Microcirculatory damage of the periosteum
From clinical case history to animal experiments and back to the bedside

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List of full papers related to the subject of the thesis


List of abstracts related to the subject of the thesis


## CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>List of papers related to the subject of the thesis</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>List of abbreviations</td>
<td>4</td>
</tr>
<tr>
<td>1.</td>
<td>BACKGROUND</td>
<td></td>
</tr>
<tr>
<td>1.1.</td>
<td>The periosteum</td>
<td>5</td>
</tr>
<tr>
<td>1.2.</td>
<td>Role of the periosteum in fracture healing</td>
<td>6</td>
</tr>
<tr>
<td>1.3.</td>
<td>Vascularity of fracture healing</td>
<td>7</td>
</tr>
<tr>
<td>1.4.</td>
<td>Avascular bone necrosis in joint dislocations</td>
<td>9</td>
</tr>
<tr>
<td>1.5.</td>
<td>Microcirculatory consequences of ischemia-reperfusion injury</td>
<td>10</td>
</tr>
<tr>
<td>1.6.</td>
<td>Significance of volume therapy in circulatory disturbances</td>
<td>11</td>
</tr>
<tr>
<td>2.</td>
<td>MAIN GOALS</td>
<td>17</td>
</tr>
<tr>
<td>3.</td>
<td>MATERIALS AND METHODS</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>A case history</td>
<td>18</td>
</tr>
<tr>
<td>3.2.</td>
<td>Animal experiments</td>
<td>18</td>
</tr>
<tr>
<td>3.2.1.</td>
<td>Surgical procedures</td>
<td>18</td>
</tr>
<tr>
<td>3.2.2.</td>
<td>Hemodynamic measurements</td>
<td>19</td>
</tr>
<tr>
<td>3.2.3.</td>
<td>Experimental protocol</td>
<td>19</td>
</tr>
<tr>
<td>3.2.4.</td>
<td>Intravital videomicroscopy</td>
<td>20</td>
</tr>
<tr>
<td>3.2.5.</td>
<td>Video analysis</td>
<td>21</td>
</tr>
<tr>
<td>3.2.6.</td>
<td>Myeloperoxidase measurements</td>
<td>21</td>
</tr>
<tr>
<td>3.2.7.</td>
<td>Statistical analysis</td>
<td>21</td>
</tr>
<tr>
<td>4.</td>
<td>RESULTS</td>
<td>22</td>
</tr>
<tr>
<td>4.1.</td>
<td>Human study</td>
<td>22</td>
</tr>
<tr>
<td>4.2.</td>
<td>Experimental study I. Effects of ischemia-reperfusion on the postischemic periosteal microcirculatory changes</td>
<td>22</td>
</tr>
<tr>
<td>4.2.1.</td>
<td>Changes in microperfusion</td>
<td>22</td>
</tr>
<tr>
<td>4.2.2.</td>
<td>Leukocyte-endothelial cell interactions</td>
<td>24</td>
</tr>
<tr>
<td>4.3.</td>
<td>Experimental study II. Effects of colloid therapy on the consequences of ischemia-reperfusion</td>
<td>26</td>
</tr>
<tr>
<td>4.3.1.</td>
<td>Changes in macrohemodynamic parameters</td>
<td>26</td>
</tr>
<tr>
<td>4.3.2.</td>
<td>Changes in microperfusion variables</td>
<td>28</td>
</tr>
<tr>
<td>4.3.3.</td>
<td>Changes in leukocyte accumulation in the soft tissue</td>
<td>33</td>
</tr>
<tr>
<td>5.</td>
<td>DISCUSSION</td>
<td>34</td>
</tr>
</tbody>
</table>
5.1. Human study 34
5.2. Experimental study I. 36
5.3. Experimental study II. 37
6. SUMMARY OF NEW FINDINGS 41
7. REFERENCES 42
8. ACKNOWLEDGMENTS 49
9. ANNEX 50

List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALB</td>
<td>albumin</td>
</tr>
<tr>
<td>CO</td>
<td>cardiac output</td>
</tr>
<tr>
<td>DEX</td>
<td>dextran</td>
</tr>
<tr>
<td>ET</td>
<td>endothelin</td>
</tr>
<tr>
<td>FCD</td>
<td>functional capillary density</td>
</tr>
<tr>
<td>GEL</td>
<td>gelatine</td>
</tr>
<tr>
<td>HES</td>
<td>hydroxyethyl starch</td>
</tr>
<tr>
<td>HR</td>
<td>heart rate</td>
</tr>
<tr>
<td>I-R</td>
<td>ischemia-reperfusion</td>
</tr>
<tr>
<td>LR</td>
<td>lactated Ringer’s solution</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>RBCV</td>
<td>red blood cell velocity</td>
</tr>
</tbody>
</table>
1. BACKGROUND

Direct and indirect trauma forces leading to fractures usually involve injury to the periosteum, the outer covering layer of the skeleton. Stability and controlled micro-motions of the bones are very important components of trauma therapies, but the biological response of the periosteal sheet is also an essential factor in the repair mechanism of the bony architecture. Indeed, it has been shown that the periosteum not only protects and feeds the bone cortex, but also strongly influences osteogenesis (Macnab 1974).

Apart from traumas, periosteal damage can be a consequence of clinical interventions. Various emergency and elective orthopedic operations are performed under artificially reduced blood flow conditions, leading to regional ischemia differing in degree and duration. As an example, vascularized bone autografts are frequently used in reconstructive surgery for the replacement of large bone defects. Harmful consequences of the re-establishment of the vascular supply have often been observed after technically successful replantations or free flap transplantations (Rücker 1999, 2002, 2003). Today it is well established that the primary ischemic damage (when the bloodless period exceeds the critical tolerance duration) is significantly aggravated by secondary events during reperfusion. The ischemia-reperfusion (I-R) phenomenon includes various pathophysiological cascade mechanisms which enhance local and remote tissue injuries (Schoenberg 1985; Boros 2003). Tourniquet methods per se are I-R events, which can cause healing disorders, including delayed bone healing, pseudoarthrosis or sequester formation (Gustilo 1990; Esterhai 1991; Utvag 1998).

Apart from local occlusion-induced circulatory derangements, similar I-R events may occur in any type of circulatory shock (Schlag 1988), which can influence the microperfusion of the periosteum and the bone. The microcirculation in general can be analyzed in vivo by intravital microscopy, a dynamic technique used to observe and quantify the microcirculatory alterations in well-defined structures in living tissues. However, as opposed to other organs, characterization of the human bone or periosteal microcirculation is still incomplete. Some observations have suggested that the periosteal microcirculation may be a good indicator of the perfusion changes of the whole bone (Rücker 1998), but the exact microcirculatory components involved in the pathogenesis of skeletal I-R remain unknown, mainly because of methodological limitations.

The few animal studies that have been reported have focused mainly on the effects of a flow reduction or soft tissue trauma-induced local microcirculatory reactions of the periosteal microcirculation (Rücker 2001, 2003; Menger 2003; Schaser 2003). For this reason, there is a clear need for the investigation and characterization of periosteal microcirculatory...
reactions in animal models of clinical I-R conditions, such as bone autotransplantation or
tourniquet ischemia.

Trauma events can lead relatively frequently to avascular bone necrosis, a time- and
region-dependent ischemic injury resulting from temporary or permanent loss of the blood
perfusion to the bones. Although this can occur in any bone, there are special sites where the
risk of avascular necrosis is very high, e.g. the peritalar region. The talus is the only bone in
the lower extremity without any muscular attachments, making it somewhat vulnerable in the
event of injury. Talus fractures can often be accompanied by partial or total talus necrosis.
Most talus body or neck fractures are observed relatively frequently and this injury can also
accompany supra- or subtalar sprains.

As concerns trauma care, elimination of the factors obstructing the bone and periosteal
microcirculation is the primary goal. The following important step is the use of
pharmacological treatment to improve the blood supply to the injured region. In line with this,
effective fluid therapy is of the utmost importance in critically ill patients with trauma-
associated injury and blood loss, in order to restore the impaired tissue perfusion. The
administration of intravenous fluid is indicated to avoid dehydration, to maintain an effective
circulating volume, and to prevent inadequate tissue perfusion. These aims should all be
considered core elements of perioperative practice (Grocott 2005). 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 global circulatory effects of colloid or crystalloids
fluid therapy have been examined in several studies (Boldt 2006), but their influences on the
periosteum have not been analyzed yet.

On the basis of this background, this thesis focuses on the recognition of periosteal
microcirculatory damage and its importance in the clinical practice of traumatology. Analysis
of the experimental post-ischemic changes in the periosteal microcirculation could provide
important information via which to improve our knowledge not only on the complications of
fractures or bone autotransplantation, but also on other trauma cases accompanied by
temporary bloodless conditions.

1.1. The periosteum

The periosteum is a double-layered membrane covering the outer surface of bones,
apart from those parts enclosed in joint capsules. It is composed of an outer fibrous, and an
inner osteogenic cellular layer. The functions of the periosteum include the isolation of the
bone from the surrounding tissues, providing a route for the circulatory and the nervous
supply. The elastic and contractile features preserve the shape of the bone, and it also plays a role in the maintenance of the metabolic and electrochemical gradients between the sides of the membrane. Moreover, a proprioceptive role has been attributed to the periosteum.

Periosteal healing begins within days of the injury, but it requires an adequate blood supply and low local mechanical strain (Malizos 2005). As for the blood supply, the bone cortex exhibits predominance as compared with that of the centromedullary part, since about 70-80% of the arterial supply is directed toward the cortex (Chanavaz 1995). The importance of the periosteal microcirculation is hallmarked by the observation that restoration of the periosteal microcirculation per se guarantees the survival of the bone graft even in an environment of a moderate blood supply (Berggren 1982). For this reason, the periosteal microcirculation is a good indicator of the perfusion changes of the whole bone, particularly during the early reperfusion phase after bone autotransplantation (Rücker 2003).

1.2. Role of the periosteum in fracture healing

An adequate blood supply and sufficient mechanical stability are necessary for timely fracture healing. Damage to vessels impairs the blood supply and the transport of oxygen, an essential metabolite for the cells involved in repair. The degree of mechanical stability determines the mechanical conditions in the healing tissues. The mechanical conditions can influence tissue differentiation and may also inhibit revascularization. The importance of the intraosseal blood vessels in the healing of fractures has long been known, but the blood supply of the periosteum has been relatively ignored. Lexer first suggested the importance of the periosteal integrity in the normal healing of fractures (Lexer 1925). Consequently, if the periosteum is the foremost osteogenic factor in the regeneration of bone, the blood supply of the periosteum should be of paramount importance in the normal healing of fractures.

The healing process in the event of a fracture of a normal bone begins with periosteal callus formation. The endosteum is not able to participate in the callus formation until the intraosseal blood circulation recovers by formation of an anastomosis between the central portion of the nutrient artery and the metaphyseal blood vessels. Only after this anastomosis has formed does the endosteum begin to produce endosteal callus. If the periosteal blood vessels have been destroyed by separation of the surrounding tissues, periosteal osteogenesis can not take place until the blood supply has returned to normal. Endosteal osteogenesis can not begin before the intraosseal circulation has recovered.

Fracture healing under unstable conditions is characterized by the proliferation of mesenchymal cells with the formation of external callus. The first step in the reparative
process is the organization of the fracture hematoma. This plays hardly any mechanical role in immobilizing the fracture, but serves as a fibrin network for the proliferation and migration of the osteogenic cells (Cruess 1975). Mizuno et al. demonstrated different properties of the fracture hematoma at different intervals following the fracture (Mizuno 1990). The influence of the periosteum on new bone formation has been addressed by many authors (Siffert 1955; Finley 1978). It is now generally accepted that the periosteum alone does not induce new bone formation; it does so in conjunction with the fracture hematoma. In secondary union, the periosteum is the principal source of blood vessels (Mindell 1971). On the other hand, additional damage to the periosteum can be due to surgical stripping by open methods, which may be critical for ischemic bone cells and lead to excessive necrosis at the fracture site. Furthermore, it has been shown that even small variations in the microenvironment around the fracture influence the healing process (Bassett 1962). Among other factors, a low oxygen tension leads to the formation of cartilage instead of bone tissue (Heppenstall 1976). Fractures associated with extensive soft tissue trauma are known to heal slowly. This is most probably due to high-energy damage to the soft tissue envelope around the fracture. In addition to fracture healing, mesenchymal cells must also aid in the healing of the soft tissues (Hulth 1989). Kernek and Perry compared open versus closed pin insertion in tibial fractures, and found better healing with the closed method in low-energy fractures, but not in severely displaced fractures (Kernek 1981). They suggested that surgery in high-energy fractures probably does not add any significant further damage to the soft tissues. The importance of an intact periosteum has been demonstrated by Macnab and De Haas (Macnab 1974). They showed that, when the periosteal seal was destroyed, fibrous tissue derived from the soft tissue surroundings could infiltrate between the bone ends, with a tendency to fibrous union. Destruction of the periosteal seal also makes it possible for the local hematoma to escape into the soft tissue with the diffusion of mesenchymal cells.

Fracture healing is a complex process that requires the recruitment, proliferation and differentiation of mesenchymal stem cells into chondrocytes and osteoblasts, and involves both endochondral ossification, whereby bone formation occurs through a cartilage intermediate, and intramembranous ossification, in which bone forms directly from differentiated osteoblasts (Einhorn 1988). Healing occurs when the bone gap is bridged by woven bone and is completed with remodeling and the formation of mature lamellar bone.

Immediately following a skeletal injury, a sequence of biochemical and cellular events commences to induce an inflammatory response. A myriad of factors, including growth factors, cytokines and prostaglandins, are released. These factors are likely to play essential
roles in initiating the healing response that leads to new bone formation (Probst 1997). The crucial events in adult bone formation are the recruitment, proliferation and differentiation of mesenchymal stem cells with endochondral and intramembranous bone formation at the injury site (Bruder 1994). In endochondral ossification, mesenchymal cells first differentiate into chondrocytes, which subsequently undergo terminal differentiation and apoptosis, leading to calcification of the matrix. The calcified matrix then serves as a template for primary bone formation, whereby osteoblasts deposit bone directly onto calcified cartilage. In intramembranous bone formation, mesenchymal cells differentiate directly into osteoblasts (de Crombrugghe 2001). Thus, both endochondral and intramembranous bone formation are dependent upon osteoblast differentiation from mesenchymal stem cells. Although the mechanisms of recruitment and stimulation of mesenchymal stem cell differentiation during adult bone regeneration are largely unknown, there is evidence suggesting that local inflammation plays an important role in the process.

1.3. Vascularity of fracture healing

Successful fracture healing depends upon reconstitution of the disrupted vascular supply at the fracture site. The vascular supply of intact adult long bones arises from the nutritive arteries and the epiphyseal and metaphyseal vessels (Hooper 1987). There are three major sources of blood flow:

a. The nutrient artery enters the cortical diaphysis and immediately divides, sending a main branch proximally and distally within the endosteal canal.

b. Smaller metaphyseal arteries enter the bone near its ends. These arteries supply the metaphyseal region and form an anastomotic system with the endosteal supply coming from the nutrient artery.

c. The bone is also perfused by small vessels rising from the periosteum that are adherent to the outer surface of the bone. Smaller contributors include vessels entering the bone in the epiphyseal region that also add to the endosteal circulation.

In normal, uninjured bone, the endosteal anastomotic circulation perfuses approximately the inner two-thirds of the cortex. Most of the metaphyseal bone is also perfused by endosteal circulation arising from the metaphyseal arteries. The outer one-third of the cortex is perfused by the periosteal vasculature. Fractures completely disrupt the endosteal blood supply. The periosteal blood supply is also disrupted locally around the fracture. The severity of the injury is thought to be directly related to the severity of the disruption of the
periosteal blood supply. This results in an early period of ischemia that leads to necrosis of the fractured ends of the bone.

Local tissue necrosis and inflammation send potent signals to initiate a vigorous revascularization of the fractured bone and surrounding callus. There are marked increases in blood flow rate within weeks of the fracture. The endosteal circulation is reconstituted across the fracture prior to reconstruction of the periosteal circulation. There is strong evidence that the revascularization process is sensitive to the local mechanical environment, especially in the periosteal region (Malizos 2005). Excessive interfragmentary motion has been shown to reduce the density of new blood vessels.

The vascular response can be considerably altered by surgical intervention. Intramedullary fixation decreases the endosteal blood flow, and the periosteal circulation is dramatically affected by open damage to the soft tissue surrounding the fracture during surgery. Although it has been demonstrated experimentally that the local vascularity can be altered by surgical intervention, the majority of these studies have not revealed differences in fracture healing (Miclau 1997). Indeed, most of them have indicated that bone has a pronounced ability to reconstitute its blood supply.

1.4. Avascular bone necrosis in joint dislocations

Avascular necrosis is a disease resulting from the temporary or permanent loss of the blood supply to the bones. Without blood, the bone tissue dies and causes the bone to collapse. Although it can occur in any bone, avascular necrosis most commonly affects the ends of long bones such as the femur, after hip joint dislocation. When joints are injured, blood vessels may be damaged. The increase in intraarticular pressure causes the blood vessels to narrow, making it difficult for them to deliver enough blood to the bone cells. Repair of the damage to the circulation is generally achieved spontaneously within a few hours after the closed repositioning of the joint. However, there are special sites where the risk of avascular necrosis is very high, e.g. the peritalar region.

The talus is the only bone in the lower extremities without any muscular attachments. Despite this fact, pure total dislocation without any associated fractures is rare, due to the strong ligamentous attachment of the talus to the adjoining midfoot bones and probably to the amount of force necessary for such an injury. 75% of these injuries are usually open, or the skin may be so tented over the talus that sloughing ultimately results.

The specific mechanism of this injury has not been described. It is thought that total talar dislocation is the endpoint of maximum pronation or supination injuries, the final result
of a continuum of forces that begin with dislocation of the subtalar joint. Injuries can occur after falling from a height or in motor vehicle accidents.

The considerations include disruption of the vascular supply to the surrounding soft tissue, and possible damage to the lymphatics, ligaments, joint capsule and tendons, all of which increase the risk of infection and necrosis of both the bone and the soft tissue.

The vascular supply of the talus has been well described. Although there is a variation in anatomy, five major vessels supply the talus. The extraosseous vascular supply comes from the anterior tibial, the posterior tibial and the perforating peroneal artery. The sinus tarsi is supplied by anastomoses of the lateral tarsal artery and the perforating peroneal artery. Further anastomoses occur with the artery of the tarsal canal, a branch of the posterior tibial artery, forming an anastomotic ring about the talar neck. The talar body is mainly supplied by the artery of the tarsal canal and the deltoid artery, a branch of the posterior tibial artery. The posterior process is supplied by small branches of the peroneal artery and calcaneal branches from the posterior tibial artery.

The amount of articular cartilage covering the talus limits the area that remains for the penetration of nutrient arteries. The likelihood of avascular necrosis is determined by the amount of soft tissue damage about the talus and by how many of the numerous anastomoses remain intact. The numerous anastomoses among all the arteries in the talus are one of the reasons why total talar avascular necrosis is relatively uncommon. Moreover, the occurrence of bone necrosis in the presence of residual intact vessels is determined by the duration of ischemia. In the event of such an injury, immediate closed repositioning is the only chance to restore the residual blood supply.

1.5. Microcirculatory consequences of ischemia-reperfusion injury

I-R initiates a cascade of pathophysiological events which in turn enhance the local and remote tissue injury. With severe blood flow deficits and impaired oxygen consumption, the oxidative phosphorylation and metabolic functions are deranged (Menguy 1974; Martin 1987; Sodeyama 1992). I-R injury has immediate and local effects, and there is substantial evidence that the generation of oxygen free radicals and disturbances of the local microcirculation are involved in this syndrome (Kurose 1997; Boros 2003). The generation of reactive oxygen species subsequent to reoxygenation inflicts tissue damage and initiates a cascade of deleterious cellular responses leading to inflammation, cell death and ultimate organ failure. Organ hypoperfusion and reperfusion generate a local inflammatory
environment that primes circulating leukocytes which provoke distant organ injury (Moore 1994).

The most convincing data concerning the consequence of this reaction derive from intravital microscopy studies. The technology allows real-time imaging of the microcirculation and the exact determination of the consequences of I-R. Disturbances of the microcirculatory perfusion are characterized by changes in the functional capillary density (FCD) and the red blood cell velocity (RBCV, μm s⁻¹). The FCD is defined as the length of red cell-perfused capillaries in relation to the observation area, which accurately describes the decrease in the efficacy of tissue perfusion when the corresponding area is unchanged (Tsai 1995). The RBCV is determined primarily by the blood flow and the cross-section of the circulatory area. The main causes of microcirculatory disturbances are as follows:

1. Changes in perfusion and vasoactivity

Microcirculatory dysfunctions seem to be mediated by endothelial cell damage and an imbalance of vasoconstrictor and vasodilator molecules, such as endothelin, reactive oxygen species and nitric oxide. These pathophysiological events can result in decreases in FCD and RBCV. The hypoxia-induced extensive release of vasoconstrictor mediators can lead to significant vasoconstriction of the precapillary sphincters, i.e. a considerable proportion of the inflowing blood returns to the venules without passing the capillaries. Precapillary vasoconstriction can also account for the relatively small decrease in arteriolar RBCV, which is determined primarily by the blood flow and the cross-section of the circulatory area. In addition to precapillary vasoconstriction, other reperfusion-related factors can also contribute to the reduction of FCD and RBCV. An aggravating consequence of I-R, the no-reflow phenomenon, may develop as a result of interstitial edema formation and external compression of the capillaries, or it may be a result of intraluminal plug formation (Filep 1992).

2. Cellular activity: leukocyte-endothelial interaction

Investigations utilizing intravital microscopy have demonstrated that the recruitment of inflammatory cells into the perivascular tissue involves a complex cascade mechanism. The adhesion process consists of several steps, beginning with the rolling of the polymorphonuclear leukocytes (PMNs) on the endothelial surface of the postcapillary venules until they have slowed down to such a degree that they stick to the endothelium. At this point, the leukocytes are sequestered from the main vascular flow, and firm adherence to the endothelial cells may follow. Subsequently, the leukocytes pass an intercellular junction between the endothelial cells and reach the abluminal side.
In addition, capillary “no-flow” (as a result of capillary plugging) with prolonged ischemia and “no-reflow” (as a result of endothelial cell swelling and microcirculatory occlusion by leukocytes) may per se initiate PMN activation (Barroso-Aranda 1988). Reperfusion injury appears to be mediated in part by PMN leukocyte-derived oxygen free radicals (Parks 1983; Hernandez 1987) and can result from the accumulation of toxic oxygen radicals generated by xanthine oxidase in the tissues themselves. During the ischemic phase, xanthine dehydrogenase, an enzyme found in many cell types, undergoes irreversible conversion to xanthine oxidase, which on the re-establishment of perfusion forms the superoxide anion from hypoxanthine and molecular oxygen. Oxygen radicals have been implicated in several toxic pathways, including damage to cellular lipids, proteins and DNA (Freeman 1982; Powell 1992).

As opposed to other organs, the periosteal changes in response to I-R have been only poorly characterized. The few studies examining the periosteal microcirculation have focused on the effects of flow reduction or the soft tissue trauma-induced local microcirculatory reactions (Rücker 2001, 2003; Menger 2003, Schaser 2003).

1.6. Significance of volume therapy in circulatory disturbances

Absolute or relative blood volume deficits are often observed in the surgical, trauma or intensive care patient. Bleeding causes absolute volume deficits, and vasodilation mediated by vasoactive substances is involved in the production of relative volume deficits. Volume deficits also develop in the absence of obvious fluid losses secondary to generalized impairment of the endothelial barrier, resulting in diffuse capillary leakage, e.g., during inflammation (Boldt 2006).

The administration of intravenous fluid to avoid dehydration, maintain an effective circulating volume and prevent inadequate tissue perfusion should be considered a core element of perioperative practice (Grocott 2005). Apart from head and chest injuries, the epidemiology of death in trauma includes bleeding in up to 30% of the cases, emphasizing the need for adequate fluid resuscitation. However, there is an ongoing debate with regard to the choice of the optimal type and timing of fluid resuscitation and the most appropriate endpoint of resuscitation. Besides trauma-associated blood loss in patients at the scene, effective fluid therapy is of utmost importance in critically ill patients with circulatory instability. Normovolemic also plays an essential role in perioperative fluid management (Vollmar 2004). The overall goal of fluid therapy is to ensure an adequate oxygen supply for the organs.
Tissue injury results in I-R, the release of mediators leading to increases in vascular permeability and tissue edema. On top of this, a concurrent hemorrhage causes a further reduction in intravenous volume. The initial fluid redistribution that occurs following trauma is related more to the degree of tissue trauma and ischemia than to blood loss *per se*. With mild hypovolemia, blood in the venous capacitance vessels is mobilized to ensure an adequate venous return. When this is depleted, fluid from the interstitial space is shifted to the intravascular space (autotransfusion) and the gradient between the oncotic pressure and hydrostatic pressure decreases. If there is further blood loss, hemorrhagic shock results. Decreases in cardiac output and arterial oxygen content lead to decreased oxygen delivery. The cellular mechanisms fail, as do the sodium potassium adenosine pumps, causing water to shift into the intracellular space, further depleting the intravascular fluid, cellular swelling occurs and ultimately cell death ensues if the process is not reversed (Bickell 1998).

Research is ongoing to define the ideal fluid for trauma and hemorrhage and for intraoperative volume support. In general, crystalloids and colloids, with either isotonic/normoncotic or hypertonic/hyponcotic modifications, and also blood, blood substitutes and oxygen therapeutics, are available (Boldt 2005). Resuscitation with isotonic (270-310 mosmol l$^{-1}$) crystalloid solutions, *i.e.* lactated Ringer’s (LR) solution or normal (0.9%) saline, is the current standard and predominates over the use of all other fluids for resuscitation. The advanced trauma life support guideline (American College of Surgeons, Committee on Trauma, 1997), which is followed by most physicians in the developed countries, comprises an aggressive regimen with rapid application of up to 2 l of crystalloid solution. Basically, this guideline recommends replacement of each ml of blood lost with 3 ml of crystalloid fluid (this is known as the 3 for 1 rule). In the case of blood pressure stabilization, resuscitation is continued with crystalloid solution. Crystalloids are distributed throughout the body and primarily fill the interstitial space without any preference for the intravascular space. Thus, the plasma-expanding effect is poor and is considered to be only 10-20% (Rizoli 2003). However, fluid resuscitation with crystalloids can enhance interstitial edema, in consequence of the reperfusion injury to the capillary interstitial membrane. The obvious risk of development of tissue edema upon crystalloid application is further increased by the dilutional decrease in plasma colloid osmotic pressure (Orlinsky 2001) and a rise in osmotic pressure in the extracellular spaces (glucose is primarily responsible for the latter). Tissue edema can decrease oxygenation, delay healing and lead to subsequent sepsis (Bickell 1998; Orlinsky 2001).
When higher volume expansion is sought, when concerns over edema formation arise, or when the patient responds only transiently and even remains hypotensive, crystalloids are replaced by colloids, or colloids are added. In the event of blood pressure stabilization, resuscitation is continued with crystalloid solution. In general, solutions of dextran (DEX), starch, albumin (ALB) and gelatin (GEL) are available, hydroxyethyl starch (HES) being the most common artificial colloid used. By virtue of their water-binding capacity, colloids prolong the circulatory effect by retaining water in the vascular space. Colloids may vary extensively in colloid osmotic pressure, due to their concentration and molecular weight (Vollmar 2004). The most commonly applied colloid solutions are as follows:

*Albumins:* These are natural (protein-containing) colloids, distributed in 5% or 20% stripping. The term for a favorable effect is 4-6 h. ALBs increase the plasma colloid osmotic pressure and the intravasal volume by retaining water. In cases of enhanced capillary permeability, a significant proportion of the ALBs effuse into the interstitial space, increasing edema formation. Meta-analyses in recent years have yielded conflicting data, but ALB administration can certainly be beneficial in hypalbumenic patients (Boldt 2005, 2006).

*Gelatins:* These are polydispersed polypeptides produced by the degradation of bovine collagen (bone, sinew, skin), with an average size of 35,000 Da. They contain many lower molecular weight (MW) components, which move freely into the extravascular space, and thus the intravascular volume-increasing effect of GELs is short, 1-2 h. They have relatively few side-effects (in higher doses, they inhibit thrombocyte function and worsen the quality of clot formation), but this is counteracted by an increased risk of allergic reactions or theoretical possibility of BSE (Boldt 2005, 2006).

*Dextrans:* They are biosynthetized commercially from sucrose by the use of *Leuconostoc* bacteria with the enzyme dextran sucrase. The resulting high-MW DEXs are then cleaved by acid hydrolysis and separated by repeated ethanol fractionation into a final product with a restricted MW range. The products of this process are D-glucose polymers joined largely by α-1,6 bonds into predominantly linear macromolecules. They are defined by their number-averaged MW: DEX 40 and DEX 70 have number-averaged MWs of 40,000 and 70,000 Da, respectively. DEXs are polydisperse, and their clearance is dependent on their MW. DEX molecules below 55,000 Da are freely filtered at the renal glomerulus, and approximately 70% of an administered dose of DEX 40 will be excreted into the urine within 24 h. Larger molecules are excreted through the gut or metabolized by endogenous dextranases in the reticuloendothelial cells (Boldt 2005, 2006).
Hydroxyethyl starches: These are a family of synthetic colloid solutions, which are modified natural polysaccharides similar to glycogen. HES is derived from amylopectin, a highly branched starch obtained from maize or potatoes. Polymerized D-glucose units are joined primarily by 1-4 linkages with occasional 1-6 branching linkages. The substitution of hydroxyethyl for hydroxy groups results in highly increased solubility and retards the hydrolysis of the compound by amylase, thereby delaying its breakdown and elimination from the blood. The hydroxyethyl groups are introduced mainly on carbon atoms \( C_2, C_3 \) and \( C_6 \) of the anhydroglucose residues. Unlike the DEXs, which are mainly characterized by their concentration and the weight-averaged mean MWs, the pharmacokinetics of HES preparations is also characterized by other patterns:

a. the concentration (6%, 10%);

b. the weight-averaged mean MW (the arithmetic mean of the MW of all HES molecules);

c. the molar substitution (the molar ratio of the total number of hydroxyethyl groups to the total number of glucose units);

d. the degree of substitution (the ratio of substituted glucose units to the total number of glucose molecules);

e. the \( C_2/C_6 \) hydroxyethyl group ratio: there is convincing evidence that the \( \alpha \)-amylase activity depends on the positions of the hydroxyethyl groups on the glucose molecule (\( C_2, C_3 \) and \( C_6 \)); the ratio of \( C_2:C_6 \) hydroxyethylation appears to be an important factor for the pharmacokinetic behavior of HES and possibly also for its side-effects (e.g. accumulation, tissue accumulation and bleeding complications).

At present, numerous HES preparations with different combinations of concentration, MW, degree of substitution, and hydroxyethylation pattern (\( C_2/C_6 \)-ratio) are available. However, it is important to distinguish between the different HES preparations, because the extent and duration of plasma volume expansion, and also their effects on the blood rheology, the coagulation system and other likely clinical variables differ with respect to the specific physico-chemical properties of a HES preparation. Recently, the most commonly used solution is a newly designed third-generation medium MW HES with a molar substitution of 0.4, i.e. it has 4 hydroxyethyl groups for every 10 glucose units. The water-binding capacity of HES ranges between 20 and 30 ml g\(^{-1}\). HES solutions therefore have a good plasma volume-expanding capacity. Hence, HES 130/0.4 in a dose of 50 ml kg\(^{-1}\) day\(^{-1}\) could be given to non-bleeding patients. The duration of the intravascular volume-expansion effect is 4-6 h. Anaphylactic reactions are caused less often by HES solution than by other colloids (Boldt 2005, 2006).
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 (Hoffmann 2002; Boldt 2006), but also in the potential to restore tissue oxygenation (Marx 2004; Rittoo 2004) and additionally in the potential to modulate inflammatory activation at the microcirculatory level (Menger 1993; Kaplan 2000; Jaeger 2001; Feng 2007). Anti-inflammatory actions and microcirculatory consequences are in fact interrelated events. Interruption of the adhesion between PMNs and endothelial cells ameliorates or prevents microcirculatory dysfunctions (Kurose 1994), and these reactions have been implicated as critical pathogenetic factors in a variety of low flow-induced tissue injuries (Granger 1994).

Our understanding of the pivotal role of activated PMNs 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 DEX, low-MW HES and GEL, have been characterized and compared in the same setup.

2. MAIN GOALS
The main goals of the present studies were:
1. To demonstrate the significance of the duration of ischemia as concerns the recovery of a peritalar sprain through a human case history.
2. To design an experimental rat model of complete limb ischemia in order to elucidate the periosteal microcirculatory alterations caused by I-R. This included examinations of the efficacy of the tissue perfusion, the primary and secondary leukocyte-endothelial cell interactions, and the tissue sequestration of PMNs.
3. I-R injuries often arise after clinical traumas, tourniquet application or vascular flap surgery, and plasma expander, isovolemic hemodilution therapy is commonly used in these conditions. A further aim was to characterize the effects of DEX (6%; 60 kDa MW), low MW HES (6%; 130 kDa/0.4) and GEL (4%; 35 kDa) on PMN reactions by using standardized in vivo microscopic methods to demonstrate their relative therapeutic benefits in ameliorating I-R-induced inflammatory activation.
3. MATERIALS AND METHODS

3.1. Human clinical study: medical attendance of injury of the peritalar region. A case history

A 23-year-old sportswoman suffered a peritalar sprain (a complete talus sprain) in her right leg, while participating in a long jump rally. Since the sports-ground was located near the place of treatment (a trauma care unit), following quick transport, the injury was diagnosed only a few minutes after the event. During transport, the complete sprain of the talocrural joint predominated in the clinical situation and the bones of the ankle almost perforated the skin medially (from the inside). Blood supply disturbances were clearly recognizable in this region.

Immediate repositioning was attempted without anesthesia prior to the X-ray radiogram, but this intervention was unsuccessful. Temporarily, a Kramer rail was fixed on the injured limb, following which a radiological examination was performed. The X-ray revealed a sprain of the subtalar region and subluxation of the talus in the talocrural joint. Closed repositioning of the sprain of the calcaneonavicular joint was subsequently carried out under anesthesia. After successful repositioning, a lower shank split concentric plaster was applied.

3.2. Animal experiments

These experiments were performed in accordance with the National Institutes of Health Guidelines (Guide for the Care and Use of Laboratory Animals); the study was approved by the Animal Welfare Committee of the University of Szeged.

3.2.1. Surgical procedures

The experiments were performed in two main series on male Wistar rats (average weight 300±35 g) housed in an environmentally controlled room with a 12-h light-dark cycle; they were deprived of food, but not water, 12 h before the experiments. The rats were anesthetized with sodium pentobarbital (45 mg kg⁻¹ ip). The right jugular vein and carotid artery were cannulated for fluid and drug administration and for the measurement of arterial pressure, respectively.

To determine macrohemodynamic variables, such as cardiac output (CO) changes, the left common carotid artery was also dissected and a thermistor-tip catheter (PTH-01 Experimetria Ltd., Budapest, Hungary) was introduced into the ascending aorta to measure the CO by a thermodilution technique. An ultrasonic flow probe (1-RS; Transonic Systems
Inc., Ithaca, NY, U.S.A.) was placed around the exposed femoral artery to measure the blood flow.

The animals were placed in a supine position on a heating pad to maintain the body temperature between 36 and 37 °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 (Wolfárd 2002), 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.

3.2.2. Hemodynamic measurements

Pressure signal (BPR-02 transducer; Experimetria Ltd., Budapest, Hungary) and femoral artery flow signals (T206 Animal Research Flowmeter; Transonic Systems Inc., Ithaca, NY, U.S.A.) were measured continuously and registered with a computerized data-acquisition system (SPEL Advanced Haemosys 2.72, Experimetria Ltd., Budapest, Hungary). The heart rate (HR) was calculated from the pulse waves of arterial pressure curve. The CO was determined by a thermodilution technique, using a Cardiostar CO-100 computer (Experimetria Ltd., Budapest, Hungary) and its values were recorded as the cardiac index (the CO/body weight ratio). The total peripheral vascular resistance was calculated via the standard formula. Arterial blood gases and hematocrit were measured with a blood gas analyzer (AVL Compact 2, Graz, Austria) from arterial blood samples.

3.2.3. Experimental protocol

In the first series of experiments (Study I), I-R-induced microcirculatory changes in the tibial periosteum were analyzed with the aid of fluorescence intravital microscopy. After a 30-min stabilization period, the baseline cardiovascular and microhemodynamic parameters were determined (baseline; \( t = -60 \) min). The animals were allotted into one or other of two experimental groups. The first group \((n=5)\) served as sham-operated controls to exclude microcirculatory changes relating solely to the anesthesia and surgery. In group 2 \((n=5)\), complete hindlimb ischemia was induced by clamping the femoral artery with an atraumatic vascular miniclip (Mehdorn clip; Aesculap AG, Germany) and placing a tourniquet around
the femur, immediately after the occlusion of the vessel. After ischemia for 60 min, the
tourniquet and the artery clip were removed, and the reperfusion was observed for 180 min.
The periosteal microcirculation was observed hourly during the 180-min reperfusion period.

In the second series of experiments (Study II), the effects of volume resuscitation with
crystalloid and different colloid solutions on the I-R-related microcirculatory disturbances of
the periosteum were examined. In these experiments, LR solution was infused at a rate of 10
ml kg⁻¹ h⁻¹ during the surgical procedures and for 50 min during the ischemic phase of the
experiments. The first group (n=10) served as LR-treated, sham-operated controls, where the
microcirculatory variables were recorded for 240 min to exclude changes relating solely to the
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. The 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 (Voluvan 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 (Boldt 2005). 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.

At the end of the experiments, muscle biopsies (m. gracilis anterior) were taken from
the operated and contralateral hindlimbs for the determination of leukocyte accumulation
(myeloperoxidase (MPO) activity).

3.2.4. Intravital videomicroscopy

The right hindlimb with the exposed tibia was positioned horizontally on an adjustable
stage and superfused with 37 °C saline. The microcirculation of the distal tibia was visualized
by intravital microscopy (Zeiss Axiotech Vario 100HD microscope, 100 W HBO mercury
lamp, Acroplan 20x water immersion objective), using fluorescein isothiocyanate (Sigma
Chemicals, U.S.A.-)labeled erythrocytes (Ruh 1998) (0.2 ml iv) for red blood cell staining
and rhodamine-6G staining (Sigma, St. Louis, MO, U.S.A.; 0.2%, 0.1 ml iv) for leukocytes.
The microscopic images were recorded with a charge-coupled device videocamera (AVT HORN-BC 12) attached to an S-VHS videorecorder (Panasonic AG-MD 830) and a personal computer.

3.2.5. 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). Periosteal capillaries were located according to the description of Menger et al. (Menger 1997). The FCD, i.e. the length of the perfused nutritive capillaries per observation area (cm\(^{-1}\)), and the RBCV (\(\mu m\) s\(^{-1}\)) were measured in 5 separate fields in 5 capillaries at each time point of each experiment. Leukocyte-endothelial cell interactions were analyzed within 5 postcapillary venules (diameter between 11 and 20 \(\mu m\)) 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. 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 a percentage of the number of nonadherent leukocytes passing through the observed vessel segment within 30 s.

3.2.6. Myeloperoxidase measurements

The tissue MPO activity, as a marker of tissue leukocyte infiltration, was measured in muscle biopsies by the method of Kuebler et al. (Kuebler 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.

3.2.7. Statistical analysis

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). Nonparametric methods were used. 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 the Figures and Tables, median values and 75th and 25th percentiles are given. \(P\) values < 0.05 were considered significant.
4. RESULTS

4.1. Human study

As a result of resting for a few days with suspended extremities and diuretic therapy, the initial edema disappeared, and on day 6 the initial split plaster fixing could be replaced by a closed concentric plaster. During physiotherapy, the patient was taught to walk with crutches, without using her injured leg.

At the control examination in the 6th week, the plaster binding could be removed. The clinical examination showed stability of the talocrural joint. The X-ray demonstrated congruity on the surface of the joints and verified the stability of the joints. However, the complementary MR record demonstrated a localized, intraosseal circulatory disturbance (Annex 1, Figs 3-6).

At this time, for amelioration of the blood supply and joint motion, cautious physiotherapy was started (active/passive gymnastics) with continued strict loading prohibition. This led to the range of motion of the patient improvings and the elimination of pain in the course of time.

After the 16th week, MR examination showed revascularization at the site of the earlier circulatory disturbance in the talus. Clinical examination indicated the same range/extent of motion as on the intact side.

In view of the favorable examination results, partial (and after the 20th week full loading of the extremity was allowed. After the 20th week, the patient was completely pain-free.

The annual control showed the perfect healing of the affected bone parts (Annex 1, Figs 9-11).

4.2. Experimental study I. Effects of I-R on postischemic periosteal microcirculatory changes

4.2.1. Changes in microperfusion

The periosteal microcirculation was characterized by the RBCV and the FCD \( i.e. \) the length of the perfused nutritive capillaries per observation area (cm\(^{-1}\)) (Fig. 1).

Intravital microscopy revealed homogenous microvascular perfusion in the periosteum in both groups under the baseline conditions. The RBCV was similar in the different groups (median values ranging between 560 \( \mu \text{m s}^{-1} \) and 620 \( \mu \text{m s}^{-1} \)) and did not change over time in the sham-operated group. I-R, however, led to a significantly decreased RBCV during the reperfusion period (Fig. 2).
Figure 1. Periosteal microcirculation under the intravital microscope (red blood cell staining)

The periosteal FCD did not change significantly in the sham-operated group. However, I-R caused a significant decrease in FCD, which decreased gradually from the beginning of the reperfusion and reached the lowest value (60% of the baseline) at 120 min of reperfusion (Fig. 3).

Figure 2. Changes in red blood cell velocity (RBCV, $\mu$m s\(^{-1}\)) in the tibial periosteum in the sham-operated group (empty circles) and the I-R group (black squares). * $P < 0.05$ within the groups, as compared with the preischemic value ($^X P < 0.05$) between the sham-operated and the I-R group.
Figure 3. Functional capillary density (FCD, cm$^{-1}$) of the periosteum in the sham-operated group (empty circles) and the I-R group (black squares). * \( P < 0.05 \) within the groups, as compared with the preischemic value (\( \times P < 0.05 \)) between the sham-operated and the I-R group.

4.2.2. Leukocyte-endothelial cell interactions

Leukocyte-endothelial cell interactions were characterized by the number of rolling and adherent (stickers) leukocytes (Fig. 4).

Figure 4. Periosteal leukocyte adherence under the intravital microscope (PMN staining)

A maximum of 30% of the nonadherent PMNs rolled along the endothelial lining of the postcapillary venules under the baseline conditions in the different groups (Fig. 5). In the sham-operated control group, there were no significant changes in the numbers of rolling and
adherent PMNs at any of the observation points throughout the experiments (Figs 5 and 6). The 60-min ischemia and reperfusion was accompanied by a significant increase in leukocyte-endothelial cell interactions. Both the percentage of rolling cells and the number of adherent PMNs were increased as compared with the preischemic values or the values for the sham-operated group at matching time points (Figs 5 and 6).

**Figure 5.** Percentage of rolling leukocytes in the postcapillary venules in the periosteum, in the sham-operated group (empty circles) and the I-R group (black squares). *P < 0.05 within the groups, as compared with the preischemic value (X P < 0.05) between the sham-operated and the I-R group.
Figure 6. Number of sticking leukocytes (mm\(^{-2}\)) in the postcapillary venules in the periosteum, in the sham-operated group (empty circles) and the I-R group (black squares). * \(P < 0.05\) within the groups, as compared with the preischemic value (\(\chi^2 P < 0.05\)) between the sham-operated and the I-R group.

4.3. Experimental study II. Effects of colloid solutions on the consequences of I-R

4.3.1. Changes in macrohemodynamic parameters

The baseline values of the macrohemodynamic variables (including the HR and the mean arterial pressure) did not differ significantly in the different groups, and there were no significant hemodynamic changes as compared with the baseline values during the experimental period in any of the groups (Table 1; HR data are not shown).

Table 1. Effects of lactated Ringer’s solution (LR), gelatin (GEL), dextran 60 (DEX) and hydroxyethyl starch 130/0.4 (HES) solutions on the mean arterial pressure (mmHg) after limb ischemia-reperfusion (I-R).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Parameters Base</th>
<th>R 0 min</th>
<th>R 60 min</th>
<th>R 120 min</th>
<th>R 180 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham+LR</td>
<td>Median 106</td>
<td>100</td>
<td>100</td>
<td>109</td>
<td>109</td>
</tr>
<tr>
<td></td>
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<td>86; 117</td>
<td>99; 122</td>
<td>105; 125</td>
<td>97; 113</td>
</tr>
<tr>
<td>I-R+LR</td>
<td>Median 120</td>
<td>110</td>
<td>110</td>
<td>106</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>25p, 75p 112; 129</td>
<td>102; 125</td>
<td>98; 139</td>
<td>101; 115</td>
<td>100; 120</td>
</tr>
<tr>
<td>I-R+GEL</td>
<td>Median 135</td>
<td>120</td>
<td>110</td>
<td>100</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>25p, 75p 105; 145</td>
<td>108; 143</td>
<td>96; 123</td>
<td>91; 116</td>
<td>100; 139</td>
</tr>
<tr>
<td>I-R+DEX</td>
<td>Median 117</td>
<td>113</td>
<td>118</td>
<td>117</td>
<td>121</td>
</tr>
<tr>
<td></td>
<td>25p, 75p 110; 137</td>
<td>112; 122</td>
<td>95; 125</td>
<td>111; 127</td>
<td>117; 126</td>
</tr>
<tr>
<td>I-R+HES</td>
<td>Median 134</td>
<td>130</td>
<td>128</td>
<td>119</td>
<td>119</td>
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<tr>
<td></td>
<td>25p, 75p 120; 146</td>
<td>125; 134</td>
<td>101; 138</td>
<td>100; 122</td>
<td>102; 128</td>
</tr>
</tbody>
</table>

The CO and femoral artery blood flow were measured to characterize the peripheral and local hindlimb perfusions. There was a nonsignificant (approximately 22%, 17%, 20% and 14%) tendency to a decreased CO at the end of the ischemic period in all the ischemic groups (the I-R + LR, I-R + GEL, I-R + DEX and I-R + HES groups, respectively). The volume replacement therapies normalized the CO in all the groups, and there were no significant differences in these values within or between the groups during the reperfusion period (Fig. 7).
Figure 7. Changes in cardiac output in response to sham operation (open circles) or 60 min of total hindlimb ischemia followed by 180 min of reperfusion in rats treated with lactated Ringer’s solution (LR, black squares), hydroxyethyl starch 130/0.4 (HES, open triangles), gelatin (GEL, gray triangles), or dextran 60 (DEX, black triangles).

Figure 8. Changes in femoral artery blood flow in response to sham operation (open circles) or 60 min of total hindlimb ischemia followed by 180 min of reperfusion in rats treated with lactated Ringer’s solution (LR, black squares), hydroxyethyl starch 130/0.4 (HES, open triangles), gelatin (GEL, gray triangles), or dextran 60 (DEX, black triangles). * $P < 0.05$ vs the baseline.
The femoral artery blood flow increased transiently during the first hour of reperfusion in all the I-R groups. Although the highest flow increase was observed in the HES-treated animals, this change was again statistically not significantly different between the treated groups during the reperfusion period (Fig. 8).

The colloid infusion-caused changes in volume expansion were assessed by hematocrit measurement. As compared with the baseline values, there was an increase in hematocrit at the end of the ischemia (before volume replacement) in all the ischemic groups, but this was followed by a complete restoration to the baseline values in the later experimental phase (Table 2).

Table 2. The effects of lactated Ringer’s solution (LR), gelatin (GEL), dextran 60 (DEX) and hydroxyethyl starch 130/0.4 (HES) solutions on the hematocrit changes (%) after limb ischemia-reperfusion (I-R). * \( P<0.05 \) vs baseline.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Parameters</th>
<th>Baseline</th>
<th>R 0 min</th>
<th>R 180 min</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Median</td>
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<td>41</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>25p, 75p</td>
<td>39.5; 46.5</td>
<td>38; 46</td>
<td>36; 41</td>
</tr>
<tr>
<td>I-R + LR</td>
<td>Median</td>
<td>42</td>
<td>47*</td>
<td>45*</td>
</tr>
<tr>
<td></td>
<td>25p, 75p</td>
<td>40; 45</td>
<td>45.5; 50.5</td>
<td>43; 47</td>
</tr>
<tr>
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<td>45.5</td>
<td>38</td>
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<tr>
<td></td>
<td>25p, 75p</td>
<td>42; 45</td>
<td>43; 48</td>
<td>37; 40</td>
</tr>
<tr>
<td>I-R + DEX</td>
<td>Median</td>
<td>42.6</td>
<td>47*</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>25p, 75p</td>
<td>42; 44</td>
<td>45; 49</td>
<td>36.5; 42</td>
</tr>
<tr>
<td>I-R + HES</td>
<td>Median</td>
<td>43</td>
<td>48*</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>25p, 75p</td>
<td>42; 44.5</td>
<td>46; 49.5</td>
<td>38; 46</td>
</tr>
</tbody>
</table>

4.3.2. Changes in microperfusion variables

Intravital microscopy revealed homogenous microvascular perfusion in the periosteum in all groups under the baseline conditions. The RBCV was similar in the different groups at the baseline (approximately 600 \( \mu \text{m s}^{-1} \)), and did not change over time in the sham-operated group (Fig. 9A-C). In the LR-treated group, however, I-R led to a significant RBCV decrease (by 39%) during reperfusion. The microcirculatory perfusion did not improve in response to GEL; in this group, lower RBCV values were observed than in the sham-operated group or under the baseline conditions (Fig. 9A). After a transient restoration during reperfusion, DEX treatment did not influence the RBCV changes induced by ischemia (Fig. 9B). HES treatment...
prevented the postischemic deterioration of RBCV nearly completely, and a complete restoration to the baseline values was observed after the first hour of reperfusion (Fig. 9C).

**Figure 9.** Changes in red blood cell velocity (RBCV) in response to sham operation (open circles) or 60 min of total hindlimb ischemia followed by 180 min of reperfusion in rats treated with lactated Ringer’s solution (LR, black squares), gelatin (GEL, A), dextran 60
(DEX, B) or hydroxyethyl starch 130/0.4 (HES, C) (open triangles). * \( P < 0.05 \) vs the baseline; ^ \( P < 0.05 \) vs sham-operated group; # \( P < 0.05 \) vs I-R + LR group.

**Figure 10.** Functional capillary density (FCD) in the tibial periosteum subjected to sham operation (open circles) or 60 min of total hindlimb ischemia followed by 180 min of reperfusion in rats treated with lactated Ringer’s solution (LR, black squares), gelatin (GEL, A), dextran 60 (DEX, B) or hydroxyethyl starch 130/0.4 (HES, C) (open triangles). * \( P < 0.05 \)
In the sham-operated group, the periosteal FCD did not change significantly, but was greatly and permanently deteriorated in the LR-treated I-R group after 60 min of reperfusion. The GEL treatment caused a transient postischemic improvement, but this was followed by a progressive deterioration after 60 min of reperfusion (Fig. 10A). DEX infusion did not lead to any amelioration of this parameter throughout the entire reperfusion phase (Fig. 10B). However, a considerable protection against I-R-induced capillary perfusion failure was provided by HES, which caused a complete restoration of the FCD, similar to that seen under the sham-operated conditions. This difference was statistically significant as compared with the effects of LR, DEX and GEL after I-R (Fig. 10C).

In the sham-operated group, the numbers of firmly adherent leukocytes (Fig. 11) did not change significantly during the experiments. In the I-R group, the proportion of rolling leukocytes 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. 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 (data not shown), 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 throughout the entire reperfusion period (Fig. 11C). In contrast, significant deteriorations in this parameter were observed in the first hour of reperfusion in the GEL- and DEX-treated groups (Fig. 11A,B), similarly 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 (Fig. 11A).
Figure 11. Secondary leukocyte-endothelial cell interactions (sticking) in submucosal 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 squares), gelatin (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 or 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.
4.3.3. Changes in leukocyte accumulation in the soft tissue

I-R caused a significant increase in the leukocyte accumulation (demonstrated by measurement of the MPO activity in the surrounding soft tissues) in the LR-treated, GEL and DEX groups as compared with that of the sham-operated animals (approximately 3-fold) and the contralateral non-ischemic limb. In the HES-treated group, however, the MPO activity remained at the normal level (Fig. 12).

**Figure 12.** Muscle myeloperoxidase (MPO) activity was assessed at the end of the 240-min observation period in limbs subjected to sham operation in the presence of vehicle (open box) or to 60-min complete ischemia followed by 180-min reperfusion in the presence of lactated Ringer’s (black box), gelatin (grey box), HES (hatched box), or dextran 60 (checked box) treatment, respectively. Data are compared with those for the intact contralateral limbs. The plots demonstrate the median values and the 25th (lower whisker) and 75th (upper whisker) percentiles. * $P < 0.05$ between groups vs saline-treated control group values, $P < 0.05$ vs the contralateral limb, * $P < 0.05$ vs the corresponding sham-operated group.
5. DISCUSSION

The case history embedded into this thesis clearly demonstrates that minimization of the duration of ischemia can lead to the successful recovery of a peritalar sprain even in cases of conservative treatment. The presented results also show that prolonged warm I-R evoke a decrease in periosteal FCD and increase the accumulation of PMNs. Finally, it has been shown that these potentially detrimental changes can be influenced significantly by appropriate adjuvant volume therapy initiated during reperfusion.

Today many traumatological interventions are performed under temporarily reduced blood flow conditions. The tourniquet methods per se cause hypoperfusion and reperfusion of the extremities. Transient ischemia characterizes graft surgery too and, despite a meticulous microvascular surgical technique and well-functioning feeding vascular anastomoses, I-R events might induce severe perfusion failure and secondary tissue damage. The mechanisms underlying these microcirculatory changes are still a subject of debate, but several observations suggest that PMN activation is critically linked to the derangement of perfusion in the reperfused tissues (Peter 1998).

5.1. Human study

Traumatological events lead relatively frequently to avascular necrosis, which is a time- and bone region-dependent ischemic injury. If the abnormal situation is not solved rapidly, the circulatory disturbances may result in aseptic bone necrosis. Although it can occur in any type of bone, avascular necrosis most commonly affects the ends of long bones such as the femur, after hip joint dislocation. However, the peritalar region is of special interest, because here the risk of avascular necrosis is also very high. The talus is the only bone in the lower extremity without any muscular attachments, making it somewhat vulnerable in the presence of injury. Furthermore, talus fractures are often accompanied by partial or total talus necrosis. Principally fracture of the talar neck or body occurs, often associated with supra- and subtalar dislocations. A rare variant involves the total dislocation of the talus, with a concomitant disruption of the tibiotalar relationship, in which the talus is completely dislocated from the ankle, and with a simultaneous dislocation of both the subtalar and the talonavicular joints. Total dislocation of the talus (peritalar dislocation or in other words a missing talus) is uncommon, accounting for approximately 2% of all traumatic talus injuries. The majority of these injuries are accompanied by fracture of the talus (occurring mainly as an open fracture). Total dislocation of the talus has a 90% probability of a poor prognosis and osteonecrosis. These facts must be taken account at the beginning of the management.
Impairment of the blood supply of the bone, originating from a talus dislocation, is one of the most challenging problems encountered in reconstructive surgery. In total devascularization of the talus, removal of the entire bone is indicated. In these cases, the site of the removed talus must be supplied with a free vascularized composite flap. The goal is to maintain the length of the limb, thereby preserving the static and dynamic functions of the foot, and ensuring a painless condition for the patient. Management of a closed dislocation must involve a closed repositioning. If this fails, an open repositioning must be applied. However, a further impairment of the blood supply may occur. Following repositioning, the extremity must be stabilized with external or internal fixation. For the healing process, the extremity must be placed at rest, but it is also essential to begin remobilization at the right time, so as to regain the proper function. The time of the mobilization, and then that of the full-weight bearing, depend on the recovery of the blood supply of the bone. For the evaluation of the intraosseal blood circulation, MR examination is the appropriate method.

The clinical case reported here demonstrated a very rare peritalar dislocation (total dislocation of the talus), where neither partial nor complete necrosis of the talus developed. The management strategy was based on the early diagnosis and MR documentation. Additionally, the following factors could play a pivotal role in the absence of complications:

1. Repositioning could be performed quickly, owing to the rapid transport between the trauma care unit and the scene of the accident. Although the blood supply of the talus seemed to be definitely impaired, secondary damage to the circulation could be surely prevented through prompt cessation of the dislocation.

2. The duration of the plaster cast fixation applied to maintain the result of the repositioning was as short as possible so as to avoid soft tissue damage, it lasted only until the ligament stability of the affected joints was achieved.

3. Non-weight-bearing active and passive physiotherapy had a beneficial effect on the impairment of the intraosseal circulation detected subsequently to the plaster cast fixation. After the recovery of the blood supply of the talus was demonstrated by the control MR examination, weight-bearing mobilization of the extremity could begin. The application of MR tomography in the diagnosis and the therapy of this injury was highly advantageous.

In summary, appropriate diagnosis and documentation of the circulatory state by means of MR tomography, and early treatment of the ischemic injury, resulted in the most advantageous management.
5.2. Experimental study I

In these experiments, we employed a rat tibia model to investigate the periosteal microcirculatory consequences of a standardized I-R challenge. Although complete vascular occlusion disturbs the perfusion of all the tissues of the exposed limb, microcirculatory deterioration predominates in the periosteum, while the surrounding muscle layers exhibit much lower ischemic sensitivity (Rücker 1998). Similarly, it has been recognized that maintenance of an adequate blood flow to the covering periosteal membrane is critical for the survival and function of the transplanted bone, and the microvascular blood supply is necessary for the further osteogenic and fibrogenic activity of the periosteum (Berggren 1982).

Our results demonstrated that a 60-min period of ischemia induces capillary perfusion failure and leukocyte-endothelial cell interactions in the periosteal microcirculation. During the reperfusion phase, the proportion of perfused capillaries decreased significantly, and thus a large proportion of the inflowing blood returned to the venules without passing the capillaries. The reason for this shunt circulation may be precapillary vasoconstriction, but other reperfusion-related factors can also contribute to the reduction of the FCD. It has been shown that ischemia time-dependently increases endothelin-1 (ET-1) production and this could lead to severe consequences in the bone microcirculation (Kato 1998). Wolfárd et al. reported that these changes were significantly influenced by antagonizing the ET-A receptor-mediated effects during reperfusion (Wolfárd 2002). ET-1 is the most powerful vasoconstrictor substance known to date; the vasoconstrictive effects are mediated predominantly via the ET-A receptors present on the vascular smooth muscle cells (Rubanyi 1994). The hypoxia-induced predominant release of vasoconstrictor mediators can lead to significant vasoconstriction of the precapillary sphincters.

Another possible cause of the reduction of the FCD is the capillary no-reflow phenomenon, which may develop as a result of external compression induced by interstitial edema formation, or it may be due to intraluminal plug formation (Menger 1997). Indeed, Filep et al. have shown that ET-1 causes dose-dependent increases in vascular permeability through the activation of ET-A receptors as a consequence of disruption of the endothelial barrier (Filep 1992).

Additionally, we have observed increased leukocyte-endothelial cell interactions in the postcapillary venules. In the periosteal vessels with smaller diameter, the adherent and rolling leukocytes form typical leukocyte plugs, thereby probably leading to obstruction of the venules. The adhesion process consists of several steps, beginning with the rolling of the
PMNs on the endothelial surface of the postcapillary venules until they have slowed down to such a degree that they stick to the endothelium. As the enhanced leukocyte-endothelial cell interactions eventually lead to leukocyte extravasation, this process plays an important role in reperfusion-associated late tissue injury too.

It has been shown that I-R is associated with physical membrane defects, phosphatidylcholine and the exhaustion of endogenous phosphatidylcholine sources (Ghyczy 2008). These findings suggest that this ubiquitous membrane-forming entity may become exhausted in response to noxious stimuli, and replenishment of the endogenous phosphatidylcholine pool could be of importance under critical circumstances (Ghyczy 2008). In line with this, it has been evidenced that phosphatidylcholine supplementation could ameliorate the harmful consequences of periosteal I-R (Gera 2007).

5.3. Experimental study II

This study investigated the anti-inflammatory actions of major artificial colloid classes in the tibial periosteal microcirculation, by comparing their effects on the perfusion characteristics and the leukocyte-endothelial interactions. In this tissue compartment, the significantly reduced FCD and capillary RBCV were accompanied by characteristic leukocyte-mediated reactions in the reperfusion phase. Of the examined solutions, these potentially harmful changes were abolished only by the HES infusion.

The volume-expanding properties of the three colloid solutions might differ due to their differing MWs and vascular retentions, 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 (LR) 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 1999) and cause a hypercoagulable state, probably due to hyperchloremia (Roche 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 (Kaplan 2000; Pascual 2001; Rittoo 2004;) and other indirect parameters of a microcirculatory improvement, such as the restored tissue oxygenation (Marx 2004; Rittoo 2004) after HES treatment. Information is also available concerning the better efficacy of HES in comparison with GEL or DEX in influencing these parameters (McGrath 1996; Allison 1999). In local ischemic insults, such as limb I-R, an imbalance between vasoconstrictor/vasodilator forces predominates and is responsible for the reduced efficacy of tissue perfusion in the postischemic tissues (Wolfárd 2002). It is of importance that, from among the currently examined resuscitation fluids, DEX triggers the release of endogenous ET-1 (Otsuka 1990), and this potent vasoconstrictor signal may explain the inefficacy of DEX in restoring the postischemic periosteal microcirculation.

Colloid infusions are routinely administered to patients with systemic circulatory disorders when a sufficient CO and restored organ perfusion are the general therapeutic goals. These pathologies are regularly accompanied by regional blood flow abnormalities and spatial redistribution of the intra-organ microcirculation (Szabó 2004; Vajda 2004); hence, re-establishment of the microvascular perfusion is a convincing manifestation of the therapeutic benefit of volume therapy. A microvascular perfusion deficit could be well described by RBCV and FCD changes, but from a methodological point of view it should be emphasized that evaluation of the average RBCV gives an exact measure of the microvascular perfusion only if all capillaries in a given area are perfused. However, in response to most insults, the inefficacy of the capillary bed is also manifested by FCD changes, as seen in this study. The FCD is a parameter of perfusion heterogeneity, and a decreased ratio somewhat modifies the value of RBCV measurements (Szabó 2004), since estimation of the average RBCV stems from individual measurements, including perfused capillaries only, and obviously excludes nonperfused capillaries from the calculations. Thus, these parameters should be taken into account simultaneously, as otherwise the severity of the microvascular impairment caused by I-R would be underestimated. Moreover, the therapeutic potential of HES is even more noteworthy if its dual effect on the RBCV and FCD changes is considered.

We found that the primary and secondary forms of PMN-endothelial interactions (rolling and firm adherence) were significantly increased in the postcapillary venules during reperfusion. In general, activated PMNs reach their final site of action after selectin-mediated rolling and subsequent firm adherence (sticking) to the endothelium. Anchorage during this phase is achieved by means of PMN-derived integrins (CD18/CD11) and their endothelial ligands: intercellular adhesion molecules and vascular cell adhesion molecules (Springer
In this respect, it has been shown that artificial colloids indeed moderate hypoxia-induced endothelial dysfunctions (Steinbauer 1998; Kaplan 2000), and interfere with the expression of adhesion molecules that mediate leukocyte-endothelial cell interactions (Akgur 1999; Pascual 2001; Tian 2004; Nohé 2005).

We have presented evidence that PMN sticking is inhibited by HES throughout the entire reperfusion phase, and HES interferes significantly with the PMN-derived CD11b integrin expression, whereas the other colloids did not affect this parameter (Varga 2008). This finding might refer to a situation where an anti-inflammatory compound decreases systemic PMN activation after a local inflammatory challenge. Indeed, others have found that various doses of HES inhibit the tissue nuclear factor-κB activation and systemic tumor necrosis factor-α elevation after local and systemic inflammatory insults (Tian 2004; Feng 2007).

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-DEX reduced the expression of CD11b in human settings (Rizoli 2003), but the reduced oxidative burst of the PMNs has been attributed to the hypertonic component of the solution (Fahrner 2002). We did not find DEX to be more effective than HES (Menger 1993) and we could not demonstrate any benefits on the FCD and/or RBCV changes, as described by Steinbauer et al. (Steinbauer 1998). However, it is recognized that, while the size and MW 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-MW HES solution which has been shown to possess more effective anti-inflammatory properties than those of medium- or high-MW HES (Jaeger 2001).

It has also been suggested that colloid solutions affect PMN adherence directly, by influencing the adhesion molecule expression independently of microhemodynamic changes (Pascual 2001). However, microhemodynamics and leukocyte adhesion are interrelated phenomena. Not only endothelium-derived vasoactive substances (typically nitric oxide and ET-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 or the dynamics and half-lives of molecular bonds (Marshall 2003). It has been shown that the reduced velocity of PMNs near the vessel wall increases the probability of PMN adhesion (Abbitt 2003) and, likewise, an increased shear rate can reduce the number of adherent cells (Kubes 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 2003). In the case of HES, nearly complete restoration of the baseline RBCV was observed during reperfusion, whereas the other colloids were ineffective. The shear rate could not be quantified in our study because of technical constraints, but the directly quantified microcirculatory parameters (i.e. RBCV and FCD) provided evidence of the improved perfusion of the capillary network. 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.

In conclusion, the minimization of the ischemic duration and quick restoration of the injured periosteal circulation can promote the avoidance of avascular necrosis in cases of an extreme peritalar sprain. In addition, this special case history suggests that the application of animal models for the clarification of human clinical problems is of enormous benefit. Our rat model allowed in vivo visualization of the microcirculation of the rat tibial periosteum in an experimental setting that aimed at simulating the clinical situation of vascularized bone autotransplantation. The beneficial effects of HES on the microvascular perfusion were paralleled by local anti-inflammatory actions and the prevention of systemic PMN activation, whereas the other colloid solutions did not influence these parameters. Although any extrapolation to clinical applications should be attempted only with caution, it appears reasonable to suggest that isovolemic hemodilution with HES provides a therapeutic advantage in this setting.
6. SUMMARY OF NEW FINDINGS

1. Our *in vivo* experiments permitted quantification of the microcirculatory alterations caused by limb I-R in a clinically relevant animal model.

2. The I-R injury was manifested in a deterioration of the efficacy of the periosteal microvascular perfusion and the PMN-endothelial interactions are critically linked to the microcirculatory derangement in the reperfused tissues.

3. The different colloid solutions exert diverse microcirculatory effects after limb ischemia. Only HES displayed a significant alleviating effect locally and in the prevention of systemic PMN activation, whereas the other colloid solutions (DEX and GEL) did not induce similar protection. This underlines the advantages of the use of HES in the care after trauma surgery.
7. REFERENCES


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9. ANNEX


