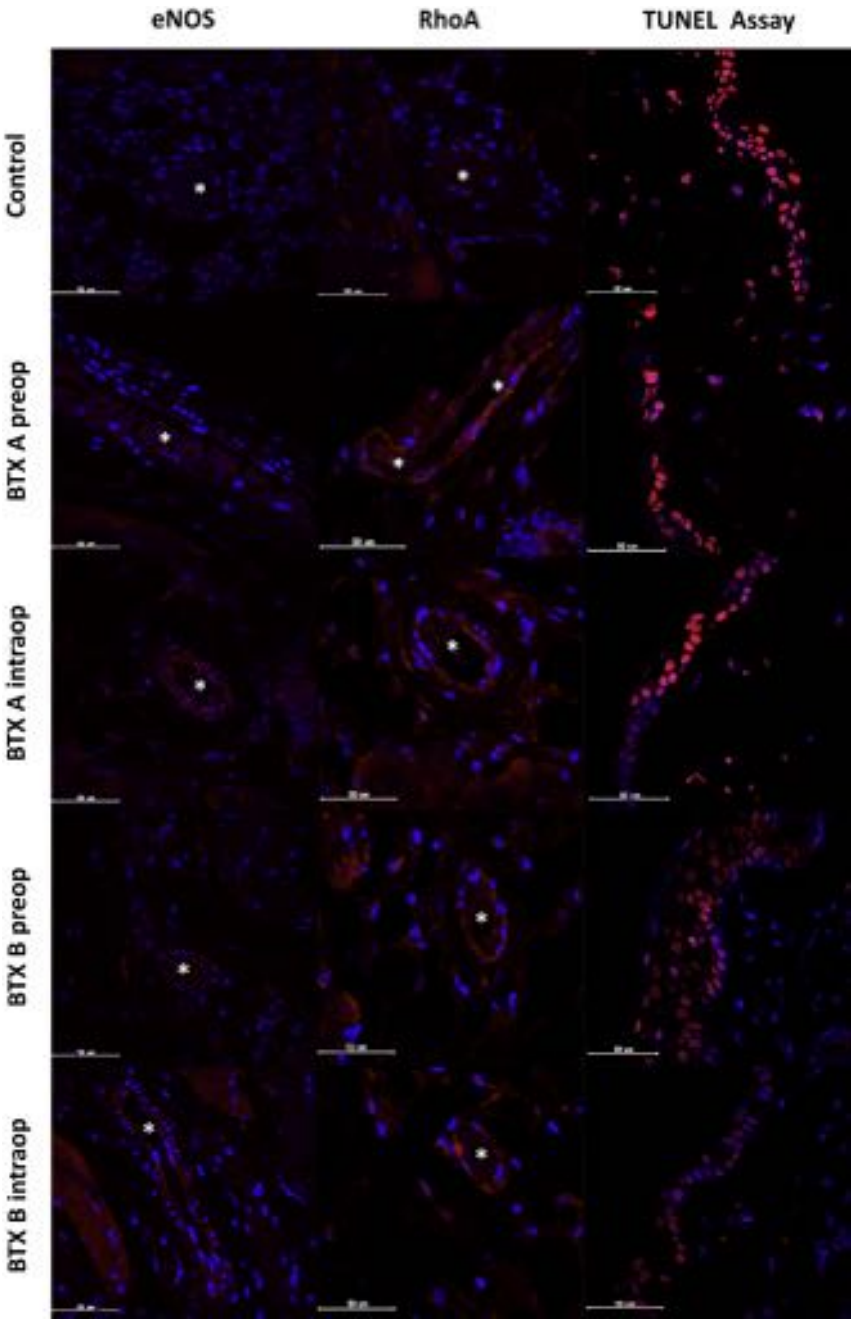


Fig. 5 – Immunofluorescence. eNOS and RhoA staining and TUNEL assay for tissue samples taken at day 5. Control, BTX A preoperative (preop) and intraoperative (intraop), BTX B preop and intraop. Asterisks (*) show the lumen of arterioles. (Color version of figure is available online.)

In our study BTX A and B revealed an increase of blood flow within 24 h after flap surgery. This early augmentation of microcirculation might be crucial for the improved outcome of flap viability. We could demonstrate an association with eNOS expression.

From a pharmacologic point of view, BTX is rapidly and irreversibly bound to the presynaptic neuron [28]. At the neuromuscular junction the release of acetylcholine is inhibited. This leads to a chemical denervation. Although there are eight serologically different types of BTX (A, B, Cα, Cβ, D, E, F, and G), the structure and function are similar [28].

The primary molecular effects of BTX are effective at the terminal end of cholinergic neurons. The BTX heavy chain specifically binds to glycoproteins at the presynaptic membrane. The light chain of both BTX agents cleaves the soluble SNARE complex, which is responsible for the release of acetylcholine from the cytosol of the axon terminal end into the synaptic cleft. This effects an irreversible chemical denervation of the muscle [29].

The vascular tonus is regulated through sympathetic neurons, which maintain a certain level of vascular tension through frequent stimulation [30]. Morris *et al.* emphasize that

Table – Quantitative data for eNOS and RhoA staining and TUNEL assay for tissue samples taken at day 5: control, BTX A preoperative and intraoperative, BTX B preoperative and intraoperative; quantitative analysis with comparison of all groups.

	eNOS [AFU]	RhoA [AFU]	TUNEL assay [AFU]
Control	20.0 ± 2.0	31.0 ± 10.2	2.2 ± 0.3
BTX A preop	27.5 ± 2.2*	52.1 ± 12.3*	1.6 ± 0.1**
BTX A intraop	33.7 ± 3.3**	67.3 ± 12.6**	2.1 ± 0.3
BTX B preop	28.9 ± 2.4**	57.3 ± 20.6**	1.4 ± 0.3**
BTX B intraop	30.9 ± 2.6**	54.8 ± 11.4**	1.7 ± 0.2*

AFU = arbitrary fluorescence units; Intraop = intraoperative; Preop = preoperative.
* $P < 0.05$.
** $P < 0.01$.

stimulation of these sympathetic vasoconstrictor neurons is likely to involve different calcium channels [31]. This exocytosis also involves other co-transmitters such as norepinephrine and neuropeptide Y or regulatory proteins such as calcium-dependent activator protein for secretion, which selectively leads to exocytosis of larger vesicles [31–33]. Yet there is no definitive clue as to how BTX would influence exocytosis, as some subtypes of calcium channels have also been demonstrated to be insensitive to BTX [31].

As demonstrated previously, BTX interacts with the Rho/Rho kinase. BTX inactivates Rho kinase and inhibits smooth muscle cell constriction directly through interference with the Ca^{2+} sensitivity of vascular smooth muscle cells and the NO system [34,35], which leads to vasodilation. The efficacy of BTX treatment on tissue perfusion, oxygen delivery, and increased tissue survival can therefore be derived from its indirect regulation of the vascular tonus. As we could also demonstrate RhoA expression in the BTX groups, we hypothesize that BTX inactivates RhoA only, but does not inhibit its expression as a protein. It remains speculative that RhoA is compensatorily overexpressed, due to blocking its function through BTX. An additional way of action recently discovered is that BTX A is inhibiting sympathetic vasoconstriction through attenuation of norepinephrine release in autonomic neurons [31], which might also have an effect in our setting, but was not studied.

Murakami *et al.* [36] found BTX C to prevent arterial grafts from spasm with fast onset of relaxation and long-lasting action. Under conditions of chronic critical perfusion, some groups have evaluated BTX A for Raynaud disease [6,7]. They reported a positive effect on perfusion and healing ulcers of the fingertips in a majority of patients; however, in some patients this concept was unsuccessful. Potentially these patients might be resistant to BTX A, as reported in the literature [37,38]. The significant increase of tissue survival by BTX in our own experiment is the highest reported in the literature so far. In two previous studies random pattern flaps were used. These studies could demonstrate a limited effect of BTX A on flap viability, which might be due to limitations of the model used. Random pattern perfusion might result in higher rates of necrosis with limited potential for compensatory mechanisms due to lack of larger arterioles present in the flap basis [18,19]. The model used in a third publication was an axial pattern flap model including three consecutive vascular networks [17]. No positive effect of BTX on the development of flap necrosis could be demonstrated [17]. This study was limited to a single time point endpoint and a complex flap design with second-order dependent collateralized vascular areas. The significant increase of tissue perfusion, oxygen distribution, and tissue survival in our experiments might be due to the model, with a defined spared vascular axis and therefore high potential of increased blood flow through vasodilation after direct local BTX application. Prior to future clinical applications, long-term durability of the observed effects should be investigated.

In our experiments BTX A and B showed equal efficacy. The pharmacologic advantage of BTX B with a faster onset could, however, only be demonstrated as a trend, without reaching statistical significance. Superiority of preoperative application was seen in the TUNEL assay only.

In conclusion, this is the first study to demonstrate that BTX application to the vascular pedicle of an axial pattern flap leads to significantly better flap perfusion, oxygenation, tissue metabolism, and tissue viability. In addition, we were able to demonstrate data supporting a mechanism for BTX A and B through the Rho/Rho kinase system and eNOS expression.

Acknowledgment

Drs Rieben, Eberli, Erni, and Plock are recipients of research grants from the Swiss National Science Foundation (SNF No. 122222, 135272, 100272).

The authors are thankful to Ms C. Fleurkens and Ms J. Denoyelle for technical assistance.

REFERENCES

[1] Maria G, Brisinda G, Bentivoglio AR, Cassetta E, Gui D, Albanese A. Botulinum toxin injections in the internal anal sphincter for the treatment of chronic anal fissure: long-term results after two different dosage regimens. Ann Surg 1998; 228:664.
[2] Schulte-Baukloh H. [Botulinum toxin for neurogenic bladder dysfunction]. Urologe A 2012;51:198.
[3] Patel S, Martino D. Cervical dystonia: from pathophysiology to pharmacotherapy. Behav Neurol 2013;26:275.
[4] Winner PK, Sadowsky CH, Martinez WC, Zuniga JA, Poulette A. Concurrent onabotulinumtoxinA treatment of cervical dystonia and concomitant migraine. Headache 2012; 52:1219.
[5] Olver J, Esquenazi A, Fung VS, Singer BJ, Ward AB. Botulinum toxin assessment, intervention and aftercare for lower limb disorders of movement and muscle tone in adults: international consensus statement. Eur J Neurol 2010; 17(Suppl 2):57.
[6] Sycha T, Graninger M, Auff E, Schnider P. Botulinum toxin in the treatment of Raynaud's phenomenon: a pilot study. Eur J Clin Invest 2004;34:312.
[7] Neumeister MW. Botulinum toxin type A in the treatment of Raynaud's phenomenon. J Hand Surg Am 2010;35:2085.
[8] Aoki KR. Pharmacology and immunology of botulinum neurotoxins. Int Ophthalmol Clin 2005;45:25.

[9] Dressler D, Benecke R. Pharmacology of therapeutic botulinum toxin preparations. Disabil Rehabil 2007;29:1761.
[10] Schlosser S, Wirth R, Plock JA, Serov A, Banic A, Erni D. Application of a new laser Doppler imaging system in planning and monitoring of surgical flaps. J Biomed Opt 2010; 15:036023.
[11] Schweizer R, Merz K, Schlosser S, et al. Morphology and hemodynamics during vascular regeneration in critically ischemic murine skin studied by intravital microscopy techniques. Eur Surg Res 2011;47:222.
[12] Plock JA, Rafatmehr N, Sinovcic D, et al. Hemoglobin vesicles improve wound healing and tissue survival in critically ischemic skin in mice. Am J Physiol Heart Circ Physiol 2009; 297:H905.
[13] Contaldo C, Plock J, Djonov V, Leunig M, Banic A, Erni D. The influence of trauma and ischemia on carbohydrate metabolites monitored in hamster flap tissue. Anesth Analg 2005;100:817. table of contents.
[14] Plock JA, Tromp AE, Contaldo C, et al. Hemoglobin vesicles reduce hypoxia-related inflammation in critically ischemic hamster flap tissue. Crit Care Med 2007;35:899.
[15] Vihanto MM, Plock J, Erni D, Frey BM, Frey FJ, Huynh-Do U. Hypoxia up-regulates expression of Eph receptors and ephrins in mouse skin. FASEB J 2005;19:1689.
[16] Ross SM. Peirce's criterion for the elimination of suspect experimental data. J Eng Tech 2003;Fall 2003.
[17] Arnold PB, Merritt W, Rodeheaver GT, Campbell CA, Morgan RF, Drake DB. Effects of perivascular Botulinum Toxin-A application on vascular smooth muscle and flap viability in the rat. Ann Plast Surg 2009;62:463.
[18] Kim TK, Oh EJ, Chung JY, Park JW, Cho BC, Chung HY. The effects of botulinum toxin A on the survival of a random cutaneous flap. J Plast Reconstr Aesthet Surg 2009;62:906.
[19] Kim YS, Roh TS, Lee WJ, Yoo WM, Tark KC. The effect of botulinum toxin A on skin flap survival in rats. Wound Repair Regen 2009;17:411.
[20] Aoki KR. A comparison of the safety margins of botulinum neurotoxin serotypes A, B, and F in mice. Toxicon 2001;39:1815.
[21] Kranz G, Paul A, Voller B, et al. Long-term efficacy and respective potencies of botulinum toxin A and B: a randomized, double-blind study. Br J Dermatol 2011;164:176.
[22] Dressler D, Adib Saberi F, Benecke R. Botulinum toxin type B for treatment of axillar hyperhidrosis. J Neurol 2002;249:1729.
[23] Flynn TC. Myobloc. Dermatol Clin 2004;22:207. vii.
[24] Ward AB. Spasticity treatment with botulinum toxins. J Neural Transm 2008;115:607.
[25] Costa J, Espirito-Santo C, Borges A, et al. Botulinum toxin type B for cervical dystonia. Cochrane Database Syst Rev 2005;4:CD004315.
[26] Flynn TC, Clark RE 2nd. Botulinum toxin type B (MYOBLOC) versus botulinum toxin type A (BOTOX) frontalis study: rate of onset and radius of diffusion. Dermatol Surg 2003;29:519. discussion 522.
[27] Yamauchi PS, Lowe NJ. Botulinum toxin types A and B: comparison of efficacy, duration, and dose-ranging studies for the treatment of facial rhytides and hyperhidrosis. Clin Dermatol 2004;22:34.
[28] Klein AW. Complications, adverse reactions, and insights with the use of botulinum toxin. Dermatol Surg 2003;29:549. discussion 556.
[29] Dressler D, Saberi FA, Barbosa ER. Botulinum toxin: mechanisms of action. Arq Neuropsiquiatr 2005;63:180.
[30] Jänig W. Pre- and postganglionic vasoconstrictor neurons: differentiation, types, and discharge properties. Annu Rev Physiol 1988;50:525.
[31] Morris JL, Jobling P, Gibbins IL. Botulinum neurotoxin A attenuates release of norepinephrine but not NPY from vasoconstrictor neurons. Am J Physiol Heart Circ Physiol 2002;283:H2627.
[32] Langley K, Grant NJ. Are exocytosis mechanisms neurotransmitter specific? Neurochem Int 1997;31:739.
[33] Tandon A, Bannykh S, Kowalchyk JA, Banerjee A, Martin TFJ, Balch WE. Differential regulation of exocytosis by calcium and CAPS in semi-intact synaptosomes. Neuron 1998;21:147.
[34] McMurtry IF, Abe K, Ota H, Fagan KA, Oka M. Rho kinase-mediated vasoconstriction in pulmonary hypertension. Adv Exp Med Biol 2010;661:299.
[35] Fukata Y, Kimura K, Oshiro N, Saya H, Matsuura Y, Kaibuchi K. Association of the myosin-binding subunit of myosin phosphatase and moesin: dual regulation of moesin phosphorylation by Rho-associated kinase and myosin phosphatase. J Cell Biol 1998;141:409.
[36] Murakami E, Iwata H, Imaizumi M, Takemura H. Prevention of arterial graft spasm by botulinum toxin: an in-vitro experiment. Interact Cardiovasc Thorac Surg 2009;9:395.
[37] Borodic G. Immunologic resistance after repeated botulinum toxin type a injections for facial rhytides. Ophthal Plast Reconstr Surg 2006;22:239.
[38] Greene P, Fahn S, Diamond B. Development of resistance to botulinum toxin type A in patients with torticollis. Mov Disord 1994;9:213.

Overall discussion and outlook

The present thesis demonstrates that activation of EC and the plasma cascade systems i.e. complement, coagulation, fibrinolysis plays an important role in the pathogenesis of peripheral I/R injury in skeletal muscle and bone. C1 inhibitor (C1 INH), a multifunctional serine protease inhibitor, significantly reduced EC activation as well as activity of the complement, coagulant and fibrinolytic systems in peripheral I/R injury. Furthermore, a simple in vitro system was established to grow and investigate EC under physiological flow conditions in the context of xenotransplantation. It was feasible to reproduce key findings of complement activation-mediated hyperacute rejection in xenotransplantation models. Complement inhibitors C1 INH, APT070, and DXS prevented activation of complement and EC in a xenotransplantation setting.

Activation of EC and the plasma cascades in I/R injury (Paper II and III) was consistent with our previous reperfusion injury studies on skeletal muscle and heart I/R [1, 2]. In particular, activity of the fibrinolytic cascade was down regulated in reperfusion injury models of hind limbs and tibia bones as reflected by expression of PAI-1 and tPA in tissue. Thereby, endothelium was transformed into a proinflammatory, pro-coagulant, and anti-fibrinolytic phenotype in I/R injury. Pre-treatment with C1 INH significantly reduced I/R injury by preventing endothelial heparan sulfate shedding and inhibiting activation of the plasma cascades in our rat hind limb I/R injury with 2h ischemia and 24h reperfusion. C1 INH may therefore be a promising strategy to prevent I/R injury in the clinical setting of elective surgery on extremities, including replantation or transplantation.

Also activation of EC and the complement cascades (Paper IV) was confirmed in our pig-to-human xenoperfusion study, which was performed in a 3D microfluidic system under physiological flow conditions, clinically mimicking some aspects of pig-to-primate xenotransplantation. Our data were in line with the previous findings by our own group in vitro on PAEC and ex vivo in pig limbs perfused with human blood [3, 4]. The novelty of this microfluidic system is its three-dimensional structure mimicking small to medium sized arteries and its ability to culture PAEC under physiological shear flow conditions. More importantly, the ratio of EC surface to blood volume is 120 cm^2/ml, which would allow the exploitation of the natural, anticoagulant properties of EC when working with non-anticoagulated whole blood.

The fraction of inspired oxygen (FiO_2) (Paper I) is a factor that influences the outcome of peripheral I/R injury. We found that 40% FiO_2 led to a more severe and more physiologically relevant reperfusion injury. Higher FiO_2 in the anesthetic gas provided a certain protection for

I/R injury, as reported in an earlier study [5]. This was probably caused by a decreased vasodilator response during hind limb reperfusion with 100% FiO_2 in the anesthetic, leading to slower reperfusion and thus a lesser extent of I/R injury [6, 7]. Based on this finding, 40-50% FiO_2 was used throughout our animal experiments.

In addition, the effect of botulinum toxin (BTX) A and B (Paper V) was investigated in a critically ischemic skin flap model. Both pre-operative and intra-operative application of BTX A and B to the vascular pedicle of an axial pattern flap leads to significantly better flap perfusion, oxygenation, tissue metabolism, and tissue viability. The mechanisms of BTX A and B occurred through the Rho/Rho kinase system and eNOS expression.

Taken together, plasma cascades and EC activation play an important role in the pathogenesis of I/R injury as well as xenotransplantation. Therapeutics targeting both plasma cascades and EC activation by inhibitors, or combinations of inhibitors of the plasma cascades and/or EC activation, but not completely, may be promising candidates to ameliorate or even prevent I/R injury. In addition, our newly established in vitro system mimics physiological conditions in vivo, with the potential for exploring underlying mechanisms and drug screening, for therapeutics in xenotransplantation, atherosclerosis and many other disease models. Within the microchannels the ratio of EC-surface to blood volume is 120 cm^2/ml, which would allow exploitation of the natural, anticoagulant properties of EC when working with non-anticoagulated whole blood. Compared to conventional 96-well plate assays our system provides a >350 times larger ratio of fluid volume exposure to EC surface, equaling in vivo conditions. In the future, coating of silicon tubing and connectors with heparin might allow the use of whole, non-anticoagulated blood for perfusion of the EC-microchannels and further improve the model. Also we can culture and analyze complement regulatory transgenic PAEC such as human CD46 (hCD46), CD55, CD59, HLA-E, human thrombomodulin (hTM) variants in this system to explore their potential functions in a pig-to-human xenotransplantation setting with reduced numbers of animals used in in-vivo experiments.

References

1. C. Duehrkop, Y. Banz, R. Spirig, S. Miescher, M. W. Nolte, M. Spycher, R. A. Smith, S. H. Sacks, and R. Rieben, "C1 esterase inhibitor reduces lower extremity ischemia/reperfusion injury and associated lung damage," *PloS One,* vol. 8, no. 8, pp. e72059, 2013.
2. Y. Banz, O. M. Hess, S. C. Robson, D. Mettler, P. Meier, A. Haeberli, E. Csizmadia, E. Y. Korchagina, N. V. Bovin, and R. Rieben, "Locally targeted cytoprotection with dextran sulfate attenuates experimental porcine myocardial ischaemia/reperfusion injury," *European Heart Journal,* vol. 26, no. 21, pp. 2334-43, 2005.
3. A. K. Bongoni, D. Kiermeir, H. Jenni, A. Wunsch, A. Bahr, D. Ayares, J. D. Seebach, E. Wolf, N. Klymiuk, M. A. Constantinescu, E. Vogelin, and R. Rieben, "Activation of the lectin pathway of complement in pig-to-human xenotransplantation models," *Transplantation,* vol. 96, no. 9, pp. 791-9, 2013.
4. A. K. Bongoni, D. Kiermeir, H. Jenni, A. Bahr, D. Ayares, N. Klymiuk, E. Wolf, E. Voegelin, M. A. Constantinescu, J. D. Seebach, and R. Rieben, "Complement dependent early immunological responses during ex vivo xenoperfusion of hCD46/HLA-E double transgenic pig forelimbs with human blood," *Xenotransplantation,* vol. 21, no. 3, pp. 230-43, 2014.
5. I. Sukhotnik, V. Brod, M. Lurie, M. A. Rahat, S. Shnizer, N. Lahat, J. G. Mogilner, and H. Bitterman, "The effect of 100% oxygen on intestinal preservation and recovery following ischemia-reperfusion injury in rats," *Critical Care Medicine,* vol. 37, no. 3, pp. 1054-61, 2009.
6. P. Crawford, P. A. Good, E. Gutierrez, J. H. Feinberg, J. P. Boehmer, D. H. Silber, and L. I. Sinoway, "Effects of supplemental oxygen on forearm vasodilation in humans," *J Appl Physiol (1985),* vol. 82, no. 5, pp. 1601-6, 1997.
7. D. L. Bredle, W. E. Bradley, C. K. Chapler, and S. M. Cain, "Muscle perfusion and oxygenation during local hyperoxia," *J Appl Physiol (1985),* vol. 65, no. 5, pp. 2057-62, 1988.

Acknowledgements

I am very grateful for all the people who have helped me during my MD-PhD study. First of all, I wish to express my utmost gratitude for my supervisor Prof. Dr. Robert Rieben for his scientific guide, continuous support, and spiritual encouragement throughout my thesis. He gave me the chance to work in his lab, which enriches my scientific life for my MD and brings me to the world with both research and clinical thoughts.

I also wish to thank PD Dr. med. Jan A Plock and Dr. Deborah Stroka, who agreed to accept as my co-advisor and mentor, respectively.

Guide and support received from collaborators namely Drs. Jan A Plcok, Yara Banz, Rolf Spirig, Marc Nolte, Carlos Wotzkow, Peter J. Cowan, Willy Hofstetter, Oliver Thierry Guenat, Fabian Blank, Mark Siegrist, Sylvia Miescher, Anders Waas, and Richard Smith, contributed a lot to the projects during my MD-PhD. I am very grateful for their collaboration and support.

I sincerely wish to express my deepest thanks for help and support from our group colleagues namely Dr. Adriano Taddeo, Mai Moustafa Abdelhafez, Riccardo Sfriso, Dzhuliya Dzhonova, Jane Shaw-Boden, Rahel Klosser, Oliver Steck, Alain Despont, Yvonne Roschi, and Sai Pavan Garimella. Also my wholehearted thanks go to my former colleagues namely Drs. Claudia Dührkop, Anjan Bongoni, Pranitha Kamat, Thusitha Gajanayake, and Julie Denoyelle. In particular, I want to express my sincere gratitude for Jane Shaw-Boden, who consistently and fully supports me throughout the thesis.

Finally, special thanks go to my family including my parents, my sisters, and my brother, as well as my fiance (Liangang Xiao), who always support me to do what I want and consistently encourage me to positively overcome all the difficulties in life and work.

Curriculum vitae

Personal Details

Name	Shengye Zhang
Date of Birth	08.07.1981
Nationality	China
Language	Mandarin, English

Education Background

Period	Where	Degree	Field of Study
2011 - 2016	University of Bern Switzerland	MD-PhD	Immunology
2008 - 2011	University of Lanzhou China	Master	Internal Medicine
2003 - 2008	Medical University of Binzhou China	Bachelor	Clinical Medicine

Education & Training

Year	Course / Training
2015	Advanced course in experimental microsurgery European Meeting on Complement (Uppsala, Sweden) Course flow cytometry Scientific communication (University of Bern) Scientific writing (University of Bern) Swiss Experimental Surgery symposium (SESS)
2014	Continuing training for performance in animal experiments (SGV meeting)

Swiss Experimental Surgery symposium (SESS)

2013 European Meeting on Complement (Jena, Germany)
Basic statistics and analysis of small and large data set
Swiss Experimental Surgery symposium (SESS)
Continuing training for performance in animal experiments (SGV meeting)
Vascular Cell Biology

2012 Confocal microscopy
Book Club – Cellular and Molecular Immunology
LTK1 – Introductory Course in Laboratory Animal Science
Swiss Experimental Surgery symposium (SESS)

Poster presentations at national and international meetings

Shengye Zhang, Claudia Duehrkop, Jan Plock, and Robert Rieben. Effect of the fraction of inspired oxygen on hind limb ischemia/reperfusion injury in a rat model – 10th Swiss Experimental Surgery symposium (SESS) – Fribourg, Switzerland, 2015

Shengye Zhang, Claudia Duehrkop, Jan Plock, and Robert Rieben. Effect of the fraction of inspired oxygen on complement activation in a model of rat hind limb ischemia/reperfusion injury. 15th European meeting on complement in human disease – Uppsala, Sweden, 2015

Shengye Zhang, Carlos Wotzkow, Mark Siegrist, Willy Hofsetter, Robert Rieben. Role of the complement system in a rat model of bone ischemia/reperfusion injury. 15th European meeting on complement in human disease – Uppsala, Sweden, 2015

Shengye Zhang, Rahel Klossner, Colette Andrea Bichsel, Oliver Thierry Guenat, Robert Rieben. Investigation of complement and coagulation cascades in a vessel-like microfluidic system under physiological flow conditions - 15th European meeting on complement in human disease – Uppsala, Sweden, 2015

Scholarship

Period	Scholarship
2011 - 2015	China Scholarship Council (CSC)
2008 - 2011	Yearly excellent graduate scholarship
2006	Excellent national undergraduate scholarship
2003 - 2006	Second prize of campus scholarship

Skills/methods

- In vivo experiments
  - Rat hind limb ischemia/reperfusion injury
  - Establishing animal models
  - Laser Doppler Imaging (Moor LDI)
  - Intra vital Microscopy
- Tissue engineering (Construction of circular microchannels mimicking small to medium sized vessels)
- Cell culture (PAEC, PJVEC, HUVEC)
- Immunofluorescence, immunohistochemistry, histology
- Microscopic analysis by using confocal microscopy
- Microscopic analysis by using immunofluorescence and light microscopy
- ELISA
- Western blot
- CH50
- Basics in flow cytometry
- Basics in Bio-Plex
- Basics in R (Language and environmental for statistical computing and graphics)
- Certain Clinical skills

List of Publications

1. **Shengye Zhang**, Claudia Duehrkop, Jan A. Plock, Robert Rieben. Inhalation anesthesia of rats: Influence of the fraction of inspired oxygen on limb ischemia/reperfusion injury. Lab Anim. 2015 Sep 7. pii: 0023677215604531.
2. Riccardo Schweizer, Pranitha Kamat, Dennise Schweize, Cyrill Dennler, **Shengye Zhang**, Jonus T. Schnider, Souzan Salemi, Pietro Giovanoli, Daniel Eberli, Volker Enzmann, Dominique Erni, Jan A. Plock. Bone marrow-derived mesenchymal stromal cells improve vascular regeneration and reduce leukocyte-endothelium activation in critical ischemic murine skin in a dose-dependent manner. Cytotherapy. 2014. S1465-3249(14)00606-9
3. Dennis F.Schweizer, Riccardo Schweizer, **Shengye Zhang**, Pranitha Kamat, Claudio Contaldo, Robert Rieben, Daniel Eberli, Pietro Giovanoli, Dominique Erni, Jan A. Plock. Botulinum toxin A and B raise blood flow and increase survival of critically ischemic skin flaps. Journal of Surgical Research. 2013, 184:1205–1213
4. **Shengye Zhang**, Jane Shaw-Boden, Yara Banz, Anjan K. Bongoni, Adriano Taddeo, Rolf Spirig, Peter J. Cowan, Robert Rieben. Effects of C1 INH on endothelial cell activation in a rat hind limb ischemia / reperfusion injury model. In submission to Mediators of Inflammation
5. **Shengye Zhang**, Carlos Wotzkow, Anjan K. Bongoni, Jane Shaw-Boden, Mark Siegrist, Adriano Taddeo, Fabian Blank, Willy Hofstetter, Robert Rieben. Role of the plasma cascade systems in ischemia/reperfusion injury of bone. In submission to Bone
6. **Shengye Zhang**, Colette Andrea Bichsel, Rahel Klossner, Oliver Steck, Alain Despont, Oliver Thierry Guenat, Robert Rieben. 3D culture and analysis of endothelial cells under physiological flow conditions in a xenotransplantation setting. In submission to ALTEX
7. **Shengye Zhang**, Yan Huang, Qian Zhao, Huan Niu, Yijia Li, Hongtao Yin. Effects of aplelin-13 on the rat model of adriamycin-induced heart failure and its relation with nitric oxide pathway [J]. Chinese Journal of Geriatric Heart Brain and Vessel Diseases, 2011,13(5), 452-455 *(In Chinese)*
8. **Shengye Zhang**, Yan Huang, Jin Zhang, Huan Niu, Qian Zhao, Yijia li. Dynamic

change of endogenous erythropoietin after intervention of acute myocardial infarction, as well as the function of cardiovascular protection and anti-inflammation [J]. The Journal of Practical Medicine, 2011, 27(17), 3124 - 3127*(In Chinese)*

9. **Shengye Zhang**, Yan Huang, Jin Zhang, Huan Niu, Qian Zhao, Yijia li. Dynamic Change of erythropoietin and hemorheology in patients with CHD before and after treatment and effect on blood viscosity [J]. Chinese Journal of Geriatric Heart Brain and Vessel Diseases, 2011,13(10), 870 – 873 *(In Chinese)*
10. Qian Zhao, Yan Huang, **Shengye Zhang**, Huan Niu. Effect of Exogenous Apelin-13 on Expression of Plasma Angiotensin-II and Adrenomedulin in Heart Failure Rats [J]. Progress in Modern Biomedicine, 2011,11(8), 1464 -1466 *(In Chinese)*
11. Huan Niu, Yan Huang, **Shengye Zhang**, Qian Zhao. bFGF increases expression of β-catenin in the damaged cardiac fibroblasts induced by hypoxia / reoxygenation [J]. Basic and Clinical Medicine, 2010,30(10), 1085-1088 *(In Chinese)*

Declaration of Originality

Last name, first name: Zhang Shengye

Matriculation number: 11-117-157

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.

All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.

I am aware that in case of non-compliance, the Senate is entitled to withdraw the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 69, of 7 June 2011.

Place, date

Bern, 18.03.2016

Signature

Shengye Zhang