Inflammatory activation after experimental limb ischemia-reperfusion.

The possibilities of therapy

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CONTENTS

LIST OF ABBREVIATIONS

DEX	dextran
GEL	gelatine
HES	hydroxyethyl-starch 130/0.4
ICAM-1	intercellular adhesion molecule-1
IPC	ischemic preconditioning
I-R	ischemia-reperfusion
IVM	intravital videomicroscopy
MAP	mean arterial pressure
MPO	myeloperoxidase
PC	phosphatidylcholine
PMN	polymorphonuclear leukocyte
ROI	reactive oxygen intermediate
TNF-α	tumor necrosis factor-alpha

LIST OF FULL PAPERS RELATING TO THE SUBJECT OF THE THESIS

Varga R, Gera L**,** Török L, Kaszaki J, Szabó A, Nagy K, Boros M: Effects of phosphatidylcholine therapy after hindlimb ischemia and reperfusion. *Magyar Sebészet* 59(6):429-36, 2006.

Gera L, **Varga R**, Török L, Kaszaki J, Szabó A, Nagy K, Boros M: Beneficial effects of phosphatidylcholine during hindlimb reperfusion. *Journal of Surgical Research* 139(1):45-50, 2007. IF (2007): 1.836

Varga R, Török L*****, Szabó A, Kovács F, Keresztes M, Varga G, Kaszaki J, Boros M: Effects of colloid solutions on ischemia-reperfusion-induced periosteal microcirculatory and inflammatory reactions: comparison of dextran, gelatin, and hydroxyethyl starch. *Critical Care Medicine* 36(10):2828-2837, 2008. IF (2007): 6.283 *** = Equal contribution**

Szabó A, **Varga R**, Keresztes M, Vízler C, Németh I, Rázga Z, Boros M: Ischemic limb preconditioning downregulates systemic inflammatory activation. *Journal of Orthopedic Research* (2009, accepted for publication) IF (2007): 2.437

Abstracts relating to the subject of the thesis

Varga R, Gera L, Kaszaki J, Szabó A, Ghyczy M, Boros M: Phosphatidylcholin treatment improves the periosteal microcirculation following hindlimb ischemia and reperfusion. *European Surgical Research* 36:S14, 2004.

Kaszaki J, **Varga R**, Török L, Szabó A, Boros M: Effects of colloids on the periosteal microcirculation after ischemia-reperfusion. *European Surgical Research* 37:S1, 2005.

Varga R, Szabó A, Keresztes M, Kaszaki J, Németh I, Rázga Zs, Boros M: Inflammatory changes after short-term ischemic preconditioning of the rat hindlimb. *European Surgical Research* 39:S57, 2007.

1. INTRODUCTION

1.1. Free radicals and reperfusion injury

Local transient limb ischemia occurs in different traumatological cases (fractures) and during therapeutic interventions (e.g. tourniquet method or flap-surgery). In general, ischemia-reperfusion (I-R) initiates a cascade of pathophysiological events which in turn enhance local and remote tissue injury. Ischemia is a state of tissue oxygen deprivation accompanied by an anoxic injury and a decrease in mitochondrial energy production (ATP synthesis, oxidative phosphorylation) and thus a fall in cellular energy (ATP) content. Despite the unequivocal benefit of reperfusion of blood to an ischemic tissue, reperfusion itself can elicit a cascade of adverse reactions that paradoxically injure tissue. Reperfusion of the ischemic tissues with oxygen-rich blood induces a cascade of chemical reactions and cellular events (Parks DA *et al.* 1986). Cell injury upon reperfusion, especially in the early reperfusion phase, may be a direct consequence of intracellular alterations that occurred in the ischemic phase. For instance, in the ischemic phase electron-transferring enzymes, such as those of the mitochondrial respiratory chain, may be damaged. Upon reperfusion of the still viable cells, the reduction of molecular oxygen to form toxic metabolites (superoxide, hydrogen peroxide and hydroxyl radical) appears to be a key chemical event (Granger DN *et al.* 1981). Once formed in postischemic tissues, the reactive oxygen intermediates (ROI) cause oxidation of nucleic acids, enzymes, receptors and membrane lipids (Dizdaroglu M *et al.* 1993, Cochrane CG *et al.* 1991). Indeed, ROI have been implicated in several toxic pathways, including damage to cellular lipids, proteins, and DNA (Freeman BA *et al.* 1982, Jones DP 2008). Furthermore, due to the impaired oxygen consumption, oxidative phosphorylation and metabolic functions are deranged (Martin GP *et al.* 1987). The two potential sources of ROI that have received most attention are xanthine oxidase (Granger DN *et al.* 1986) (found in endothelial and different epithelial cells) and NADPH oxidase (Grisham MB *et al.* 1986)

(found in phagocytic cells such as neutrophils). Xanthine oxidase, a rate-limiting enzyme in nucleic acid degradation, possesses the ability to generate both hydrogen peroxide and superoxide when converted from its xanthine dehydrogenase form in ischemic tissue.

Reperfusion phase also initiates an inflammatory response which is triggered by constituents of the damaged cells (cell debris) or by the disrupted tissue matrix via the activation of the complement cascade or of macrophages, endothelial, dendritic, and other cells. A major characteristic of these reactions is the recruitment of various inflammatory cells, including polymorphonuclear leukocytes (PMNs) into the formerly ischemic tissues. Once adhering to the endothelial surface, PMNs become activated and release a variety of enzymes (NADPH oxidase, myeloperoxidase; MPO), elastase and collagenase that injure these cells and neighboring parenchymal cells.

1.2. Microcirculatory inflammatory reactions during ischemia-reperfusion injury

PMN adhesion to the endothelium is significantly enhanced within minutes after the onset of reperfusion and remains elevated for hours (Harris AG *et al.* 1996). Several adhesion molecules present on the surface of PMNs and/or endothelial cells, such as immunoglobin-like adhesion receptors (ICAM-1, PECAM-1, VCAM-1), integrins (CD11/CD18), and selectins (E-, P-, L-selectin), are involved in the multistep process of PMN accumulation and emigration (Eppihimer MJ *et al.* 1997, Springer TA 1990). Soluble mediators released during I-R exert chemotactic actions and direct PMN accumulation to the site of injury. These chemotactic substances include proinflammatory cytokines/chemokines such as interleukins, tumor necrosis factor-alpha (TNF-α), platelet-activating factor, and leukotrienes released by the postischemic endothelium but also by resident leukocytes and non-leukocyte cells (Wanner GA *et al.* 1996, Serrick A *et al.* 1994, Kurose I *et al.* 1997). In addition, radicals generated during reperfusion and the reintroduction of molecular oxygen are believed to contribute significantly to I-R-induced microvascular dysfunction. Both cytokines and ROI

increase the expression of adhesion molecules by initiating surface mobilization of preformed adhesion proteins from intracellular stores (see later). In addition, oxidants and cytokines induce gene transcription and *de novo* synthesis of adhesion molecules by activating the nuclear transcription factor AP-1 or NFκB, (Roebuck KA *et al.* 1995, Ichikawa H *et al.* 1997, Kacimi R *et al.* 1998, Sawa Y *et al.* 1997) altogether leading to PMN accumulation. Upon adhesion/activation, recruited PMNs in turn release ROI and proinflammatory mediators (e.g. PAF, interleukins, leukotrienes), thus initiating a vicious circle at the level of the microcirculation. PMNs are thought to be important players in the manifestation of microvascular and tissue injury. The degree of tissue damage during I-R strongly correlates with the number of recruited PMNs (Harris AG *et al.* 1996). At the same time, prevention of PMN accumulation in postischemic tissues by depletion with anti-neutrophil serum or leukocyte filters results in a marked attenuation of reperfusion injury (Korthuis RJ *et al.* 1988). The mechanism of PMN-caused tissue injury is less clear. The impairment of reflow by capillary plugging, the production of oxidants, increased permeability of the vascular endothelial barrier as a result of PMN adhesion and emigration and in particular, the release of proinflammatory mediators and proteolytic enzymes by PMNs favoring leakage of macromolecules are thought to play predominant roles (Harris AG *et al.* 1997, Kurose I *et al.* 1994a).

1.3. The roles of different adhesion molecule expression and endothelial dysfunction in the ischemia-induced microcirculatory inflammatory changes

The inflammatory aspect of I-R injury relies on the role of PMNs and leukocyte adhesion molecules in the I-R-related events (see Figure 1). The initial step during reperfusion is characterized by a loss of endothelium-derived nitric oxide, rapidly leading (i.e. within 2–5 min) to endothelial dysfunction (Tsao PS *et al.* 1990). The next step in the reperfusion process is up-regulation of the expression of adhesion P-selectin on the endothelial surface of the

affected area 10–20 min following reperfusion (Weyrich AS *et al.* 1995). This leads to increased number of PMNs to the dysfunctional and selectin up-regulated endothelium (Ma XL *et al.* 1993). The selectin family of leukocyte adhesion molecules consists of three known members: L, P, and E-selectins, respectively, each type plays key role in the cell adhesion cascade of inflammation. Basically, the selectins initiate PMN rolling along the endothelium, the first step in PMN recruitment to activated endothelium at a site of tissue injury. L-selectin is a cell surface glycoprotein expressed constitutively on a wide variety of leukocytes. L-selectin plays a role in the emigration of lymphocytes into peripheral lymph nodes and sites of chronic inflammation and of PMNs into acute inflammatory sites. P-selectin is cell surface glycoprotein that also plays a critical role in the emigration of PMNs into tissues. P-selectin is constitutively stored in the Weibel-Palade bodies of endothelial cells and in the alpha granules of platelets (Lorant DE *et al.* 1991). It is expressed on the cell surface within minutes after exposure to stimuli such as thrombin. E-selectin expression is largely restricted to endothelial cells activated by different stimuli such as endotoxin and the pro-inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF) (Chamoun F *et al.* 2000).

In areas of inflamed or injured tissue, PMN adhesion and transendothelial migration are observed in the postcapillary venules rather than in the precapillary arterioles (Atherton A *et al.* 1973, Ley K *et al.* 1991). To emigrate into tissue, PMNs initially tether to and roll on the vascular endothelium. This relatively loose adhesion is followed by firmer adhesion mediated through integrin adhesion molecules (e.g., CD18) and their receptors (e.g., intercellular adhesion molecule-1, ICAM-1). The primary sequences of ICAM-1, ICAM-2, VCAM-1, and PECAM-1 revealed that these molecules belong to the immunoglobulin (Ig) supergene family of cell surface molecules (Beekhuizen H *et al.* 1993, Springer TA 1990, Williams AF *et al.* 1988). All Ig superfamily members have a variable number of repetitive extracellular Ig-like domains, followed by a transmembrane domain and a short cytoplasmic

sequence. The Ig-like domains specifically recognize heterodimeric glycoproteins, designated as integrins. PMN-specific integrins - are composed of specific α chains (CD11) and a common β2 chain which exclusively expressed on the PMNs - are required for neutrophil adhesion and transmigration across the activated endothelium. ICAM-1 is abundantly expressed on the endothelium, where it interacts with neutrophils via CD11a/CD18 (LFA-1) or CD11b/CD18 (Mac-1), respectively (Springer TA 1990). CD11a/CD18 is expressed on the PMNs constitutively, while the expression of the latter one is induced by ischemia-reperfusion injury (Table 1).

Although the microcirculatory inflammatory consequences of many locations of I-R are well characterized, periosteal changes in response to I-R are not nearly so well characterized. For this reason, we set out to characterize the postischemic periosteal microcirculatory reactions in order to create a model of clinically applied interventions, such as bone autotransplantation and tourniquet-induced circulatory reactions.

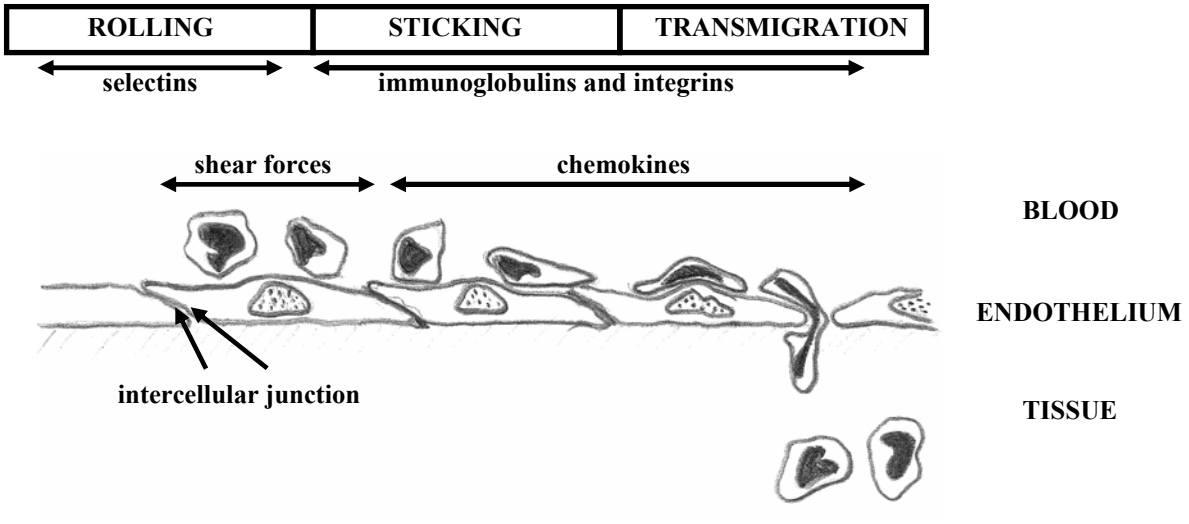

Figure 1. Scheme of PMN-endothelial interactions.

Table 1. Major ligands of primary (rolling) and secondary (firm adhesion) PMN-endothelial interactions.

Phases	Endothelial ligand	Leukocyte ligand
Rolling	Selectins/*ligands* E-selectin (endothelium) P-selectin (endothelium and platelets) *GlyCAM-1*	Selectins/*ligands* + ESL-1 + PSGL-1 + L-selectin
Adhesion (sticking)	Immunoglobulin family VCAM-1 (vascular adhesion molecule 1) ICAM-1 (intercellular adhesion molecule 1) ICAM-1	Integrins + VLA-4 = β1 integrin + LFA1 (CD11a/CD18) + MAC1 (CD11b/CD18) = β2 integrin
Transmigration	Endothelial PECAM-1 ("platelet-endothelial cell adhesion molecule" = CD31)	Leukocyte PECAM-1 = CD31

1.4. Periosteal damage in the clinical practice

As dark side of civilization, widespread motorization has led to increasing frequency of severe traumatological injuries in the last decades. Since many traumatological interventions are performed under reduced blood flow conditions (elicited by the tourniquet), limb hypoperfusion-reperfusion is a frequent consequence of these operations too. Apart from the bones, the periosteum is also involved in this process which can lead to different degrees of bone healing disorders (delayed bone healing, pseudoarthrosis or sequester formation).

Apart from fractures, the radical resection of tumors can also lead to bone defects. During such reconstructions, autotransplantation via vessel anastomoses is often used since the survival and incorporation degree of the transferred bone can be greatly enhanced by these interventions. During these procedures, the bone is subjected of iatrogenic I-R injury.

Bone healing following fractures with severe soft tissue is critically affected by impaired periosteal perfusion. The importance of periosteum is highlighted by the clinical observation where extensive soft tissue injury and periosteal stripping typically precedes delayed fracture repair. This scenario is frequently characterized by non-union or manifest

pseudarthrosis (Gustilo RB *et al.* 1984, 1990, Esterhai JL *et al.* 1991, Kowalski MJ *et al.* 1996, Utvag SE *et al.* 1998).

1.5. The effects of ischemic preconditioning on I-R injury

Ischemic preconditioning (IPC), i.e. repeated brief periods of ischemia have been shown to significantly increase the resistance to the harmful consequences of a subsequent severe ischemic insult (Murry CE *et al.* 1986). Apart from the local effects, the protection of remote tissues can also be achieved through brief periods of arterial occlusions of the intestine, kidneys and many other organs (Gho BC *et al.* 1996, Cheung MM *et al.* 2006). Although the remote effects of intra-abdominal IPC may be notable, the extremities provide better opportunities for clinical purposes. IPC of the limbs in humans can be easily performed, the risk of surgical complications is low, and moreover, the large tissue mass may theoretically provide strong defense-triggering IPC signals. Hence, we hypothesized that IPC is associated with potential therapeutic benefits against the local and distant effects of limb ischemia.

Transient limb ischemia has been shown to cause microvascular disturbances in the periosteum (Wolfárd A *et al.* 2002). Although several lines of evidence have suggested that IPC can effectively influence the perfusion of different organs (Wang WZ *et al.* 1999, Mallick IH *et al.* 2005), the periosteal microcirculatory response in this condition is as yet largely undefined.

1.6. Biological and anti-inflammatory effects of endogenous phosphatidylcholine

Phosphatidylcholine (PC) is the most common and essential membrane forming of the body. It has been shown however, that I-R is associated with physical membrane defects which results in PC degradation and the exhaustion of endogenous PC sources (Bruhl A *et al.* 2004, Jones RL *et al.* 1989, Gross RW 1992). This observation suggests that PC supplementation can be beneficial is various diseases. This is further supported by the notion

that ischemic preconditioning restores the membrane stability with the simultaneous prevention of phospholipid degradation (Bruhl A *et al.* 2004).

Stress induces the phospolipase D-catalyzed hydrolysis of membrane PC. This reaction leads to the endogenous production of phosphatidic acid and choline. Choline is a potent anti-inflammatory agent and is actively transported into the epithelial cells (Kuehl FA *et al.* 1957). Choline could also form part of a defense mechanism which may operate in biological systems against oxido-reductive stress (Ghyczy M *et al.* 2003). Furthermore, PC is taken up by phagocytic cells and accumulates in inflamed tissue (Cleland LG *et al.* 1979). *In vitro* studies have demonstrated that PC may protect against the membrane damage caused by bile salts (Martin GP *et al.* 1981, el-Hariri LM *et al.* 1992). Further, *in vivo* studies have revealed that choline is an essential nutrient for humans and a choline deficiency may result in hepatic steatosis (Zeisel SH *et al.* 1991, Buchman AL *et al.* 1995). PC provides protection against many chemical toxin-induced pathological conditions, and especially liver damage (Kidd PM 1996).

It has been shown that PC metabolites might relieve a potentially dangerous increase in the ratio of NADH / NAD^+ (reductive stress), a predisposing cause of oxidative damage (Ghyczy M *et al.* 2003). This reaction sequence could explain the still incompletely understood essential role of choline in diet, and its preventive efficacy in a number of experimentally induced pathologies associated with a redox imbalance. It may be assumed that the endogenous pool of these metabolites may become exhausted during exogenous provocation and that an exogenous supply might help to replenish and strengthen the endogenous protective mechanism.

Endogenous PC influences several pathways of the bone physiology, including the induction of bone formation (Han B *et al.* 2003), the modulation of resorption (Kwak HB *et al.* 2004) and calcification (Bonucci E *et al.* 1997). Exogenous PC likewise exerts beneficial

effects during ischemia (Duan JM *et al.* 1990), but its role in I-R-related microvascular changes is yet undefined. For this reason we aimed at modulating the limb I-R-induced microcirculatory alteration by PC supplementation.

1.7. Treatments of macro- and microcirculatory injury by fluid therapy

The main purpose of fluid resuscitation is effective and sustained restoration of the tissue perfusion. Attempts to achieve this goal are currently made by the administration of a variety of crystalloid and colloid solutions or an infusion of their combinations. The added benefits of colloids as compared with crystalloids include prolongation of the circulatory improvement (Hoffmann JN *et al.* 2002, Marx G *et al.* 2004) and various anti-inflammatory actions (Lang K *et al.* 2003, Tian J *et al.* 2004, Gombocz K *et al.* 2007). Although the fields of indication for the use of natural or synthetic colloids are well-established (Hoffmann JN *et al.* 2002, Boldt J 2006), the therapeutic power of different fluid replacement strategies is still a subject of debate. The volume-restoring potential of colloids is often regarded as a most important clinical feature; nevertheless, the net efficacy of ”plasma expanders” is determined by many other factors. Specifically, the various experimental and clinical data demonstrate significant differences not only in the characteristics of the macrohemodynamic responses (Boldt J 2006), but also in the potential to restore tissue oxygenation (Marx G *et al.* 2004, Rittoo D *et al.* 2004) and additionally the potential to modulate inflammatory activation at the microcirculatory level (Menger MD *et al.* 1993, Kaplan SS *et al.* 2000, Jaeger K *et al.* 2001, Feng X *et al.* 2007). Anti-inflammatory actions and microcirculatory consequences are in fact interrelated events. Interruption of the adhesion between PMN leukocytes and endothelial cells ameliorates or prevents microcirculatory dysfunctions (Kurose I *et al.* 1994b), and these reactions have been implicated as critical pathogenic factors in a variety of low flow-induced tissue injuries (Granger DN *et al.* 1994). In this respect, it has been shown that artificial colloids indeed moderate hypoxia-induced endothelial dysfunctions (Kaplan SS *et al.* 2000,

Steinbauer M *et al.* 1998), and interfere with the expression of adhesion molecules that mediate PMN-endothelial cell interactions (Tian J *et al.* 2004, Gombocz K *et al.* 2007, Akgur FM *et al.* 1999, Pascual JL *et al.* 2001, Nohé B *et al.* 2005). Our understanding of the pivotal position of inflammation in the pathogenesis of a microvascular dysfunction raises questions concerning the opportunities for fluid therapy in the prevention or treatment of this syndrome. To date, however, there have been only very few *in vivo* studies where the microcirculatory effects of the clinically most important artificial colloid classes, including dextran (DEX), low-molecular-weight hydroxyethyl starch (HES) and gelatine (GEL), have been characterized and compared in the same setup.

2. AIMS

Our main aim was to examine the local, periosteal microcirculatory inflammatory consequences of I-R in animal models of transient limb ischemia. During these studies, the estimation of the efficacy of different therapeutic implications was achieved by quantifying the changes of local periosteal PMN-endothelial interactions and the expression of adhesion molecules known to play a role in this process.

Study I. Our primary aim was to assess the degree of local microcirculatory protection that can be achieved by IPC in a rat model of experimental limb I-R using intravital IVM examination of the periosteum. We additionally aimed to examine the possibility of systemic inflammatory activation caused by a local ischemic trigger, and the extent of IPC-induced protection.

Study II. Our second aim was to modulate the outcome of an I-R-induced inflammatory reaction through the administration of exogenous PC. In addition to the intravital microscopic examination of the effects of PC, we investigated the nature and the

time course of PMN accumulation in the periosteum and skeletal muscle in relation to the magnitude of mast cell degranulation.

Study III. Thirdly, our aim was a comprehensive characterization of the pleiotropic efficacies of DEX (6%; 60 kDa MW), low molecular weight HES (6%; 130 kDa/0.4) and GEL (4%; 35 kDa) on PMN reactions at the periosteal microciculatory level by using standardized *in vivo* microscopic methods.

3. MATERIALS AND METHODS

The experiments were performed on rats (average weight 300±20 g) in accordance with the NIH Guidelines (Guide for the Care and Use of Laboratory Animals) and the study was approved by the Animal Welfare Committee of the University of Szeged.

3.1. Experimental protocol

Study I.

These experiments were performed to assess the effects of IPC in the limb ischemia-induced microcirculatory inflammatory reactions in two experimental series of Sprague-Dawley rats. The first experiments were used to determine the extent of I-R-induced microcirculatory changes in the rat tibial periosteum, using IVM. As the fluorescence technique interferes with measurements of CD11b expression changes, blood samples for the assessment of adhesion molecule expression (sICAM-1 and CD11b, see later) were taken in a second series with an identical protocol (see below).

In the first series, the first group (Sham) (n=8) served as sham-operated control; the microcirculatory variables were recorded for 280 min to exclude changes relating solely to the anesthesia and surgery. In a second group (IPC+Sham; n=6), sham-operation was combined with IPC (2 cycles of 10 min of complete hindlimb ischemia and 10 min of reperfusion). The complete hindlimb ischemia was induced by placing a tourniquet around the proximal femur,

with simultaneous occlusion of the femoral artery with a miniclip. In groups 3 (I-R; n=11) and 4 (IPC combined with I-R, n=9), complete hindlimb ischemia was induced for 60 min. The occlusions were then released (time=0 min), and the periosteal microcirculation was observed after reperfusion for 60, 120 or 180 min. At the end of the experiments, tissue biopsies from tibias together with the surrounding muscles were taken for the immunohistochemical localization of ICAM-1.

In the second experimental series, identical experimental groups were used and blood samples were taken into EDTA-coated tubes (BD Microtainer K2E, Plymouth, UK) at baseline, and at 120 and 180 min of reperfusion, for sICAM-1 and CD11b measurements (n=6-14; see later).

Study II.

In this study, the effect of PC supplementation was examined after limb ischemia. In these experiments, two groups of Wistar rats were subjected to complete hindlimb ischemia by placing a tourniquet around the proximal femur, and the femoral artery was occluded with a miniclip for 60 min. The occlusions were then released (time=0 min), and the periosteal microcirculation was observed after reperfusion for 0, 30, 60, 120 and 180 min. Group 1 (n=7) was treated with the vehicle for PC, while group 2 (n=6) received PC in a dose of 50 mg/kg iv for 10 min, starting 10 min after the beginning of reperfusion. The 5.0% PC solution (soybean lecithin, MW: 785, Phospholipon 90, Phospholipid GmbH, Cologne, Germany) was freshly prepared according to the description of the manufacturer. Further two groups served as sham-operated, vehicle- or PC-treated controls (n=7 and n=6, respectively); the microcirculatory variables were recorded for 240 min to exclude changes relating solely to the anesthesia and surgery. At the end of the experiments, muscle biopsies (m. gracilis anterior) for biochemical (MPO) and histological examinations (mast cells degranulation assessment) were taken from the operated and contralateral hindlimbs.

Study III.

There experiments were conducted to assess the effects of different clinically applied volume expanders on the limb I-R-induced inflammatory reactions. The experiments were performed in two series of Wistar rats. In the first part of the protocol I-R-induced microcirculatory changes in the tibial periosteum were analyzed with the aid of fluorescence intravital microscopy. In the first series, the first group (n=10) served as lactated Ringer-treated, sham-operated controls, where the microcirculatory variables were recorded for 240 min to exclude changes relating solely to anesthesia and surgery. In the next four groups, complete hindlimb ischemia was induced by placing a tourniquet around the proximal femur, with simultaneous occlusion of the femoral artery with a miniclip for 60 min. The occlusions were then released (t = 0 min), and the periosteal microcirculation was observed after reperfusion for 180 min. Animals in groups 2-4 were treated with LR (n=10), GEL (Gelofusine 4%; 35 kDa; B.Braun Melsungen AG, Melsungen, Germany; n=6), DEX (Macrodex 6%; 60 kDa; Baxter Deutschland GmbH, Germany; n=9) or HES (Voluven 6%; 130 kDa/0.4; Fresenius Kabi Deutschland GmbH, Homburg, Germany; n=8), respectively, in a dose of 15 ml kg-1 h-1 iv, starting during the last 10 min of ischemia. The concentrations used were based on previously reported clinical investigations of the agents (Wolfárd A 2002). In pilot studies, typical, comparable hemodynamic reactions were attained in vivo (data not shown). The infusions were maintained during the first hour of reperfusion and the doses were then decreased to 5 ml kg^{-1} h^{-1} iv in the second and third hours of reperfusion. To exclude any possibility of anaphylactic reactions, the DEX-treated group was treated with hapten (Promit; Fresenius Kabi Inc., Norge AS, Norway) in a dose of 1 ml kg^{-1} 30 min prior to the initiation of the DEX infusion, the other groups receiving saline in the corresponding volume. In the second series, identical experimental groups were used and blood samples were taken into EDTA-coated tubes (BD Microtainer K2E, Plymouth, UK) at baseline and at

120 min and 180 min of reperfusion for sICAM-1 and CD11b measurements (n=6-12).

3.2. Surgical procedures, preparation of the tibial periosteum in rats

Rats were anesthetized with sodium pentobarbital (45 mg kg^{-1} ip), the trachea was cannulated to facilitate respiration, and the right jugular vein and carotid artery were cannulated for fluid and drug administration and for the measurement of arterial pressure (a Statham P23Db transducer with a computerized data acquisition system; Experimetria Ltd., Budapest, Hungary), respectively. The animals were placed in a supine position on a heating pad to maintain the body temperature between 36 and 37 ^{o}C, and Ringer's lactate was infused at a rate of 10 ml kg^{-1} h^{-1} during the experiments, together with small supplementary doses of pentobarbital iv when necessary. The trachea was cannulated to facilitate respiration, the right femoral artery was dissected free, and the periosteum of the medial surface of the right tibia was exposed under a Zeiss 6x magnification operating microscope. By means of an atraumatic surgical technique (developed by our research group), the skin above the anterior tibia was dissected and the gracilis posterior muscle was cut through. This simple, novel, easily reproducible procedure provides a tissue window with good exposure of the proximal and medial microvascular architecture of the anterior tibial periosteum without using local microcirculatory disturbances or inflammatory reactions.

3.3. Intravital videomicroscopy

The microcirculation of the distal tibia was visualized by IVM (Zeiss Axiotech Vario 100HD microscope), using fluorescein isothiocyanate (Sigma Chemicals, USA)-labeled erythrocytes (Ruh J *et al.* 1998) 7 (0.2 ml), and rhodamine-6G staining (Sigma, St. Louis, MO, 0.2%, 0.1 ml iv) for the leukocytes. The IVM images were recorded with a charge-coupled device videocamera (Teli CS8320Bi, Toshiba Teli Corporation, Japan) attached to an S-VHS videorecorder (Panasonic AG-MD 830) and a personal computer.

3.4. Video analysis

Quantitative assessment of the microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images, using image analysis software (IVM, Pictron Ltd., Budapest, Hungary). Leukocyte-endothelial cell interactions were analyzed within 5 postcapillary venules (diameter between 11 and 20 μm) per animal. *Adherent leukocytes* (stickers) were defined in each vessel segment as cells that did not move or detach from the endothelial lining within an observation period of 30 s, and are given as the number of cells per mm^2 of endothelial surface. The *rolling leukocytes* were defined as cells moving at a velocity less than 40% of that of the erythrocytes in the centerline of the microvessel, and are given as the number of cells per second per vessel circumference (Study I) or as a percentage of the number of nonadherent leukocytes passing through the observed vessel segment within 30 s (Studies II-II).

3.5. Flow-cytometric analysis of CD11b expression changes

Surface expression of CD11b on peripheral blood granulocyte was determined with whole blood flow-cytometric analysis by a modified method of Keresztes *et al.* (Keresztes M *et al.* 2007) in duplicates. 100 μl of whole blood was incubated with 20 μl of (50 μg/ml) mouse anti-rat monoclonal antibody (BD Pharmingen, BD Biosciences, San Jose, CA) for 20 min. Negative controls were obtained by omitting the monoclonal antibody. The cells were than washed twice in Hank's buffer, and centrifuged at 13,000 rpm for 5 min and the resuspended pellet was incubated with fluorescein isothiocyanate-conjugated polyclonal rabbit anti-mouse immunoglobulin (10 μg/ml; DAKO Cytomation, Glostrup, Denmark; 20μl aliquots of reagents to 180μl aliquots of blood cells). Cells were again washed twice, the erythrocytes were lysed using a Lysing kit (Biodesign, Saco, ME), then the cells washed twice again (6.000 rpm, 5 min) and resuspended in 200 μl Hank's buffer. A computer-assisted FACStar Plus Becton-Dickinson equipment was used for cytometry, the granulocytes were gated on the basis of their characteristic forward and side-scatter features (forward scatter: to

differentiate from the lymphocytes and monocytes based on the average cell size; side scatter: based on the granular surface). Generally, 10.000 events per sample were collected, recorded, the percentage of the labelled (activated) granulocytes (% of marker-bearing cells) and the mean fluorescence intensity (average marker density) were presented.

3.6. Measurements of soluble ICAM-1 levels

Plasma sICAM-1 levels were determined with the Quantikine sICAM-1-ELISA Kit (R&D Systems Inc., Minneapolis, MN), according to the manufacturer's instructions.

3.7. Immunohistochemical analysis of tissue ICAM-1 expression

Tibias with the surrounding muscle tissues were taken at the end of the experiments and fixed in formalin for a minimum of 48 h. Histological specimen were decalcified with an electrophoretic apparatus (decalcifying solution: Sakura TDE30; Sakura Finetek Corp.; USA), embedded in paraffin, and sections stained with mouse monoclonal anti-rat ICAM-1 antibody (BD Pharmingen, BD Biosciences, San Jose, CA) as primary antibody (1:200; 30 min), followed by a biotinylated goat anti-mouse antibody conjugated to horseradish peroxidase polymer (Envision® system; Dako, Denmark) for 30 min, with 3,3'-diaminobenzidine as chromogen. Semiquantitative analysis was based on the calculation of percentage of ICAM-1-positive vessels in the periosteal and intramuscular vessels (Table 2).

Score	% of positive vessels	Staining
1	< 5%	local
2		diffuse
3	5-25%	local
4		diffuse
5	25-50%	local
6		diffuse
7	> 50%	local
8		diffuse

Table 2. Semiquantitative categories (1-8) to calculate the percentage of ICAM-1-positive vessels.

3.8. Myeloperoxidase measurements

The tissue myeloperoxidase (MPO) activity, as a marker of tissue PMN infiltration, was measured in muscle biopsies by the method of Kuebler *et al.* (Kuebler WM *et al.* 1996). Briefly, the tissue was homogenized with Tris-HCl buffer (0.1 M, pH 7.4) containing 0.1 mM polymethylsulfonyl fluoride to block tissue proteases, and then centrifuged at 4°C for 20 min at 24,000 g. The MPO activities of the samples were measured at 450 nm (UV-1601 spectrophotometer, Shimadzu, Japan), and the data were referred to the protein content.

3.9. Histological assessment of mast cell degranulation

Samples of muscle biopsies were fixed in ice-cold Carnoy's fixative, embedded in paraffin, sectioned (6 μm) and stained with hematoxylin and eosin, acidic toluidine blue (pH 0.5) or alcian blue-safranin O (pH 0.4) (Szabó A *et al.* 1997). Mast cells were counted by one investigator (RV) in coded sections in 10 fields at an optical magnification of 400. Loss of intracellular granules, and stained material dispersed diffusely within the lamina propria, were taken as evidence of mast cell degranulation.

3.10. Statistical analysis

In all studies, the statistical software package SigmaStat version 2.03 (Jandel Corporation, San Rafael, CA, USA) was used. In Study I, changes in variables within and between groups were analyzed by one-way ANOVA followed by the Bonferroni test. Data were expressed as means±standard error of the mean (SEM), p values < 0.05 were considered statistically significant. In Studies II-III, Friedman repeated measures analysis of variance on ranks was applied within the groups. Time-dependent differences from the baseline were assessed by Dunn's method. Differences between groups were analyzed with Kruskal-Wallis one-way analysis of variance on ranks, followed by Dunn's method for pairwise multiple comparison. In these studies median values and 75th and 25th percentiles were given. *P* values <0.05 were considered significant.

4. RESULTS

4.1.1. Local anti-inflammatory effects of IPC in the postischemic periosteum

In the sham group, the numbers of rolling or adherent leukocytes did not change significantly throughout the experiments. As compared with the baseline values, however, significantly increased numbers of rolling PMNs were observed throughout the entire reperfusion period, whereas increased adherent PMN counts were found at 120 and 180 min of reperfusion in the I-R group (Figure 2A and 2B).

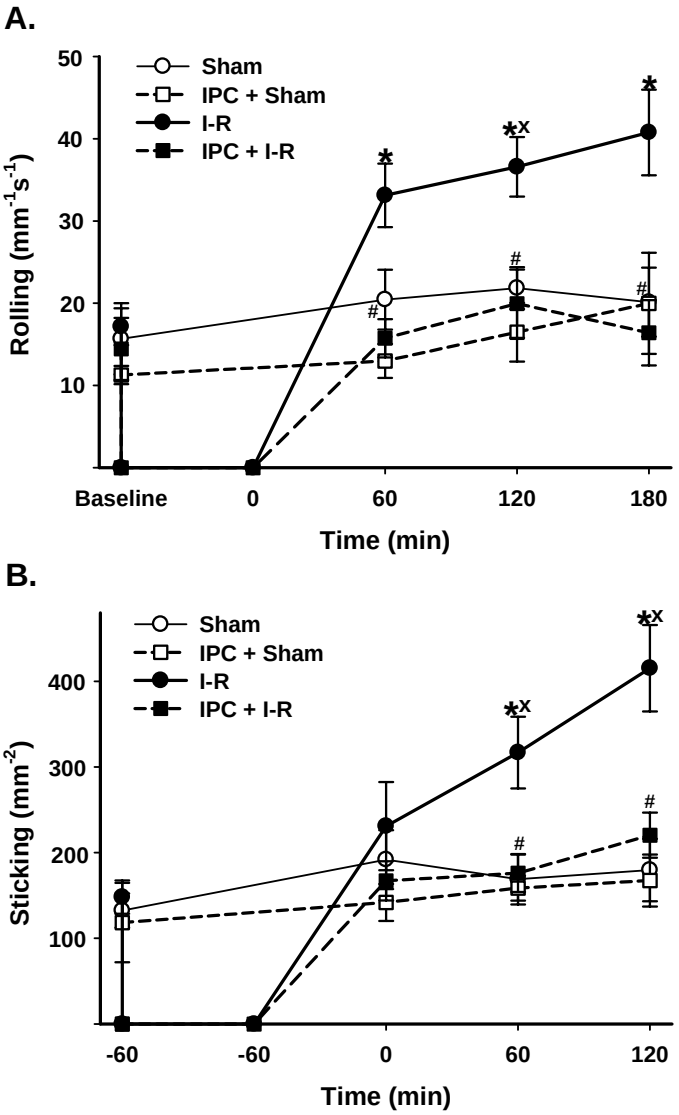

Figure 2. Primary (rolling, 2A) and secondary (sticking, 2B) leukocyte-endothelial cell interactions in postcapillary venules of the tibial periosteum after ischemia-reperfusion (I-R), sham-operation (Sham) or IPC which was followed by 60 min of ischemia/180 min of reperfusion (IPC+I-R) or sham-operation (IPC+Sham). Values are means and standard errors.* $P < 0.05$ *vs* the baseline; x $P < 0.05$ *vs* the Sham group; # $P < 0.05$ *vs* the I-R group.

IPC alone did not induce PMN rolling or sticking. The I-R-induced elevations in both parameters, however, were completely prevented by IPC during the whole of the reperfusion phase.

4.1.2. Ameliorated systemic inflammatory reactions in response to IPC after limb ischemia

The activation of PMN leukocytes, as evidenced by the surface expression of CD11b, did not change significantly in response to the sham-operation (Figure 3). Hence, we included only these time points in further evaluations. I-R caused ~1.8 and 1.72-fold elevations in this parameter ($P < 0.05$) at 120 min and 180 min of reperfusion, respectively. IPC, however, significantly prevented the I-R-induced increases of CD11b expression by the end of reperfusion (an ~1.2-fold change was noted). In the event of IPC combined with sham-operation, IPC *per se* did not affect the CD11b expression.

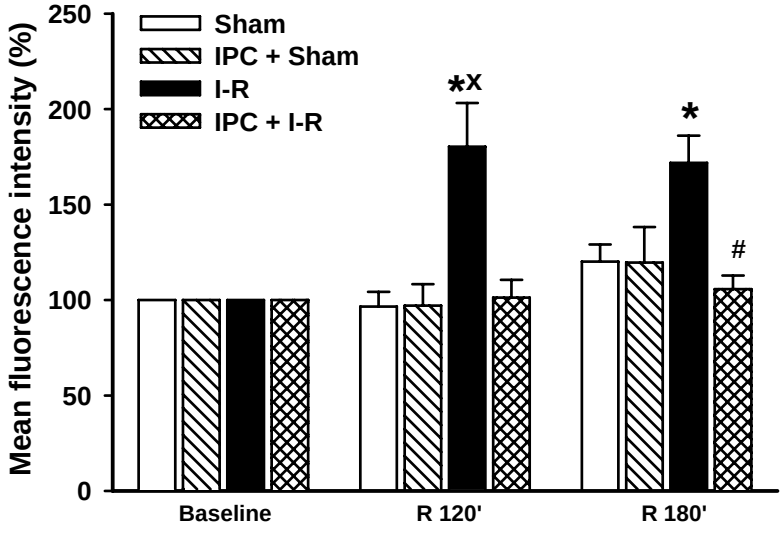

Figure 3. Changes in the expression of CD11b adhesion molecule on the surface appearance of neutrophil leukocytes in response to ischemia-reperfusion (I-R), sham-operation (Sham) or IPC (2 cycles of 10 min of complete limb ischemia and 10 min of reperfusion), which was followed by 60 min of ischemia/180 min of reperfusion (IPC+I-R) or sham-operation (IPC+Sham). Data are presented as means and standard error. * $P < 0.05$ *vs* the baseline, x $P < 0.05$ *vs* the Sham group; # $P < 0.05$ *vs* the I-R group. ANOVA followed by the Bonferroni test.

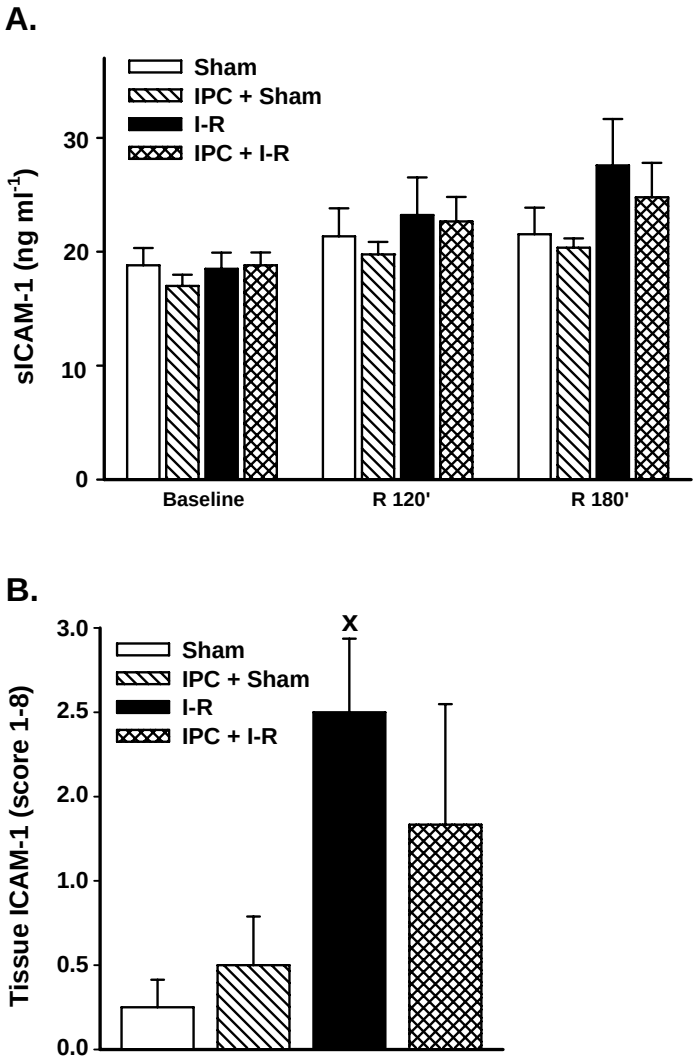

Figure 4. Changes in levels of soluble (A) and tissue (B) ICAM-1 expression induced after ischemia-reperfusion (I-R), sham-operation (Sham) or IPC (2 cycles of 10 min of complete limb ischemia and 10 min of reperfusion), which was followed by 60 min of ischemia/180 min of reperfusion (IPC+I-R) or sham-operation (IPC+Sham). Data are presented as means and standard error. x $P < 0.05$ *vs* the Sham group.

The levels of sICAM-1 (Figure 4A) increased, albeit not significantly at time points matching those for CD11b. Even though we used a sufficiently large number of animals, these parameters displayed considerable dispersion and not even the effect of I-R *vs* the sham reached the level of statistical significance.

As assessed by immunohistochemical analysis in the periosteum, however, tissue ICAM-1 density, exhibited significantly higher values in the I-R group as compared with the

Sham group, and IPC did not influence this parameter (Figures 4B and 5). There was only a moderate increase in ICAM-1 positivity in the muscle tissue (from 0.75±0.49 in the Sham group and to 1.57±0.29 in the I-R group), and IPC did not appear to influence these changes in this structure either (data not shown).

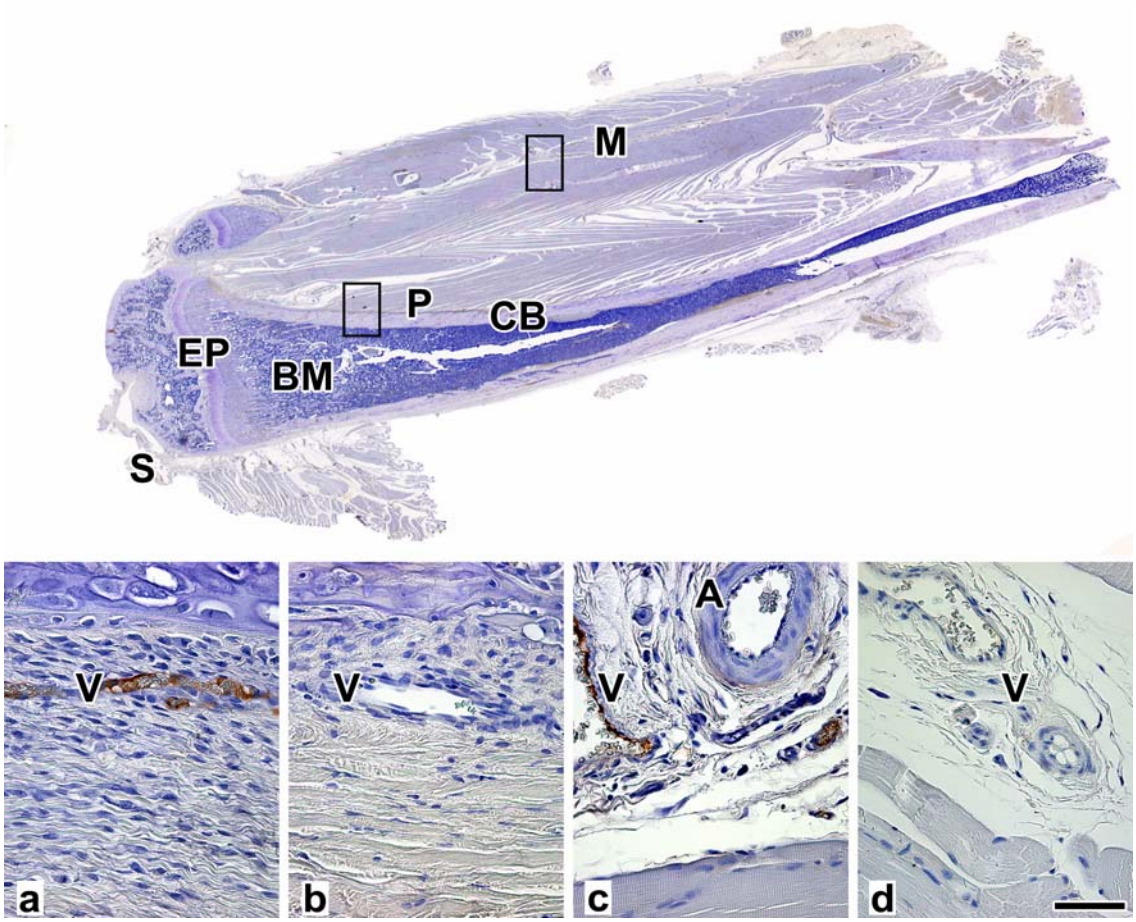

Figure 5. Representative longitudinal section of the rat tibia surrounded by soft tissues (stained with ICAM-1 antibody plus hematoxylin). Upper panel: tibial epiphysis (E), cortical bone (CB), bone marrow (BM), knee-joint synovia (S), muscle (M) and tibial periosteum (P) are indicated. Lower panel: positive staining for ICAM-1 was found in the veins (V), but not in the arteries (A). A significantly higher positivity was found in response to I-R in the periosteum (a) than in the muscle (c), whereas in the Sham groups, the staining was weak in the periosteum (b) and the muscle (d). Bar represents 50 μm.

4.2.1. Local anti-inflammatory effect of PC in the postischemic periosteum

In the sham-operated groups, the numbers of rolling and adherent leukocytes did not change significantly throughout the experiments (Figures 6A and 6B).

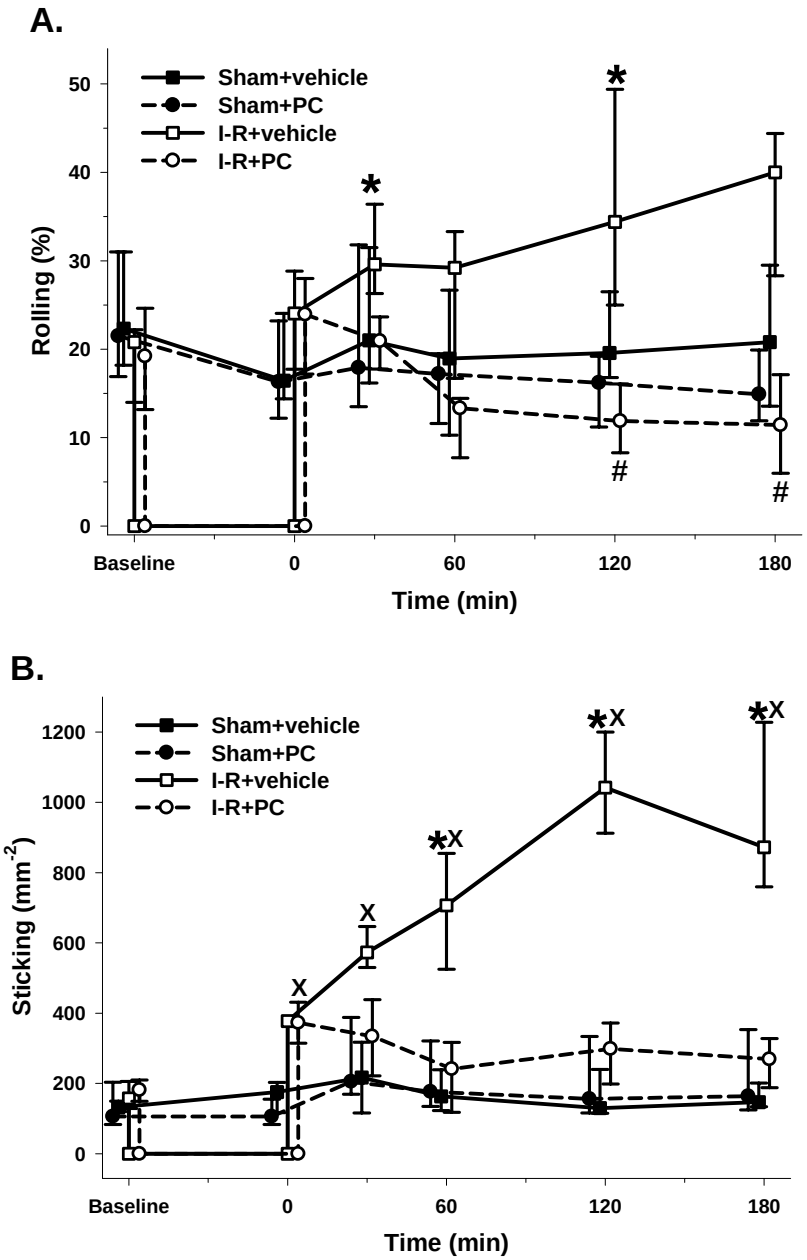

Figure 6. Primary (rolling, **A**) and secondary (sticking, **B**) leukocyte-endothelial cell interactions (rolling) in postcapillary venules of the tibial periosteum in the sham-operated vehicle (black rectangles)- and PC-treated (black circles) controls and after 60-min ischemia and 180-min reperfusion in the animals receiving vehicle (open rectangles) or an iv PC infusion (open circles) in the second 10 min of reperfusion, respectively. Values are median values, and 25th and 75th percentiles. *$P < 0.05$ vs the baseline values, # $P < 0.05$ between the I-R groups.

In the vehicle-treated group, the proportion of rolling leukocytes increased from 20.8% at baseline to 34.4% and 40.0% after 120 min and 180 min of reperfusion, respectively, and significant increases were observed in the number of firmly adherent leukocytes at 120 min and 180 min of reperfusion. In the PC-treated animals, the elevations in the numbers of rolling and firmly adherent PMNs were significantly lower than those in the control I-R group throughout the 180-min reperfusion period.

4.2.2. Other signs of ameliorated inflammatory reactions in response to PC

In the vehicle-treated I-R group, the tissue MPO level was significantly increased as compared with that of the sham-operated animals (approx. 3-fold) and the contralateral non-ischemic limb. In the PC-treated group, the MPO activity was significantly lower than in the vehicle-treated I-R group (Figure 7).

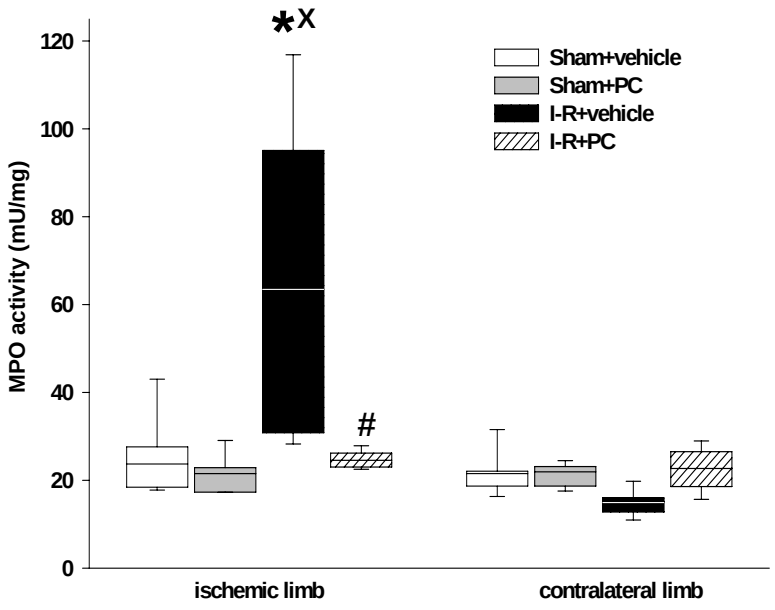

Figure 7. Muscle myeloperoxidase (MPO) activity was assessed at the end of the 240-min observation period in limbs subjected to sham operation (Sham) in the presence of vehicle (open box) or PC treatment (gray box) or to 60-min complete ischemia followed by 180-min reperfusion in the presence of vehicle (I-R + vehicle; black box) or 50 mg kg^{-1} PC treatment (I-R + PC; hatched box). Data are compared with those for the intact contralateral limbs. *P < 0.05 vs the contralateral limb, x P < 0.05 vs the corresponding sham-operated group, # P < 0.05 between the I-R groups.

In the sham-operated groups or in the contralateral, non-ischemic hindlimb, no significant increase in mast degranulation was observed in the muscle by the end of the observation period (Figure 8). I-R, however, caused a significant extent of mast cell degranulation. In these biopsies, the degree of mast cell degranulation was approximately 82.5%, whereas 7.6% degranulation was found in the contralateral limb. The PC pretreatment prevented the I-R-induced increase in mast cell degranulation (M=20%, p25=18; p75=24), and the values were not significantly different from those for the sham-operated groups or the samples from the contralateral limb.

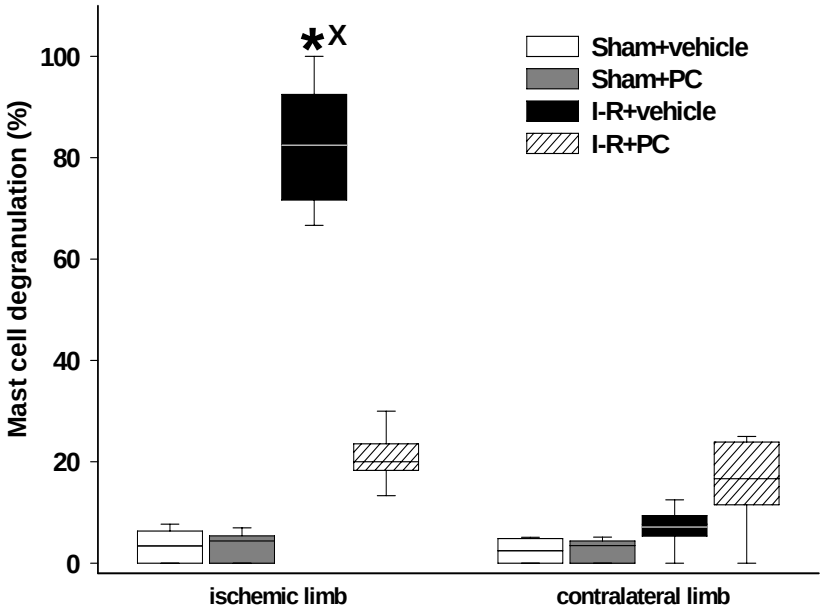

Figure 8. Changes in muscle mast cell degranulation (%) were assessed at the end of the 240-min observation period in limbs subjected to sham operation (Sham) in the presence of vehicle (open box) or PC treatment (gray box) or to 60-min complete ischemia followed by 180-min reperfusion in the presence of vehicle (I-R + vehicle; black box) or 50 mg kg^{-1} PC treatment (I-R + PC; hatched box). Data are compared with those for the intact contralateral limbs. Median (thick line in the box), 25th percentile (bottom of the box), 75th percentile (top of the box), 5th and 95th percentiles. * $P < 0.05$ vs the contralateral limb, x $P < 0.05$ vs the corresponding sham-operated group.

4.3.1. Local anti-inflammatory effects of colloid solutions in the postischemic periosteum

In the I-R group, the proportion of rolling PMNs increased from a baseline level of approximately 16.4% to around 37.1% and 42.6% after 120 min and 180 min of reperfusion, respectively (Figures 9 and 10). Significant increases were observed in the number of sticking leukocytes at 120 min of reperfusion (from approximately 140 mm^{-2} to 1000 mm^{-2}). The postischemic increases in the proportion of rolling leukocytes were not influenced by any of the colloid treatments, though the HES infusion caused a moderate improvement in the later stages of reperfusion. However, the I-R-induced firm leukocyte adherence was completely prevented by HES during the entire reperfusion period. In contrast, significant deteriorations in this parameter were observed in the first hour of reperfusion in the GEL- and DEX-treated groups, similar to that seen after I-R with LR treatment. In the case of GEL treatment, this was followed by a moderate improvement in the later experimental phase.

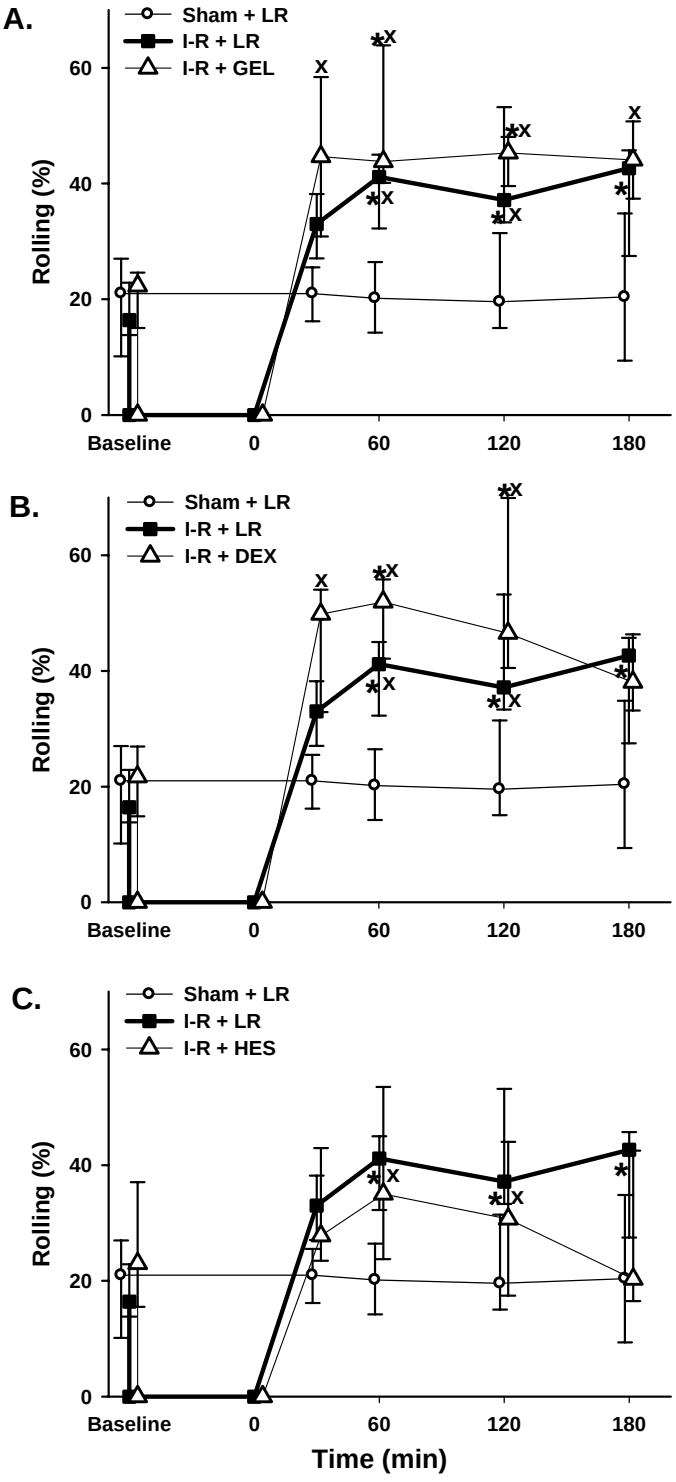

Figure 9. Primary leukocyte-endothelial cell interactions (rolling) in postcapillary venules of the tibial periosteum in the sham-operated controls (open circles), after 60-min ischemia and 180-min reperfusion in the animals receiving lactated Ringer's solution (LR, black rectangles), gelatine (GEL, **A**), dextran 60 (DEX, **B**) or hydroxyethyl starch 130/0.4 (HES, **C**) (open triangles). Observations were made at baseline and after reperfusion for 30, 60, 120 and 180 min. Values are median values, and 25p and 75p = 25th and 75th percentiles. * $P < 0.05$ *vs* baseline; x $P < 0.05$ *vs* sham-operated group.

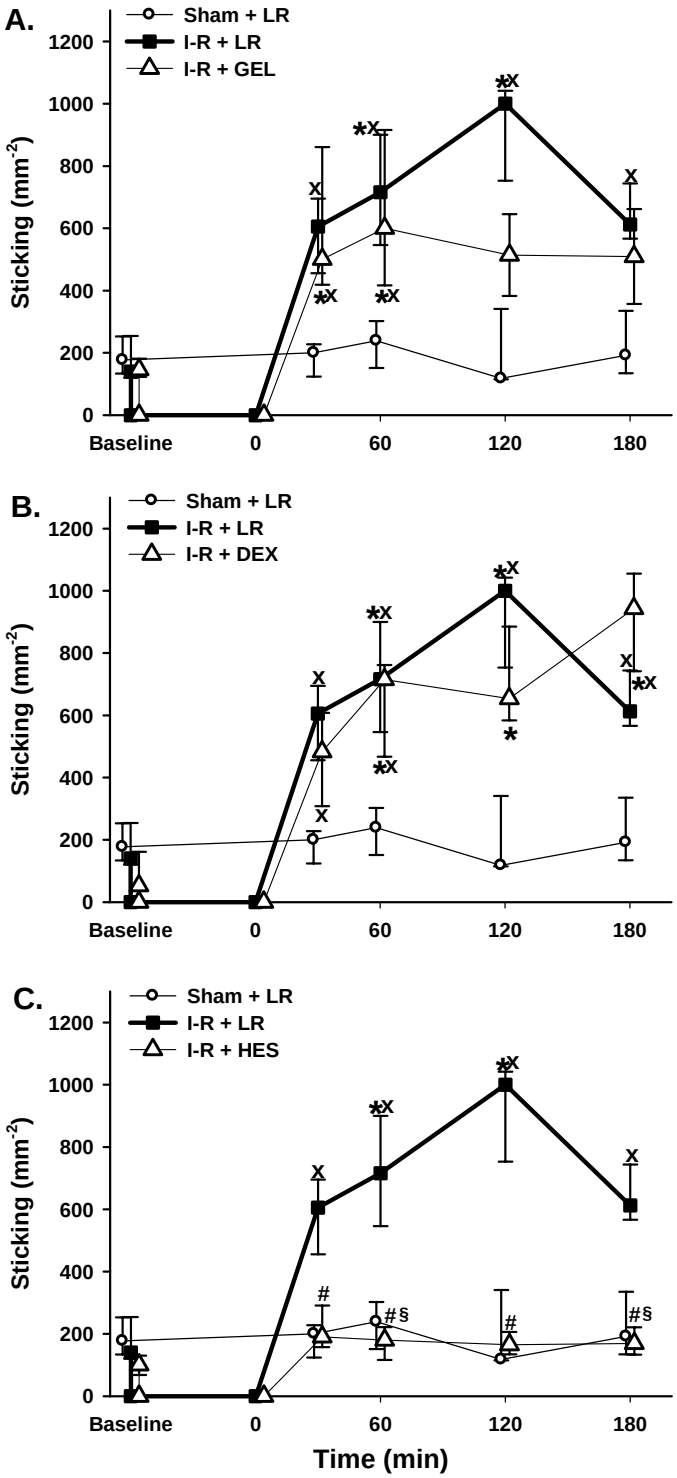

Figure 10. Secondary leukocyte-endothelial cell interactions (sticking) in postcapillary venules of the tibial periosteum in the sham-operated controls (open circles), after 60-min ischemia and 180-min reperfusion in the animals receiving lactated Ringer's solution (LR, black rectangles), gelatine (GEL, **A**), dextran 60 (DEX, **B**) or hydroxyethyl starch 130/0.4 (HES, **C**) (open triangles). Observations were made at baseline and after reperfusion for 30, 60, 120 and 180 min. Values are median values; 25p and 75p = 25th and 75th percentiles. * $P < 0.05$ *vs* baseline; x $P < 0.05$ *vs* sham-operated group; # $P < 0.05$ *vs* I-R + LR group; § $P < 0.05$ I-R + HES *vs* I-R + DEX group.

4.3.2. Signs of ameliorated systemic inflammatory reactions in response to different colloid solution therapy after limb ischemia

As evidenced by the surface expression of adhesion molecule CD11b, the activation of the PMNs was not changed significantly after the sham operation (Figure 11). In a pilot study, the dynamics of the changes in CD11b expression was characterized in response to I-R; and significant elevations were found not earlier than 120 and 180 min of reperfusion. Hence, we included only these time points in further evaluations. I-R with LR treatment caused approximately 1.8-1.9-fold elevations in this parameter ($p < 0.05$) at 120 min and 180 min of reperfusion. Similar increases were observed in the GEL- and DEX-treated groups. HES treatment, however, significantly depressed the I-R-induced increase in CD11b expression.

The levels of sICAM-1 were also evaluated at time points matching those for CD11b. This parameter displayed a rather great dispersion, and not even the effect of I-R *vs* the sham reached statistical significance. However, GEL treatment caused a significant elevation in the level of sICAM-1 at 180 min of reperfusion relative to the baseline (Table 3) and at 120 min of reperfusion resulted in a significantly higher level than those achieved following DEX or HES treatment.

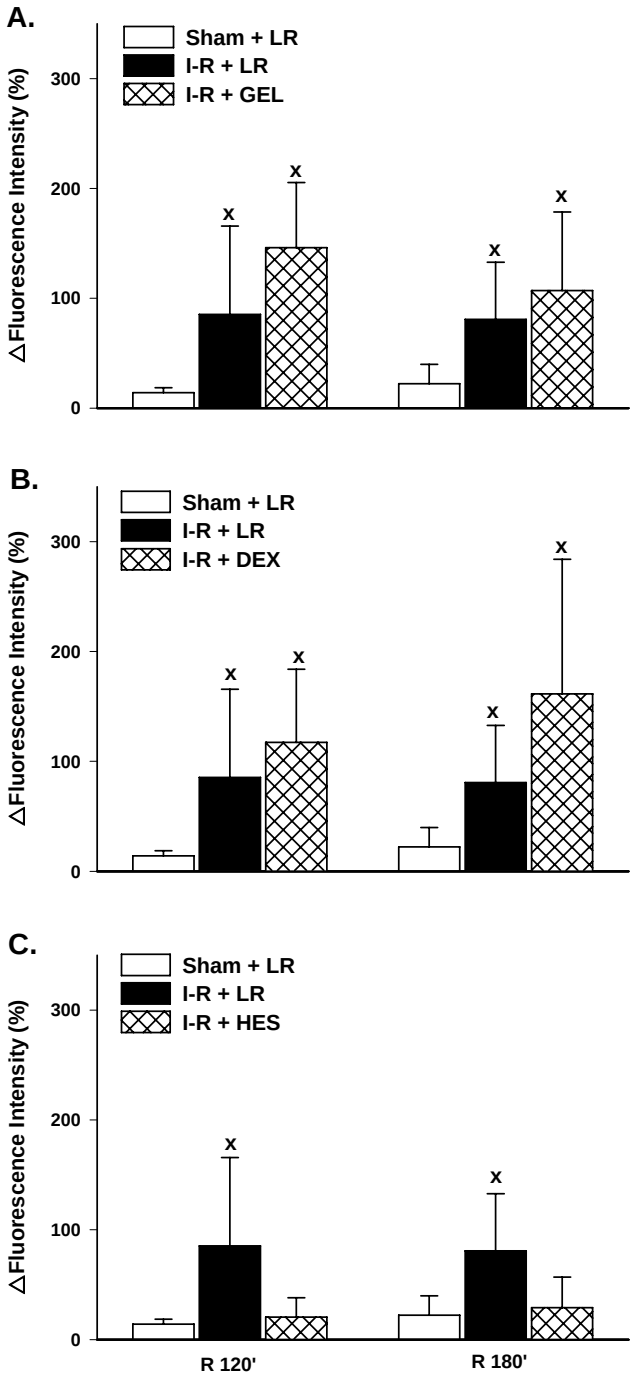

Figure 11. Changes in the expression of the CD11b adhesion molecule on the surface of PMNs in response to sham operation (white column) or 60 min of total hindlimb ischemia followed by 180 min of reperfusion in rats treated with lactated Ringer's solution (LR, black column), gelatine (GEL, **A**), dextran 60 (DEX, **B**) or hydroxyethyl starch 130/0.4 (HES, **C**) (crossed column). Measurements were made at baseline and after reperfusion for 120 and 180 min. Data are presented as median values, and 25th and 75th percentiles. x $P < 0.05$ *vs* sham-operated group.

Table 3. Effects of lactated Ringer's solution (LR), gelatine (GEL), dextran 60 (DEX) and hydroxyethyl starch 130/0.4 (HES) solutions on limb ischemia-reperfusion-induced systemic soluble ICAM-1 release (ng ml^{-1}) during 120 and 180 min of reperfusion.

Groups	**Parameters**	**Baseline**	**R 120 min**	**R 180 min**
Sham + LR	Median	17.97	22.45	21.76
	25p, 75p	*15.70; 19.83*	*14.91; 25.45*	*16.29; 24.79*
I-R + LR	Median	17.27	20.35	23.93
	25p, 75p	*15.87; 18.24*	*18.64; 24.74*	*20.83; 26.17*
I-R + GEL	Median	16.81	30.07†$	32.43*x
	25p, 75p	*15.45; 17.77*	*25.78*; *31.26*	*28.94; 34.22*
I-R + DEX	Median	19.87	17.86	24.54
	25p, 75p	*17.88; 21.72*	*15.38; 19.76*	*23.44; 25.91*
I-R + HES	Median	17.35	17.69	20.83
	25p, 75p	*15.75; 18.67*	*14.80; 19.66*	*19.32; 26.33*

* $P < 0.05$ *vs* the baseline; x $P < 0.05$ I-R + GEL *vs* Sham + LR; † $P < 0.05$ I-R + GEL *vs* I-R + DEX; $ $P < 0.05$ I-R + GEL *vs* I-R + HES groups.

5. DISCUSSION

5.1. Effect of different therapeutic interventions on PMN-endothelial interactions

Open fractures of the extremities are often associated with delayed unions or non-unions, and the accompanying periosteal stripping may contribute significantly to the morbidity. Restoration of a compromised periosteal microcirculation is essential for infection prevention and the incorporation of microvascular bone grafts (Berggren A *et al.* 1982). In our experimental studies, complete limb I-R was followed by a periosteal microcirculatory dysfunction and significant mast cell degranulation in the adjacent muscle. On the other hand, complete vascular occlusion disturbs the perfusion of all tissues of the exposed limb, microcirculatory deterioration predominated in the periosteum, while the surrounding muscle layers exhibited much lower ischemic sensitivity (Rucker M *et al.* 1998). In our model, the local injury was manifested in significant increases in the primary and secondary forms of PMN-endothelial interactions (rolling and firm adherence) in the periosteal postcapillary venules during reperfusion. These reactions can be quantitatively assessed by IVM and the efficacy of various therapeutic interventions could also objectively be judged.

First, we examined the effect of limb IPC which is a relatively easily accessible treatment modality with a low risk during the surgical interventions. On the other hand, the large tissue mass involved provides a strong signal. The low microcirculatory risk of IPC alone could be traced in these studies. Moreover, IPC has been shown to exert alleviating effects on the endothelial site of the periosteal inflammatory reactions (rolling, sticking). In other studies, IPC alleviated direct endothelial cell injury (Kaeffer N *et al.* 1996), exerted nitric oxide-dependent microvascular protection (Wang WZ *et al.* 2004) and to restored endothelium-dependent vasorelaxation in humans (Kharbanda RK *et al.* 2001). As I-R injury is a primarily intracellular sequence of oxido-reductive events, some of the protective effects of IPC are related to the modulation of this process targeting the endothelium directly or

indirectly through PMNs (Hernandez LA *et al.* 1987). As such, it has been demonstrated that the protection provided by IPC is also mediated by oxidants, low concentrations of which are necessary for this adaptation (Oldenburg O *et al.* 2002). The endothelium regulates this latter reaction, since it produces adhesion molecules which enhance PMN attachment and consequent PMN-derived oxidative damage. In our study, IPC effectively reduced the local manifestations of I-R injury in the tibial microvasculature. Our results show that IPC also exerts protection at the other component of these PMN-endothelial interactions by also affecting the PMN activation process (see later).

Major components of this complex inflammatory reaction, including leukocyte-endothelial cell interactions in the periosteum, were also effectively ameliorated by systemic PC supplementation, but these benefits were provided through potentially different mechanisms from that provided by IPC. PC is one of the most abundant compounds of the body hence the need for PC supplementation should be clarified first. It has been shown that I-R is associated with physical membrane defects, PC degradation and the exhaustion of endogenous PC sources (Bruhl A *et al.* 2004, Jones RL *et al.* 1989, Gross RW 1992), whereas ischemic preconditioning restores the membrane stability with the simultaneous prevention of phospholipid degradation (Bruhl A *et al.* 2004). These findings suggest that this ubiquitous membrane-forming entity may become depleted in response to noxious stimuli, and replenishment of the endogenous PC pool could be of importance under critical circumstances (Bruhl A *et al.* 2004). In line with this, it has been shown that PC supplementation could ameliorate the harmful consequences of myocardial I-R (Duan JM *et al.* 1990). Although exogenous PC exerts protection in various experimental scenarios, such as ethanol-induced liver injury, gastric damage (Lieber CS *et al.* 1997, Dunjic BS *et al.* 1993), esophageal inflammation (Demirbilek S *et al.* 2002), doxorubicin toxicity (Gabizon A *et al.* 1986), and sepsis (Yan JJ *et al.* 2004), its mechanism of action is not fully understood. PC is taken up by

phagocytic cells, and thus it accumulates in inflamed tissues (Cleland LG *et al.* 1979) and restores the mitochondrial function (Duan JM *et al.* 1990). It is widely believed that the biological efficacy of PC depends on the fatty acid moiety (Lieber CS *et al.* 1997). Nonetheless, some recent studies revealed that the protective role of PC may be independent of the fatty acids and the active principle is choline. Phospholipase-D is activated by nearly all stress factors, which results in the release of phospholipid metabolites, several of which could be of importance in stress-induced defense reactions (Exton JH 1999, Hansen HS *et al.* 2000). Choline is actively transported by a choline carrier described in epithelial and endothelial cells (Friedrich A *et al.* 2001). Indeed, it has been shown that PC metabolites may relieve a potentially dangerous increase in the $NADH/NAD^-$ ratio (reductive stress), a situation predisposing to oxidative damage (Ghyczy M *et al.* 2001). In this study, reperfusion of the previously ischemic hindlimb triggered an acute inflammatory reaction, with PMN accumulation and a microcirculatory dysfunction in the tibial periosteum. It is recognized that neutrophils contribute significantly to I-R injury in many organs (Chanavaz M 1995, Hernandez LA *et al.* 1987). We have recently demonstrated that PC reduces PMN accumulation in experimental esophagitis (Erős G *et al.* 2006), and similar effects were observed here in the post-ischemic periosteum and skeletal muscle. Moreover, these changes were accompanied by a reduced degree of mast cell degranulation in the muscle. Although a wide range of protective functions have been attributed to mast cells in the bone (mostly related to the mediation of early and late stages of bone healing) (Lindholm RV *et al.* 1970, Saffar JL *et al.* 1990, Lindholm RV *et al.* 1967), these cells play an additional, effector role in I-R-related tissue injuries. Mast cells undergo degranulation upon reperfusion (Szabó A *et al.* 1997, Boros M *et al.* 1989), and it has been shown that granulocyte recruitment is closely related to mast cell activation, even after remote ischemia (Kanwar S *et al.* 1997, Schmeling DJ *et al.* 1989). Our data demonstrate that decreased degranulation of mast cells in response

to PC may, at least in part, account for the reduced local inflammatory reactions seen after this treatment. Furthermore, a reduced PMN accumulation can also achieved by PC treatment after limb ischemia.

We also set out to investigate whether application of different volume expanders (DEX, HES or GEL) can beneficially influence the local microcirculatory disturbances that occur in response to tourniquet ischemia. These treatment modalities are easily accessible at the bedside, hence can routinely be applied. The volume-expanding properties of the three colloid solutions might differ due to their differing molecular weight and vascular retention, but the macrohemodynamic changes suggested quite similar volume-related effects for the examined solutions. The matching macrocirculatory endpoints indicated that other parameters, including microcirculatory changes, could be accurately compared in this setup. The macrovascular perfusion enhancement after colloid administration is usually attributed to a rheological improvement, particularly when these fluids are given together with other solutions. In our study, these compounds were tested alone, after crystalloid (lactated Ringer) infusion, somewhat differently from in the clinical situation. By this approach, perfusion reactions and possible anti-inflammatory consequences could be examined unambiguously, without contributions from other, potentially modifying factors. Crystalloid solutions could induce inflammatory activation by affecting cell-cell interactions (Akgur FM *et al.* 1999) and cause a hypercoagulable state, probably due to hyperchloremia (Roche AM *et al.* 2006). The quantification of the impact of various forms of colloid therapy on the I-R-induced endothelial dysfunction demonstrated conclusive protective effects only after the HES infusion. This is consistent with other reports on improved microvascular permeability (Rittoo D *et al.* 2004, Kaplan SS *et al.* 2000, Pascual JL *et al.* 2001) and other indirect parameters of a microcirculatory improvement, such as the restored tissue oxygenation after HES treatment (Marx G *et al.* 2004, Rittoo D *et al.* 2004). Information is also available concerning the better

efficacy of HES in comparison with GEL or DEX in influencing these parameters (Allison KP *et al.* 1999, McGrath AM *et al.* 1996). Our data provides similar evidence on the marked therapeutic benefit of HES in ameliorating the local inflammatory consequences of tourniquet ischemia.

5.2. Effects of different therapeutic interventions on adhesion molecule expressions

One of the major messages of our studies is that limb I-R was also associated with an increased CD11b expression, as a sign of systemic activation of the PMNs (Jones DH *et al.* 1988), suggesting a potential systemic risk of traumatological interventions which are accompanied by temporary ischemia to the limbs. Since the second phase of adherence is critically mediated by the integrins, we examined the effects of IPC on the surface expression of the PMN-derived CD11b (the αM part of the CD11b-CD18 complex), and its endothelial ligand, ICAM-1. CD11b is an important determinant of I-R-induced, PMN-mediated injury (Welbourn R *et al.* 1991), and it has been suggested that it plays a somewhat lesser role in the process of sticking than CD11a (Andreka G *et al.* 1993). However, it has subsequently been clarified that, although PMN adhesion to ICAM-1 is primarily CD11a-dependent, it is stabilized and promoted by CD11b (Park KM *et al.* 2001). CD11b is transferred to the cell surface from preformed intracellular pools; and its expression on circulating PMNs is increased several-fold in response to different stimuli, including local I-R injury (Addison PD *et al.* 2003, Kanoria S *et al.* 2006). Although the causal link between local and systemic inflammatory events usually remains uncertain, changes in CD11b expression level could also relate to the global status of PMN activation (Addison PD *et al.* 2003).

The results show that some of the above changes were significantly ameliorated by IPC. Similar over-expression of CD11b on the PMNs was observed in humans after the release of forearm occlusion in the systemic circulation and not in the veins draining the formerly ischemic tissues suggesting an enhanced local microcirculatory sequestration of the

PMNs (Kharbanda RK *et al.* 2001). From a clinical aspect, these observations suggest that tourniquet ischemia not only induces local inflammatory reactions, but may also result in the initiation of a systemic inflammatory reaction. The IPC protocol here and in former studies ameliorated systemic PMN activation after a local postischemic challenge (Kharbanda RK *et al.* 2001). The background of this finding is incompletely understood, but it has been shown that several intracellular and signal transduction pathways (*e.g.* NF-κB and TNF-α) are influenced by IPC (Welbourn R *et al.* 1991). Similarly, it has been suggested that plasma factors released from ischemic body compartments can induce CD11b activation in naive leukocytes (Barry MC *et al.* 1997). In this sense, the TNF-α released from an ischemic region can comprise a potential plasma factor which mediates remote injury (Welbourn R *et al.* 1991). Indeed, the extent of TNF-α release is reduced in response to IPC in the heart (Meldrum DR *et al.* 1994). It is also noteworthy, that IPC attenuated NF-κB activation and subsequently reduced TNF-α expression, which resulted in the amelioration of microcirculatory disturbances and PMN sequestration in the I-R-injured muscle (Akimitsu T *et al.* 1996). Moreover, reduced PMN priming by IPC can explain some of the beneficial remote preconditioning effects of limb IPC. As a result, ameliorated lung injury, together with a reduced tissue accumulation of PMNs in the lung tissue, was demonstrated after limb ischemia (Harkin DW *et al.* 2002).

In the other study, we have presented evidence that also HES interferes significantly with CD11b expression during reperfusion after limb ischemia, whereas the other colloids did not affect this parameter. This study did not let us examine the exact pathophysiological background of these observations, but others have shown that various doses of HES inhibit the tissue NF-κB activation and systemic TNF-α elevation after local and systemic inflammatory insults (Tian J *et al.* 2004, Feng X *et al.* 2007). Hence, an influence on signal transduction pathways provided by HES may potentially account for the reduced PMN-

endothelial interactions also locally.

The enhanced adhesion of leukocytes is also attributable to a triggering role of mast cell-derived mediators on the expression of several adhesion molecules (e.g., P-selectin, β2 integrin, and ICAM-1) (Gaboury JP *et al.* 1995, Kubes P *et al.* 1994). Similarly, it has been demonstrated that both PMNs and endothelial cells possess receptors for mast cell-derived proteases that directly modulate adhesion molecule expression and PMN-endothelial interactions (Meyer MC *et al.* 2005, Shpacovitch VM *et al.* 2004). Nevertheless, the exact mode of action of PC is still unclear. A direct effect of PC on the expression of surface adhesion molecules is also possible, as it has been shown that choline deficiency may lead to an increased ICAM-1 expression on endothelial cells (Lundberg AH *et al.* 2001). The present research protocol did not allow an assessment of mast cell degranulation in the periosteum itself, but only in the surrounding skeletal muscle. Systemic PC administration decreased both the leukocyte recruitment and the mast cell degranulation in this compartment, which strongly suggests that PC-induced mast cell stabilization was at least partially involved in the beneficial microcirculatory responses in the post-ischemic periosteum, too. This result is in line with our previous observation that PC pretreatment inhibited mast cell degranulation in a canine model of experimental esophagitis (Erős G *et al.* 2006). However, the inhibition of PMN adherence, together with the improved microvascular flow, could also contribute to the overall tissue protection in the affected area during reperfusion. The exact mechanism of action of PC on mast cell degranulation remains to be established: more direct (e.g., *in vitro*) approaches are needed to define the effects of PC on mast cell reactions. We did not examine the expression changes in CD11b in our studies where the effects of exogenous PC were examined, but based on mast cell stabilizing effect of PC, we can hypothesize that a mast cell-dependent reduction in adhesion molecule experessions (Kubes P *et al.* 1994, Gaboury JP *et al.* 1995) may account for these local anti-inflammatory effects of PC.

The endothelial ligand ICAM-1 is a counterpart of CD11b as an inducible transmembrane protein of the PMN migration process. The level of its soluble form (sICAM-1) is proportional to the expression of ICAM-1 on the cell membranes, and particularly on the endothelial cells (Cowley HC *et al.* 1994), and this parameter can therefore be used to assess endothelial activation. Nevertheless, the result was unhelpful in that the plasma concentration of this protein displayed a very large dispersion. Clinical studies have also indicated rather controversial changes in sICAM-1 expression. Specifically, not only pronounced increases (Hambsch J *et al.* 2002) but also decreases in sICAM-1 expression were reported after human surgical procedures (Spark JI *et al.* 1997). In other studies, high sICAM-1 levels could be traced locally, whereas the systemic concentration even changed in the opposite direction (Huda R *et al.* 2004). In our model, I-R affected a considerable tissue mass and induced an endothelial dysfunction (as seen by PMN-endothelial interactions) and systemic PMN activation. Hence, it is unlikely that the noxa would not cause injury to the affected vasculature. Even though we could not observe a significant increase in the soluble form of the ICAM-1, we could trace a marked increase in the tissue form (shown by immunohistochemistry in the periosteum). It is noteworthy, however, that the highest degree of positive staining was found in the periosteal vessels, and not in the muscle. This phenomenon can be explained by the greater sensitivity of the periosteum than that of the muscle (Rucker M *et al.* 1998). It is reasonable to assume that the signal of locally released ICAM-1 is blunted in the systemic circulation. None of the applied interventions influence this parameter significantly. Nonetheless, we believe that our negative ICAM-1 data do not exclude that for instance IPC preserved endothelial integrity. Although *in vitro* studies have pointed to reduced ICAM-1 signaling after IPC following an ischemic trigger (Beauchamp P *et al.* 1999), and kidney IPC reduced the expressions of ICAM-1 and TNF-α and NF-kappaB/DNA-binding activity (Jiang SH *et al.* 2007), we could not prove similar effects in

our model. As ICAM-1 expression can be initiated by proinflammatory cytokines and TNF-α (Zahler S *et al.* 2000), similarly to what was seen with CD11b, reduced TNF release by IPC can make an important contribution to this protection. Clarification of the details of the adhesion process requires further in-depth investigations.

Interestingly, GEL caused not a decrease, but rather an increase in postischemic sICAM-1 values. It should be noted that, in human studies where an elevated sICAM-1 has been reported, this reaction was effectively reduced by HES (but not by lactated Ringer) 4 h after abdominal surgery (Lang K *et al.* 2003). Even though the influence of colloids on the PMN-endothelial interaction components (i.e. endothelial cells *vs* PMN activation) can not be separated or judged unequivocally, our observations tend to support the *in vitro* findings that these resuscitation fluids target the leukocyte part without significantly influencing endothelial ICAM-1 release (Nohé B *et al.* 2005). They are also in agreement with the results of a recent clinical trial, where PMN activation was reduced by HES, but not by GEL therapy (Rittoo D *et al.* 2004). Similar data were reported on the oxidative burst of PMNs (Jaeger K *et al.* 2001) and ICAM-1 expression; low-molecular-weight HES reduced these changes, while GEL was ineffective (Feng X *et al.* 2007). Moreover, GEL has been shown to increase PMN-linked oxidative processes and CD11b expression (Welters ID *et al.* 2000). As concerns DEX, little is known regarding its effects on PMN activation, because the majority of studies involved a combination with hypertonic salt solutions. In such cases, hypertonic saline-dextran reduced CD11b expression in human settings (Rizoli SB *et al.* 2006), but the reduced oxidative burst of PMNs has been attributed to the hypertonic component of the solution (Fahrner SL *et al.* 2002). We did not find DEX to be more effective than HES, (Menger MD *et al.* 1993) and we could not demonstrate any benefits on the FCD and/or RBCV changes, as demonstrated by Steinbauer *et al.* (Steinbauer M *et al.* 1998). However, it is recognized that, while the size and molecular weight are major determinants of the effects of DEX and GEL,

different subtypes of HES solutions are characterized further by their degree of molar substitution and C2:C6 substitution ratio. Here, we used a low- molecular-weight HES solution which has been shown to possess more effective antiinflammatory properties than those of medium or high-molecular-weight HES (Jaeger K *et al.* 2001). It has also been suggested that colloid solutions affect PMN adherence directly, by influencing the adhesion molecule expression independently of microhemodynamic changes (Pascual JL *et al.* 2001). However, microhemodynamics and PMN adhesion are interrelated phenomena. Not only endothelium-derived vasoactive substances (typically nitric oxide and endothelin-1), but also perfusion changes and secondary shear stress *per se* influence PMN activation and adhesive interactions by changing the levels of expressions of adhesion molecules (CD11b/CD18, 38) or the dynamics and half-lives of molecular bonds (Marshall BT *et al.* 2003). It has been shown that the reduced velocity of PMNs near the vessel wall increases the probability of PMN adhesion (Abbitt KB *et al.* 2003) and, likewise, an increased shear rate can reduce the number of adherent cells (Kubes P 1997). Specifically, a force below the shear optimum can induce an increase in PMN rolling, while an increased wall shear stress enhances the velocity of rolling leukocytes and consequently decreases adhesion (Marshall BT *et al.* 2003). These observations might be indicative of an indirect, flow- or volume-dependent anti-inflammatory effect, as this improvement could affect the perfusion of the postcapillary venules and thereby also contribute to the reduction of PMN-endothelial interactions. Although the intravital microscopic observations together with the *in vitro* data cover a sufficiently wide field to suggest a direct anti-inflammatory effect for HES, it would be of importance to compare the effects of these colloids in shear stress-induced integrin activation *in vitro*.

Our findings have several clinical implications. Firstly, our observations suggest that periosteal microcirculation is particularly affected by limb ischemia and also that the systemic inflammatory consequences of tourniquet ischemia have to be taken into consideration during

surgical interventions. Limb IPC may exert potential therapeutic benefit not only by providing local protection for the periosteal microcirculation after tourniquet ischemia, but additionally by influencing potentially beneficial, remote processes linked to PMN activation and modulation of inflammatory cell recruitment. The use of limbs is one of the most promising possibilities of evolving both pre- and postconditioning ischemic tolerance (Loukogeorgakis SP *et al.* 2007, Andreka G *et al.* 2007). The major beneficiary of these approaches is the heart (Andreka G *et al.* 2007), but the kidney (Park KM *et al.* 2001), distant muscle (Addison PD *et al.* 2003), liver (Kanoria S *et al.* 2006), lung (Akimitsu T *et al.* 1996) or CNS (Gurcun U *et al.* 2006) can also be targeted. With respect to remote preconditioning, the present study has two messages. Firstly, even though vascular occlusion is associated with marked local and systemic inflammatory reactions, brief, repeated periods of limb ischemia did not have similar effects. Secondly, reduced PMN priming presented an important anti-inflammatory consequence of limb IPC both locally and during the remote protection in this animal model. Nonetheless, other potential mechanisms of IPC can also be involved in this protection (Murphy E *et al.* 2007).

PC supplementation also efficiently decreased the harmful consequences of limb I-R-induced microcirculatory perfusion failure and inflammatory reactions in the rat, and the reduced PMN sequestration in the post-ischemic tissues could be partly mediated by mast cell stabilization. Further studies may be warranted to clarify, whether dietary supplementation or infusion therapy with PC would beneficially influence the outcome of traumatological intervention.

Among the colloid solutions, only HES exerted marked beneficial effects of HES at the prevention of systemic PMN activation, whereas other colloid solutions did not influence these parameters. Although any extrapolation to clinical applications should be attempted only with cautions, it is reasonable to propose that isovolemic hemodilution with HES

provides an easily accessible therapeutic advantage in various postoperative settings.

6. SUMMARY OF NEW FINDINGS

1. We have shown that complete limb I-R induces not only local, but also systemic inflammatory consequences. These observations should be taken into consideration when tourniquet ischemia is used in the traumatological practice.
2. IPC of the limb may exert potential therapeutic benefit by exerting simultaneous protection against the local inflammatory consequences and systemic inflammatory cell recruitment caused by limb ischemia. Hence IPC may provide a simple and easily accessible treatment modality at the surgical site.
3. PC supplementation efficiently ameliorated the limb I-R-induced inflammatory reactions, as manifested by the reduced PMN adhesion and sequestration in the post-ischemic tissues. This effect of PC could partly be mediated by mast cell stabilization. PC administration may reduce the risk of traumatological interventions also in the clinical practice.
4. The different colloid solutions have diverse microcirculatory effects after limb ischemia. Only HES had significant alleviating effect locally and at the prevention of systemic PMN activation, whereas other colloid solutions (DEX and GEL) did not exert similar protection. This underlines the advantages of the use of HES in the postoperative care after trauma surgery.

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8. REFERENCES

Abbitt KB, Nash GB: Rheological properties of the blood influencing selectin-mediated adhesion of flowing leukocytes. Am. J. Physiol. 285:H229-H240, 2003.

Addison PD, Neligan PC, Ashrafpour H, Khan A, Zhong A, Moses M, Forrest CR, Pang CY: Noninvasive remote ischemic preconditioning for global protection of skeletal muscle against infarction. Am. J. Physiol. 285: H1435-H1443, 2003.

Akgur FM, Zibari GB, McDonald JC, Granger DN, Brown MF: Effects of dextran and pentoxifylline on hemorrhagic shock-induced P-selectin expression. J. Surg. Res. 87:232-238, 1999.

Akimitsu T, Gute DC, Korthuis RJ: Ischemic preconditioning attenuates postischemic leukocyte adhesion and emigration. Am. J. Physiol. 271:H2052-H2059, 1996.

Allison KP, Gosling P, Jones S, Pallister I, Porter KM: Randomized trial of hydroxyethyl starch versus gelatine for trauma resuscitation. J. Trauma 47:1114-1121, 1999.

Andreka G, Vertesaljai M, Szantho G, Font G, Piroth Z, Fontos G, Juhasz ED, Szekely L, Szelid Z, Turner MS, Ashrafian H, Frenneaux MP, Andreka P: Remote ischaemic postconditioning protects the heart during acute myocardial infarction in pigs. Heart 93:749-752, 2007.

Atherton A, Born GV: Relationship between the velocity of rolling granulocytes and that of the blood flow in venules. J. Physiol. 233:157-165, 1973.

Barry MC, Wang JH, Kelly CJ, Sheehan SJ, Redmond HP, Bouchier-Hayes DJ: Plasma factors augment neutrophil and endothelial cell activation during aortic surgery. Eur. J. Vasc. Endovasc. Surg. 13:381-387, 1997.

Beauchamp P, Richard V, Tamion F, Lallemand F, Lebreton JP, Vaudry H, Daveau M, Thuillez C: Protective effects of preconditioning in cultured rat endothelial cells: effects on neutrophil adhesion and expression of ICAM-1 after anoxia and reoxygenation. Circulation

100: 541-546, 1999.

Beekhuizen H, van Furth R: Monocyte adherence to human vascular endothelium. J. Leukoc. Biol. 54:363-378, 1993.

Berggren A, Weiland AJ, Ostrup LT, Dorfman H: Microvascular free bone transfer with revascularization of the medullary and periosteal circulation or the periosteal circulation alone. A comparative experimental study. J. Bone Joint Surg. Am. 64:73-87, 1982.

Boldt J: Do plasma substitutes have additional properties beyond correcting volume deficits? Shock 25:103-116, 2006.

Bonucci E, Silvestrini G, Mocetti P: MC22-33F monoclonal antibody shows unmasked polar head groups of choline-containing phospholipids in cartilage and bone. Eur. J. Histochem. 41:177-190, 1997.

Boros M, Kaszaki J, Ördögh B, Nagy S. Mast cell degranulation prior to ischemia decreases ischemia-reperfusion injury in the canine small intestine. Inflamm. Res. 48(4):193-198, 1999.

Bruhl A, Hafner G, Loffelholz K: Release of choline in the isolated heart, an indicator of ischemic phospholipid degradation and its protection by ischemic preconditioning: no evidence for a role of phospholipase D. Life Sci. 75:1609-1620. 2004.

Buchman AL, Dubin MD, Moukarzel AA, Jenden DJ, Roch M, Rice KM, Gornbein J, Ament ME: Choline deficiency; a cause of hepatic steatosis during parenteral nutrition that can be reversed with intravenous choline supplementation. Hepatology 22:1399-1403, 1995.

Chamoun F, Burne M, O'Donnell M, Rabb H: Pathophysiologic role of selectins and their ligands in ischemia reperfusion injury. Fron. Bioscience 5:103-109, 2000.

Chanavaz M: Anatomy and histophysiology of the periosteum: Quantification of the periosteal blood supply to the adjacent bone with 85Sr and gamma spectrometry. J. Oral Implantol. 21:214-219, 1995.

Cheung MM, Kharbanda RK, Konstantinov IE, Shimizu M, Frndova H, Li J, Holtby HM,

Cox PN, Smallhorn JF, Van Arsdell GS, Redington AN: Randomized controlled trial of the effects of remote ischemic preconditioning on children undergoing cardiac surgery: first clinical application in humans. J. Am. Coll. Cardiol. 47:2277-2282, 2006.

Cleland LG, Shandling M, Percy JS, Poznansky MJ: Liposomes: a new approach to gold therapy? J. Rheumatol. Suppl. 5:154-163, 1979.

Cochrane CG: Mechanisms of oxidant injury of cells. Mol. Aspects Med. 12:137-147, 1991.

Cowley HC, Heney D, Gearing AJ, Hemingway I, Webster NR: Increased circulating adhesion molecule concentrations in patients with the systemic inflammatory response syndrome: a prospective cohort study. Crit. Care Med. 22: 651-657, 1994.

Demirbilek S, Aydin G, Yucesan S, Vural H, Bitiren M: Polyunsaturated phosphatidylcholine lowers collagen deposition in a rat model of corrosive esophageal burn. Eur. J. Pediatr. Surg. 12:8-12, 2002.

Dizdaroglu M: Chemistry of free radical damage to DNA and nucleoproteins. In: Halliwell B, Aruoma OI, eds. DNA and free radicals. Chichester: Ellis Harwood, p19-39, 1993.

Duan JM, Karmazyn M: Protection of the reperfused ischemic isolated rat heart by phosphatidylcholine. J. Cardiovasc. Pharmacol. 15:163-171, 1990.

Dunjic BS, Axelson J, Ar'Rajab A, Larsson K, Bengmark S: Gastroprotective capability of exogenous phosphatidylcholine in experimentally induced chronic gastric ulcers in rats. Scand. J. Gastroenterol. 28:89-94, 1993.

el-Hariri LM, Marriott C, Martin GP: The mitigating effects of phosphatidylcholines on bile salt- and lysophosphatidylcholine-induced membrane damage. J. Pharm. Pharmacol. 44:651-654, 1992.

Eppihimer MJ, Granger DN: Ischemia/reperfusion-induced leukocyte-endothelial interactions in postcapillary venules. Shock 8:16-25, 1997.

Erős G, Kaszaki J, Czobel M, Boros M: Systemic phosphatidylcholine pretreatment protects

canine esophageal mucosa during acute experimental biliary reflux. World J. Gastroenterol. 12:271-279, 2006.

Esterhai JL, Queenan J: Management of soft tissue wounds associated with type III open fractures. Orthop. Clin. North Am. 22:427–32. 1991.

Exton JH: Regulation of phospholipase D. Biochim. Biophys. Acta 1439:121-133, 1999.

Fahrner SL, Shackford SR, Bourguignon P, Bednar M, Shatney-Leach L: Effect of medium tonicity and dextran on neutrophil function in vitro. J. Trauma 52:285-292, 2002.

Feng X, Yan W, Wang Z, Liu J, Yu M, Zhu S, Xu J: Hydroxyethyl starch, but not modified fluid gelatin, affects inflammatory response in a rat model of polymicrobial sepsis with capillary leakage. Anesth. Analg. 104:624-630, 2007.

Freeman BA, Crapo JD. Biology of disease: free radicals and tissue injury. Lab. Invest. 47(5):412-426, 1982.

Friedrich A, George RL, Bridges CC, Prasad PD, Ganapathy V: Transport of choline and its relationship to the expression of the organic cation transporters in a rat brain microvessel endothelial cell line (RBE4). Biochim. Biophys. Acta 1512:299-307, 2001.

Gabizon A, Meshorer A, Barenholz Y: Comparative long-term study of the toxicities of free and liposome-associated doxorubicin in mice after intravenous administration. J. Natl. Cancer Inst. 77:459-469, 1986.

Gaboury JP, Johnston B, Niu XF, Kubes P: Mechanisms underlying acute mast cell-induced leukocyte rolling and adhesion in vivo. J. Immunol. 154:804-813, 1995.

Gho BC, Schoemaker RG, van den Doel MA, Duncker DJ, Verdouw PD: Myocardial protection by brief ischemia in noncardiac tissue. Circulation 94:2193-2200, 1996.

Ghyczy M, Boros M: Electrophilic methyl groups present in the diet ameliorate pathological states induced by reductive and oxidative stress: A hypothesis. Br. J. Nutr. 85:409-414, 2001.

Ghyczy M, Torday C, Boros M: Simultaneous generation of methane, carbon dioxide, and

carbon monoxide from choline and ascorbic acid: a defensive mechanism against reductive stress? FASEB J. 17:1124-1126, 2003.

Gombocz K, Beledi A, Alotti N, Kecskés G, Gábor V, Bogár L, Kőszegi T, Garai J: Influence of dextran-70 on systemic inflammatory response and myocardial ischaemia-reperfusion following cardiac operations. Crit Care; 11:R87, 2007.

Granger DN, Höllwarth ME, Parks DA: Ischemia-reperfusion injury: role of oxgen-derived free radicals. Acta. Physiol. Scan. (Suppl.) 548:47-63, 1986.

Granger DN, Kubes P: The microcirculation and inflammation: modulation of leukocyteendothelial cell adhesion. J. Leukoc. Biol. 55:662-675, 1994.

Granger DN, Rutili G, McCord JM: Superoxide radicals in feline intestinal ischemia. Gastroenterol. 81:22-29, 1981.

Grisham MB, Hernandez LA, Granger DN: Xanthine oxidase and neutrophil infiltration in intestinal ischemia. Am. J. Physiol. 251:G567-574, 1986.

Gross RW: Myocardial phospholipases A_2 and their membrane substrates. Trends Cardiovasc. Med. 2:115-124, 1992.

Gurcun U, Discigil B, Boga M, Ozkisacik E, Badak MI, Yenisey C, Kurtoglu T, Meteoglu I: Is remote preconditioning as effective as direct ischemic preconditioning in preventing spinal cord ischemic injury? J. Surg. Res. 135:385-393, 2006.

Gustilo RB, Mendoza RM, Williams DN: Problems in the management of type III (severe) open fractures. J. Trauma 24:742–746, 1984.

Gustilo RB, Merkow RL, Templeman D: The management of open fractures. J. Bone Joint Surg. Am. 72:299–304. 1990.

Hambsch J, Osmancik P, Bocsi J, Schneider P, Tárnok A: Neutrophil adhesion molecule expression and serum concentration of soluble adhesion molecules during and after pediatric cardiovascular surgery with or without cardiopulmonary bypass. Anesthesiology 96:1078-

1085, 2002.

Han B, Tang B, Nimni ME: Combined effects of phosphatidylcholine and demineralized bone matrix on bone induction. Connec. Tissue Res. 44:160-166, 2003.

Hansen HS, Moesgaard B, Hansen HH, Peterson G: N-Acylethanolamines and precursor phospholipids-relation to cell injury. Chem. Phys. Lipids 108:135-150, 2000.

Harkin DW, Barros D'Sa AA, McCallion K, Hoper M, Campbell FC: Ischemic preconditioning before lower limb ischemia-reperfusion protects against acute lung injury. J. Vasc. Surg. 35:1264-1273, 2002.

Harris AG, Leiderer R, Peer F, Messmer K.: Skeletal muscle microvascular and tissue injury after varying durations of ischemia. Am. J. Physiol. 271:H2388-H2398, 1996.

Harris AG, Steinbauer M, Leiderer R, Messmer K: Role of leukocyte plugging and edema in skeletal muscle ischemia-reperfusion injury. Am. J. Physiol. 273:H989-H996, 1997.

Hernandez LA, Grisham MB, Twohig B, Arfors KE, Harlan JM, Granger DN: Role of neutrophils in ischemia-reperfusion-induced microvascular injury. Am. J. Physiol. 253:H699-H703, 1987.

Hoffmann JN, Vollmar B, Laschke MW, Inthorn D, Schildberg FW, Menger MD: Hydroxyethyl starch (130 kD), but not crystalloid volume support, improves microcirculation during normotensive endotoxemia. Anesthesiology 97:460-470, 2002.

Huda R, Solanki DR, Mathru M: Inflammatory and redox responses to ischaemia/reperfusion in human skeletal muscle. Clin Sci (Lond) 107:497-503, 2004.

Ichikawa H, Flores S, Kvietys PR, Wolf RE, Yoshikawa T, Granger DN, Aw TY: Molecular mechanisms of anoxia/reoxygenation-induced neutrophil adherence to cultured endothelial cells. Circ. Res. 81:922-931, 1997.

Jaeger K, Heine J, Ruschulte H, Jüttner B, Scheinichen D, Kuse ER, Piepenbrock S: Effects of colloidal resuscitation fluids on the neutrophil respiratory burst. Transfusion 41:1064-1068,

2001.

Jiang SH, Liu CF, Zhang XL, Xu XH, Zou JZ, Fang Y, Ding XQ: Renal protection by delayed ischaemic preconditioning is associated with inhibition of the inflammatory response and NF-kappaB activation. Cell Biochem. Funct. 25:335-343, 2007.

Jones DH, Anderson DC, Burr BL, Rudloff HE, Smith CW, Krater SS, Schmalstieg FC: Quantitation of intracellular Mac-1 (CD11b/CD18) pools in human neutrophils. J. Leukoc. Biol. 44:535-544, 1988.

Jones DP: Radical-free biology of oxidative stress. Am. J. Physiol. 295(4):C849-68, 2008

Jones RL, Miller JC, Hagler HK, Chien KR, Willerson JT, Buja ML: Association between inhibition of arachidonic acid release and prevention of calcium loading during ATP depletion is cultured rat cardiac myocytes. Am. J. Path. 135:541-556, 1989.

Kacimi R, Karliner JS, Koudssi F, Long CS: Expression and regulation of adhesion molecules in cardiac cells by cytokines: response to acute hypoxia. Circ. Res. 82:576-586, 1998.

Kaeffer N, Richard V, François A, Lallemand F, Henry JP, Thuillez C: Preconditioning prevents chronic reperfusion-induced coronary endothelial dysfunction in rats. Am. J. Physiol. 271:H842-H849, 1996.

Kanoria S, Jalan R, Davies NA, Seifalian AM, Williams R, Davidson BR: Remote ischaemic preconditioning of the hind limb reduces experimental liver warm ischaemia-reperfusion injury. Br. J. Surg. 93:762-768, 2006.

Kanwar S, Kubes P: Ischemia/reperfusion-induced granulocyte influx is a multistep process mediated by mast cells. Microcirculation 1:175-182, 1994.

Kaplan SS, Park TS, Gonzales ER, Gidday JM: Hydroxyethyl starch reduces leukocyte adherence and vascular injury in the newborn pig cerebral circulation after asphyxia. Stroke 31:2218–2223, 2000.

Kharbanda RK, Peters M, Walton B, Kattenhorn M, Mullen M, Klein N, Vallance P, Deanfield J, MacAllister R: Ischemic preconditioning prevents endothelial injury and systemic neutrophil activation during ischemia-reperfusion in humans in vivo. Circulation 103:1624-1630, 2001.

Kidd PM: Phosphatidylcholine, a superior protectant against liver damage. Altern. Med. Rev. 1:258-274, 1996.

Korthuis RJ, Grisham MB, Granger DN: Leukocyte depletion attenuates vascular injury in postischemic skeletal muscle. Am. J. Physiol. 254:H823-H827, 1988.

Kowalski MJ, Schemitsch EH, Kregor PJ, Senft D, Swiontkowski MF: Effect of periosteal stripping on cortical bone perfusion: a laser-Doppler study in sheep. Calcif. Tissue Int. 59:24–26, 1996.

Kubes P, Kanwar S: Histamine induces leukocyte rolling in postcapillary venules. A P-selectin-mediated event. J. Immunol. 152:3570-3577, 1994.

Kubes P: The role of shear forces in ischemia/reperfusion-induced neutrophil rolling and adhesion. J. Leukoc. Biol. 62:458-464, 1997.

Kuebler WM, Abels C, Schuerer L, Goetz AE: Measurement of neutrophil content in brain and lung tissue by a modified myeloperoxidase assay. Int. J. Microcirc. Clin. Exp. 16:89-97, 1996.

Kuehl FA, Jacob TA, GanleyOH, Ormond RE, Meisinger MAP: The identification of N-(2-hydroxyethyl)-palmitamide as a naturally occuring anti-inflammatory agent. J. Am. Chem. Soc. 79:5577-5578, 1957.

Kurose I, Anderson DC, Miyasaka M, Tamatani T, Paulson JC, Todd RF, Rusche JR, Granger DN: Molecular determinants of reperfusion-induced leukocyte adhesion and vascular protein leakage. Circ. Res. 74:336-343, 1994a.

Kurose I, Argenbright LW, Wolf R, Lianxi L, Granger DN: Ischemia/reperfusion-induced

microvascular dysfunction: role of oxidants and lipid mediators. Am. J. Physiol. 272:H2976-H2982, 1997.

Kurose I, Granger DN: Evidence implicating xanthine oxidase and neutrophils in reperfusion-induced microvascular dysfunction. Ann. N. Y. Acad. Sci. 723:158-179, 1994b.

Kwak HB, Lee SW, Li YJ, Kim YA, Han SY, Jhon GJ, Kim HH, Lee ZH: Inhibition of osteoclast differentiation and bone resorption by a novel lysophosphatidylcholine derivative, SCOH. Biochem. Pharmacol. 67:1239-1248, 2004.

Lang K, Suttner S, Boldt J, Kumle B, Nagel D: Volume replacement with HES 130/0.4 may reduce the inflammatory response in patients undergoing major abdominal surgery. Can. J. Anaesth. 50:1009-1016, 2003.

Ley K, Gaehtgens P: Endothelial, not hemodynamic, differences are responsible for preferential leukocyte rolling in rat mesenteric venules. Circ. Res. 69:1034-1041, 1991.

Lieber CS, Leo MA, Aleynik SI, Aleynik MK, DeCarli LM: Polyenylphosphatidylcholine decreases alcohol-induced oxidative stress in the baboon. Alcohol Clin. Exp. Res. 21:375-379, 1997.

Lindholm RV, Lindholm S, Liukko P: Fracture healing and mast cells. I. The periosteal callus in rats. Acta Orthop. Scand. 38:115, 1967.

Lindholm RV, Lindholm TS: Mast cells in endosteal and periosteal bone repair. A quantitative study on callus tissue of healing fractures in rabbits. Acta Orthop. Scand. 41:129-133, 1970.

Lorant DE, Patel KD, McIntyre TM, McEver RP, Prescott SM, Zimmerman GA: Co-expression of GMP-140 and PAF by endothelium stimulated by histamine or thrombin: a juxtacrine system for adhesion and activation of neutrophils. J. Cell. Biol. 115:223–234, 1991.

Loukogeorgakis SP, Williams R, Panagiotidou AT, Kolvekar SK, Donald A, Cole TJ, Yellon DM, Deanfield JE, MacAllister RJ: Transient limb ischemia induces remote preconditioning

and remote postconditioning in humans by a K(ATP)-channel dependent mechanism. Circulation 116:1386-1395, 2007.

Lundberg AH, Fukatsu K, Gaber L, Callicutt S, Kotb M, Wilcox H, Kudsk K, Gaber AO: Blocking pulmonary ICAM-1 expression ameliorates lung injury in established dietinduced pancreatitis. Ann. Surg. 233:213-220, 2001.

Ma XL, Weyrich AS, Lefer DJ, Lefer AM: Diminished basal nitric oxide release after myocardial ischemia and reperfusion promotes neutrophil adherence to the coronary endothelium. Circ. Res. 72:403–412, 1993.

Mallick IH, Yang W, Winslet MC, Seifalian AM: Protective effects of ischemic preconditioning on the intestinal mucosal microcirculation following ischemia-reperfusion of the intestine. Microcirculation 12: 615-625, 2005.

Marshall BT, Long M, Piper JW, Yago T, McEver RP, Zhu C: Direct observation of catch bonds involving cell-adhesion molecules. Nature 423:190-193, 2003.

Martin GP, Marriott C: Membrane damage by bile salts: the protective function of phospholipids. J. Pharm. Pharmacol. 33:754-759, 1981.

Marx G, Pedder S, Smith L, Swaraj S, Grime S, Stockdale H, Leuwer M: Resuscitation from septic shock with capillary leakage: hydroxyethyl starch (130 kd), but not Ringer's solution maintains plasma volume and systemic oxygenation. Shock 21:336-341, 2004.

McGrath AM, Conhaim RL, Myers GA, Harms BA: Pulmonary vascular filtration of starch-based macromolecules: effects on lung fluid balance. J. Surg. Res. 65:128-134, 1996.

Meldrum DR, Dinarello CA, Shames BD, Cleveland JC Jr, Cain BS, Banerjee A, Meng X, Harken AH: Ischemic preconditioning decreases postischemic myocardial tumor necrosis factor-alpha production. Potential ultimate effector mechanism of preconditioning. Circulation 98(Suppl):II214-218, 1998.

Menger MD, Thierjung C, Hammersen F, Messmer K: Dextran vs. hydroxyethylstarch in

inhibition of postischemic leukocyte adherence in striated muscle. Circ. Shock 41:248-255, 1993.

Meyer MC, Creer MH, McHowat J: Potential role for mast cell tryptase in recruitment of inflammatory cells to endothelium. Am. J. Physiol. 289:C1485-1491, 2005.

Murphy E, Steenbergen C: Preconditioning: the mitochondrial connection. Annu. Rev. Physiol. 69:51-67, 2007.

Murry CE, Jennings RB, Reimer KA: Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 74:1124-1136, 1986.

Nohé B, Johannes T, Reutershan J, Rothmund A, Haeberle HA, Ploppa A, Schroeder TH, Dieterich HJ: Synthetic colloids attenuate leukocyteendothelial interactions by inhibition of integrin function. Anesthesiology 103:759-767, 2005.

Oldenburg O, Qin Q, Sharma AR, Cohen MV, Downey JM, Benoit JN: Acetylcholine leads to free radical production dependent on K(ATP) channels, G(i) proteins, phosphatidylinositol 3-kinase and tyrosine kinase. Cardiovasc. Res. 55:544-552, 2002.

Park KM, Chen A, Bonventre JV: Prevention of kidney ischemia/reperfusion-induced functional injury and JNK, p38, and MAPK kinase activation by remote ischemic pretreatment. J. Biol. Chem. 276:1870-1876, 2001.

Parks DA, Granger DN: Contributions of ischemia and reperfusion to mucosal lesion formation. Am. J. Physiol. 250:G749-753, 1986.

Pascual JL, Ferri LE, Chaudhury P, Seely AJ, Campisi G, Giannias B, Evans DC, Christou NV: Hemorrhagic shock resuscitation with a low molecular weight starch reduces neutrophil-endothelial interactions and vessel leakage in vivo. Surg. Infect. (Larchmt) 2:275-287, 2001.

Rittoo D, Gosling P, Burnley S, Bonnici C, Millns P, Simms MH, Smith SR, Vohra RK: Randomized study comparing the effects of hydroxyethyl starch solution with Gelofusine on pulmonary function in patients undergoing abdominal aortic aneurysm surgery. Br. J.

Anaesth. 92:61-66, 2004.

Rizoli SB, Rhind SG, Shek PN, Inaba K, Filips D, Tien H, Brenneman F, Rotstein O: The immunomodulatory effects of hypertonic saline resuscitation in patients sustaining traumatic hemorrhagic shock: a randomized, controlled, double-blinded trial. Ann. Surg. 243:47-57, 2006.

Roche AM, James MF, Bennett-Guerrero E, Mythen MG: A head-to-head comparison of the in vitro coagulation effects of saline-based and balanced electrolyte crystalloid and colloid intravenous fluids. Anesth. Analg. 102:1274-1279, 2006.

Roebuck KA, Rahman A, Lakshminarayanan V, Janakidevi K, Malik AB: H2O2 and tumor necrosis factor-alpha activate intercellular adhesion molecule 1 (ICAM-1) gene transcription through distinct cis-regulatory elements within the ICAM-1 promoter. J. Biol. Chem. 270:18966-18974, 1995.

Rucker M, Roesken F, Vollmar B, Menger MD: A novel approach for comparative study of periosteum, muscle, subcutis, and skin microcirculation by intravital fluorescence microscopy. Microvasc. Res. 56:30-42, 1998.

Ruh J, Ryschich E, Secchi A, Gebhard MM, Glaser F, Klar E, Herfarth C: Measurement of blood flow in the main arteriole of the villi in the rat small intestine with FITC-labelled erythrocytes. Microcirc. Res. 56:62-69, 1998.

Saffar JL, Klapisz-Wolikow M: Changes in mast cell number during the activation phase of an induced synchronized remodeling sequence in the rat. Bone 11:369-372, 1990.

Sawa Y, Morishita R, Suzuki K, Kagisaki K, Kaneda Y, Maeda K, Kadoba K, Matsuda H: A novel strategy for myocardial protection using in vivo transfection of cis element 'decoy' against NFkappaB binding site: evidence for a role of NFkappaB in ischemia-reperfusion injury. Circulation 96(9 Suppl):II-280-284, 1997.

Schmeling DJ, Caty MG, Oldham KT, Guice KS, Hinshaw DB: Evidence for neutrophil-

related acute lung injury after intestinal ischemia-reperfusion. Surgery 106:195-201, 1989.

Serrick C, Adoumie R, Graid A, Shennib H: The early release of interleukin-2, tumor necrosis factor-alpha and interferon-gamma after ischemia reperfusion injury in the lung allograft. Transplantation 58:1158-1162, 1994.

Shpacovitch VM, Varga G, Strey A, Gunzer M, Mooren F, Buddenkotte J, Vergnolle N, Sommerhoff CP, Grabbe S, Gerke V, Homey B, Hollenberg M, Luger TA, Steinhoff M: Agonists of proteinase-activated receptor-2 modulate human neutrophil cytokine secretion, expression of cell adhesion molecules, and migration within 3-D collagen lattices. J. Leukoc. Biol. 76:388-398, 2004.

Spark JI, Chetter IC, Kester RC, Guillou PJ, Scott DJ: The regulation of neutrophil activation and adhesion during femorodistal bypass surgery. Eur. J. Vasc. Endovasc. Surg. 13:407-412, 1997.

Springer TA: Adhesion receptors of the immune system. Nature 346:425-434, 1990.

Springer TA: Traffic signals for lymphocyte recirculation and leukocyte emigration: The multistep paradigm. Cell 76:301-314, 1994.

Steinbauer M, Harris AG, Leiderer R, Abels C, Messmer K: Impact of dextran on microvascular disturbances and tissue injury following ischemia/reperfusion in striated muscle. Shock 9:345-351, 1998.

Szabó A, Boros M, Kaszaki J, Nagy S: The role of mast cells in mucosal permeability changes during ischemia-reperfusion injury of the small intestine. Shock 8:284-291; 1997.

Szabó A, Suki B, Csonka E, Eszlári E, Kucsa K, Vajda K, Kaszaki J, Boros M: Flowmotion in the intestinal villi during hemorrhagic shock. A new method to characterize the microcirculatory changes. Shock 21:320-329, 2004.

Tian J, Lin X, Guan R, Xu JG: The effects of hydroxyethyl starch on lung capillary permeability in endotoxic rats and possible mechanisms. Anesth. Analg. 98:768-774, 2004.

Tsao PS, Aoki N, Lefer DJ, Johnson G, Lefer AM: Time course of endothelial dysfunction and myocardial injury during myocardial ischemia and reperfusion in the cat. Circulation 82:1402–1412, 1990.

Utvag SE, Grundnes O, Reikeras O: Effects of lesion between bone, periosteum and muscle on fracture healing in rats. Acta Orthop. Scand. 69:177–180, 1998.

Wang WZ, Fang XH, Stepheson LL, Khiabani KT, Zamboni WA: NOS upregulation attenuates vascular endothelial dysfunction in the late phase of ischemic preconditioning in skeletal muscle. J. Orthop. Res. 22:578-585, 2004.

Wang WZ, Tsai TM, Anderson GL: Late-preconditioning protection is evident in the microcirculation of denervated skeletal muscle. J. Orthop. Res. 17:571-577, 1999.

Wanner GA, Ertel W, Müller P, Höfer Y, Leiderer R, Menger MD, Messmer K: Liver ischemia and reperfusion induces a systemic inflammatory response through Kupffer cell activation. Shock 5:34-40, 1996.

Welbourn R, Goldman G, O'Riordain M, Lindsay TF, Paterson IS, Kobzik L, Valeri CR, Shepro D, Welters ID, Spangenberg U, Menzebach A, Engel J, Menges T, Langefeld TW, Hempelmann G: The effect of different volume expanders on neutrophil granulocyte function in vitro. Anaesthesist 49:196-201, 2000.

Weyrich AS, Buerke M, Albertine KH, Lefer AM: Time course of coronary vascular endothelial molecule expression during reperfusion of the ischemic feline myocardium. J. Leuk. Biol. 57:45–55, 1995.

Williams AF, Barclay AN: The immunoglobulin superfamily--domains for cell surface recognition. Annu. Rev. Immunol. 6:381-405, 1988.

Wolfárd A, Császár J, Gera L, Petri A, Simonka JA, Balogh A, Boros M: Endothelin-A receptor antagonist treatment improves the periosteal microcirculation after hindlimb ischemia and reperfusion in the rat. Microcirculation 9:471-476, 2002.

Yan JJ, Jung JS, Lee J, Huh SO, Kim HS, Jung KC, Cho JY, Nam JS, Suh HW, Kim YH, Song DK: Therapeutic effects of lysophosphatidylcholine in experimental sepsis. Nat Med. 10:161-167, 2004.

Zahler S, Kupatt C, Becker BF: Endothelial preconditioning by transient oxidative stress reduces inflammatory responses of cultured endothelial cells to TNF-alpha. FASEB J. 14:555-564, 2000.

Zeisel SH, Da Costa KA, Franklin PD, Alexander EA, Lamont JT, Sheard NF, Beiser A: Choline, an essential nutrient for humans. FASEB J. 5:2093-2098, 1991.

9. ANNEX