Examination of the periosteal microcirculation of long bones. Consequences of estrogen deficiency

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ABSTRACT RELATING TO THE SUBJECT OF THE THESIS

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LIST OF ABBREVIATIONS

BIS	bisphosphonate
CD11b	adhesion molecule
E2	17beta-estradiol
ER-alpha	estrogen receptor-alpha
ER-beta	estrogen receptor-beta
ICAM-1	intercellular adhesion molecule-1
IPC	ischemic preconditioning
IR	ischemia-reperfusion
IVM	intravital videomicroscopy
MPO	myeloperoxidase
MRONJ	medication-related osteonecrosis of the jaws
OVX	ovariectomy
OPS	orthogonal polarization spectral imaging
PMN	polymorphonuclear leukocyte
RANKL	receptor activator of nuclear factor κB ligand
TNF-alpha	tumor necrosis factor-alpha
ZOL	zolendronate

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SUMMARY

The major aim of this thesis was to examine and modulate the local (periosteal) microcirculatory and systemic inflammatory consequnces of transient lower limb ischemia during osteoporosis – a state which affects a considerably high portion of the elderly population (during surgical management of osteoporotic fractures).

In the first study, our aim was to examine the effects of limb ischemic preconditioning (IPC, 2 cycles of 10'/10') on the local periosteal and systemic inflammatory consequences of hindlimb ischemia-reperfusion (IR) in overiectomized (OVX) rats in the presence and absence of chronic 17beta-estradiol supplementation (E2, 20 μg kg^{-1}, 5 days/week for 5 weeks). In the second study, we aimed to examine the influence of chronic zoledronate (ZOL; 80 μg/kg iv, weekly for 8 weeks) on the periosteal microcirculatory consequences of limb IR. In both studies, neutrophil leukocyte (PMN)-endothelial interactions were quantitated in tibial periosteal postcapillary venules by intravital fluorescence videomicroscopy and the expression leukocyte adhesion molecule CD11b was measured by flow cytometry. In the first study, plasma TNF-alpha (by ELISA) and plasma free radical levels (as measured by chemiluminescence) as well as periosteal estrogen receptor expressions (by RT-PCR) were also determined.

The results show that the beneficial periosteal microcirculatory effects of limb IPC (reduced PMN–endothelial interactions and PMN-derived adhesion molecule CD11b expressions) are lost after OVX, but can be reversed with chronic E2 treatment (also causing positive effects that are not present after IPC, i.e. reduced TNF-alpha and free radical levels). This suggests that the presence of endogenous estrogen is a necessary facilitating factor in the anti-inflammatory protection provided by limb IPC in females and that the IPC-independent effects of E2 on inflammatory reactions should also be taken into account.

ZOL treatment caused temporary exacerbation in the limb IR-induced increases in periosteal microcirculatory reactions without causing any increases in CD11b expression on PMNs (which latter increased only in rats not treated with ZOL). The unaltered extent of limb IR-induced local periosteal microcirculatory reactions in the presence of reduced CD11b adhesion molecule expression on circulating PMNs may be attributable to local endothelial injury/activation caused by ZOL. Overall, the results suggest that chronic BIS treatment in osteoporosis causes no considerable postischemic periosteal microcirculatory complications during traumatological interventions involving tourniquet ischemia.

1. INTRODUCTION

1.1. The importance of periosteal microcirculatory examinations in bone surgery

The periosteal membranes separate the bones from the surrounding tissues and also bind them to the elements of the skeletal system, the tendons, septa and ligaments. Although it is well recognized that the periosteum is more than simply an envelope for the bone, it is a relatively infrequent site for microcirculatory studies. Despite several decades of research, the regenerative potential of the periosteum and the distinct role of the microcirculation in a range of important physiological and pathological events are only incompletely characterized, mainly due to methodological limitations. Using intravital microscopy (IVM), however, periosteal microcirculatory reactions can be assessed in clinically relevant animal models of reconstructive surgery, orthopedic trauma interventions and systemic diseases.

1.2. Brief anatomy and physiology of the periosteum

The periosteum is composed of an outer fibrous and an inner osteogenic cellular layer (for reviews, see *Augustin G et al., 2007; Dwek JR, 2010; Lin Z et al., 2014*). From a structural aspect, the superficial portion of the outer layer is the most vascularized part, supplying the deeper periosteal layers and the superficial layer of cortical bone. The endosteum has a similar histological structure to that of the periosteum with a rich blood supply and (albeit to a lesser extent) mesenchymal stem cells (*Brighton CT et al., 1992*). Providing a highly vascular connective tissue coverage, the endosteum also plays an active role in the regulation of the metabolism and regeneration of the bone.

The application of intravital microscopic methods has led to detailed descriptions of the morphological and functional characteristics of the periosteal microvasculature in different species. The microvascular architecture has been most extensively examined in rats, in which certain differences in the organization of the microvessels can be observed. The microvascular network within the rat mandibular periosteum comprises mainly arterioles and venules (*Varga R et al., 2014*). In rabbits, the maxillary periosteum consists mostly of parallel capillaries (*Rücker M et al., 2005*). Only a few capillaries and mostly venules are present in the tibial periosteum of the rat (*Varga R et al., 2008*). The microvessel density in the mandibular periosteum is similar to that in the anteromedial tibia (180–220 mm/mm^2), whereas it is about 30% lower in the anterolateral tibia (*Rücker M et al., 2006; Varga R et al., 2014*). The calvarial periosteum has a somewhat lower microvessel density (120–130 mm/mm^2) than that of any of these other tissues (*Rana M et al., 2011*).

Due to this rich nutritive vascular plexus, the periosteum is accurately described as the "umbilical cord of the bone" (*Chanavaz M, 1995*). The inner layer, also called the cambium layer, contains many osteogenic progenitors, similar to mesenchymal stem cells (*Soleymaninejadian E et al., 2012; Tenenbaum HC and Heersche JN, 1985; Zohar R et al., 1997*) and osteoblasts, situated directly on the outer surface of the bone cortex covered by fibroblasts. Besides the osteogenic stem cell properties, the cells of these membrane sheets have been shown to retain the ability to differentiate into fibroblasts, osteoblasts, chondrocytes, adipocytes and skeletal myocytes (*Emans PJ et al., 2005*). Consequently, the reconstruction, replacement or repair of lost tissues may be performed appropriately with an autogenous periosteum in experimental settings (*Finley JM et al., 1978; Reynders P et al., 1999*). These differentiation processes are under hormonal control (e.g. estrogen, parathyroid hormone and calcitonin) and are also influenced by alimentary factors (e.g. calcium and vitamin D3 uptake) and age (*Geusens P and Lems WF, 2011*). Both regenerative properties of the periosteal progenitor cells and anabolic actions of parathyroid hormone are markedly reduced by age (*Yukata K et al., 2014*).

1.3. The periosteum in bone healing and repair

It is well known that successful healing after fractures requires the regeneration of the peri- and endosteal microcirculations (*Macnab I and Dehoas WG, 1974*). Likewise, periosteal damage leads to perturbed bone healing with resultant delayed union or pseudoarthrosis formation (*Esterhai JL Jr and Gelb I, 1991; Gustilo RB et al., 1990; Utvag SE et al., 1998*). The importance of the periosteal microcirculation was hallmarked by early studies on dogs showing that autologous tibial grafts with only the periosteal blood supply intact survived transplantation (*Berggren A et al., 1982*). In another study, free gingival autografts placed on the periosteum survived by receiving nutrients from the maxillary bone with an intact periosteum (*Yanagihara K, 1990*). Further results demonstrated that revascularization during wound healing is critically linked to the release of vascular endothelial growth factor by periosteal cells (*Bourke HE et al., 2003*). In the oral cavity, the tissues produced by cells of mesenchymal origin include cementum with periodontal ligament fibers and bone; under appropriate culture conditions, periosteal cells secrete an extracellular matrix and form a membranous structure in the periodontium (*Mizuno H et al., 2006*). Apart from the initiation of cell differentiation during the bone repair and remodeling process after traumas and invasive dental procedures (*Tran Van PT et al., 1982*), the periosteal and endosteal membranes also play significant roles in the pathogenesis of metabolic bone alterations (*Allen MR et al., 2004*). In summary, it can be concluded that adequate microcirculation is a prerequisite of the

bone metabolism and the regenerative potential of the periosteal and endosteal compartments.

1.4. Potential regional differences in periosteal functions (axial versus appendicular bones)

Although a continuous microcirculatory supply is necessary to ensure physiological remodeling, metabolism and regeneration, there are many differences within the skeletal system between axial (i.e. the skull, facial bones, vertebrae, ribs and sternum) and appendicular bones. While the appendicular long bones receive their vascular supply from the nutritive epiphyseal and metaphyseal vessels (*Findlay DM, 2007; Johnson EO et al., 2004*), the circulation of the maxillofacial bones, and especially the lower jaw, is provided by the mucoperiosteal tissue through the inferior alveolar and sublingual arteries (*Huelke DF and Castelli WA, 1965; Shannon J et al., 2011*). It should be emphasized that the jaw region is endowed with additional, particular regeneration characteristics (*Elshahat A et al., 2004; Støre G and Granström G, 1999*). Unlike long bone fractures, which heal mainly through endochondral ossification, intramembranous ossification has a much higher impact in the mandible (*Yu YY et al., 2012*). In line with this, mandible periosteum-derived stem cells have been shown to possess the highest osteogenic potential among the different anatomical locations (*Solheim E et al., 1995; Ueno T et al., 2002*), while the tibial periosteum or the bone marrow stem cells are superior in terms of chondrogenesis (*Park JB et al., 2012*). Further, there are distinct differences in the expression pattern of bone development-related genes between the mandibular and tibial osteoblasts (*Reichert JC et al., 2013*). A point of interest is that systemic disorders such as osteoporosis affect the mandible to a significantly lesser extent (*Liu H et al., 2014; Mavropoulos A et al., 2007; Yamashiro T and Takano-Yamamoto T, 1998*). It has been proposed that the intense mechanical loading of the alveolar process during mastication may protect the alveolar bone from the osteoporosis-related bone loss observed at other skeletal sites (*Mavropoulos A et al., 2007*).

1.5. Examination of the periosteal microcirculation

Besides histology, various imaging methods, such as corrosion casting, microangiography, computer tomography and to some extent scintigraphy (*Berggren A et al., 1982; Bhatt R et al., 2000; Fayad LM et al., 2005; Nobuto T et al., 1989; Pazzaglia UE, 1996; Rhinelander FW et al., 1968; Trueta J and Cavadias AX, 1955*) can be used to visualize the architecture of the periosteal microcirculation in the jaw and long bones. Laser-Doppler flowmetry, laser speckle imaging (*ElMaraghy AW et al., 1999; Swiontkowsky MF et al., 1986*) and the radioactive microsphere technique (*Barron SE et*

al., 1977) can characterize functional details of the periosteal perfusion, but only with limitations. If more accurate detection or improved spatial resolution of the microcirculation is required, traditional fluorescence IVM can provide an opportunity for real-time examination of the microcirculation of superficial layers. Conventional fluorescence IVM visualizes not only changes in the efficacy of microvascular perfusion, but also cell–cell (e.g. polymorphonuclear leukocyte (PMN)–endothelial cell) interactions, such as rolling and adhesion, vascular diameter changes or signs of apoptosis (*Abshagen F et al., 2006; Horie Y et al., 1996*) (see Fig. 1A–D). Non-fluorescence techniques, including orthogonal polarization spectral imaging (OPS) (*Groner W et al., 1999*) (Fig. 1E–F) and sidestream dark-field imaging, have also been developed for the imaging of individual vessels and cells (*Milstein DM et al., 2010*). Nevertheless, observation of the microcirculation of the periosteal compartment would still necessitate surgical exposure.

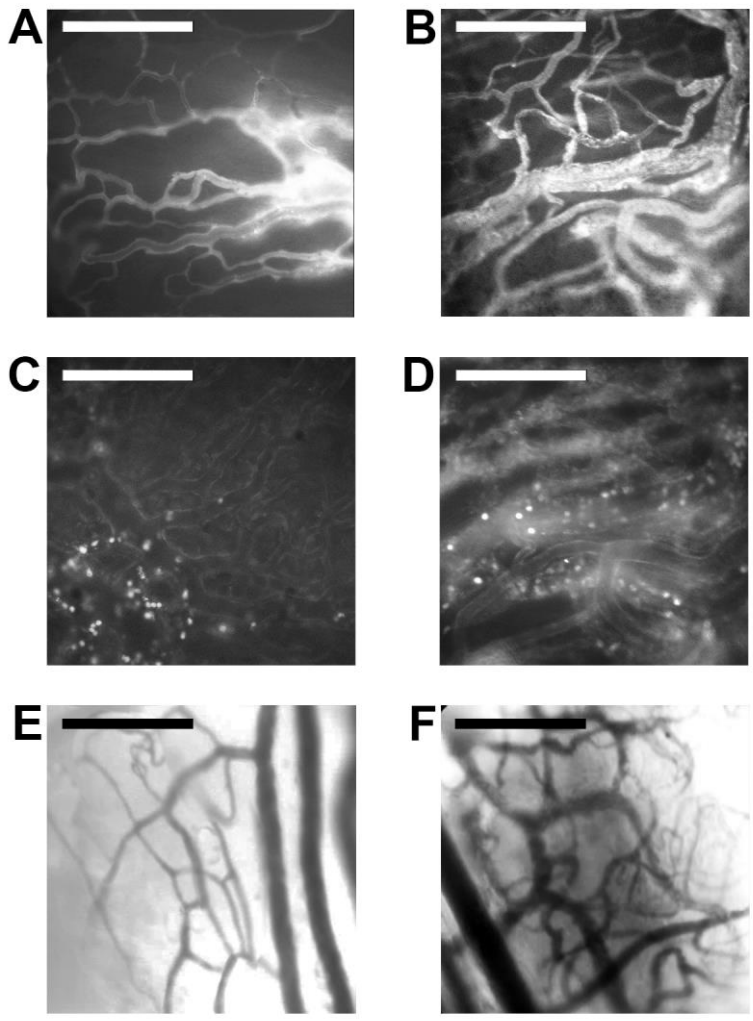

Figure 1. Fluorescence intravital microscopic (IVM) and orthogonal polarization spectral (OPS) images of the anteromedial tibial (**A, C, E**) and mandibular periosteum (**B, D, F**) in Sprague–Dawley rats. Upper two panels: IVM images; plasma labeling with fluorescein isothiocyanate-dextran (150 KDa, Sigma, St. Louis, MO, USA) (**A, B**) and rhodamine 6G (Sigma, St. Louis, MO, USA)-labeled neutrophil leukocytes (**C, D**), respectively. On the lower panels, OPS images of the tibia (**E**) and the mandible (**F**) are shown. The bar denotes 200 μm.

As concerns the appendicular bones, the anterior tibial periosteum appears to be one of the most easily accessible sites for microcirculatory examinations with IVM (see Table 1 in *Szabó A et al., 2017*, Annex). Methods have been established for the examination of osteomyocutaneous flaps, where microvascular perfusion characteristics and PMN–endothelial interactions can be dynamically assessed not only in the lateral tibial periosteum, but also in the adjacent muscle, subcutis and skin (*Rücker M et al., 1998*). In the cases of the axial bones, the calvarian periosteum in rats can be visualized under experimental conditions by means of different methods, either in acute settings (*Stoetzer M et al., 2014*) or with chronic models, applying the periosteal window and chamber methods (*Rana M et al., 2011; Stuehmer C et al., 2009; von See C et al., 2010*). The midfacial periosteum in rabbits (*Rücker M. et al., 2005*) and the mandibular periosteum in rats are also accessible for IVM, in the latter case also for OPS and confocal laser scanning microscopic assessments (*Varga R et al., 2014*).

1.6. Changes in nutritive and angiogenetic functions of the periosteum in clinically relevant trauma-orthopedic models

The direct consequences of traumas or surgical interventions on the periosteal microcirculation have been examined in a number of studies (see Table 1 in *Szabó A et al., 2017*, Annex). Soft tissue damage was induced by periosteal elevation using conventional and piezoelectric devices and the short- and long-term effects on the periosteal perfusion were compared (*Stoetzer M et al., 2014*). The extents of immediate and long-term periosteal perfusion failure were demonstrated and quantified after closed soft tissue traumas and closed tibial fractures showing an interaction between the skeletal muscle damage and periosteal microvascular injury (*Schaser KD et al., 2003; Zhang L et al., 2003*). In another study where functional capillary density changes were evaluated in the midfacial periosteum in rabbits, the risk of complications was higher after subperiosteal dissection compared to the supraperiosteal approach (*Rücker M et al., 2005*). Periosteal and subperiosteal angioneogenesis were induced with the self-inflating hydrogel expanders used for preliminary soft tissue augmentation before bone augmentation surgery (*von See C et al., 2010*).

The message of these studies is that regeneration of the periosteal microvasculature is a prerequisite of its tissue-regenerative potential. Another point for consideration is the communication of the periosteal and endosteal vascular networks. Alterations in the anteromedial and anterolateral periosteal microcirculations were recently studied with an OPS technique following standardized surgical destruction of the endosteum (*Greksa F et al., 2012*) (Fig. 1). The periosteal microvascular reorganization caused by reaming the

endomedullary cavity was not altered by implantation with titanium, a material with good osseointegrative properties, whereas the periosteal microcirculation was augmented by unstable endomedullary polyethylene nails. Although unstable implant materials can lead to inadequate restoration of the endosteal circulation, which remains insufficient to supply the cortical bone, this may be compensated for with enhanced periosteal sources (*Greksa F et al., 2012*).

1.7. Microcirculatory and inflammatory consequences of ischemia-reperfusion (IR) injury in bones and flap models

The consequences of ischemia-reperfusion (IR) and perfusion deficiencies have been extensively examined in the periosteum of bones and composite flaps. Vasomotion has been described as a compensatory reaction of the microvasculature aiding tissue survival. Critical perfusion conditions induce capillary flow motion in muscle, but not in the periosteum (*Rücker M et al., 2000*), and this potentially favorable reaction is preserved with heat shock priming (*Rücker M et al., 2005*). Heat shock has been shown to stimulate periosteal angiogenesis in the intact calvaria and in the tibia in a chronic ischemia model of microthrombosis (*Rana M et al., 2011; Rücker M et al., 2006*). The positive effects of heat shock priming are manifested with improved periosteal microvascular recanalization, recovery of perfusion rate and blood flow in the capillaries, similarly to those observed in other components of the osteomyocutaneous flap (*Rücker M et al., 2006*). In an acute composite flap ischemia model, heat shock reduced PMN adhesion in the postcapillary venules and intracellular adhesion molecule-1 (ICAM-1) expression in all examined tissues (*Rücker M et al., 2001*).

Primarily for emergency or surgical indications, transient limb ischemia is often achieved with the application of a tourniquet (i.e. wrapping a band around the extremity) in daily orthopedic trauma clinical practice. A tourniquet provides a clear operating field with reduced blood loss, but it also causes iatrogenic IR injury. Since a tourniquet around the extremity causes IR of an appreciably high amount of tissue, the venous effluent or afferent neurogenic signals lead to further systemic inflammatory reactions (i.e. activation of circulatory PMNs). The affected tissues undergo the typical biochemical and microcirculatory changes of a local IR injury, the periosteal microcirculation primarily being affected in this process. The postischemic microcirculatory inflammatory reactions include perfusion failure and activation of the PMN–endothelial interactions and the upregulation of adhesion molecule expression (e.g. ICAM-1) (*Szabó A et al., 2009*) (Fig. 2). Apart from activated PMNs (*Vega VL et al., 1999*), humoral factors (*Goldman G et al., 1990*), mediators of oxidative stress (*Koike K et al., 1992; Koike K et al., 1993*),

proinflammatory cytokines (e.g. TNF-alpha, platelet activatinf factor, IL-1 beta and IL-6) (*Lawlor DK et al, 1999*) and complement C5a (*Fleming SD et al., 2003*) have been implicated in remote organ dysfunctions and injuries (e.g. in the liver or lungs). These local processes also have relevant implications in remote organ preconditioning reactions as well. In other studies, antagonism of vasoconstriction-mediating endothelin-A receptors or pretreatment with anti-inflammatory agents (phosphatidylcholine and colloid solutions) likewise exerted some protection (*Gera L et al., 2007; Varga R et al., 2008; Wolfárd A et al., 2002*). These studies further revealed that the microcirculation of the tibial periosteum is more prone to microcirculatory inflammatory complications than that of the synovial membrane (*Hartmann P et al., 2012*).

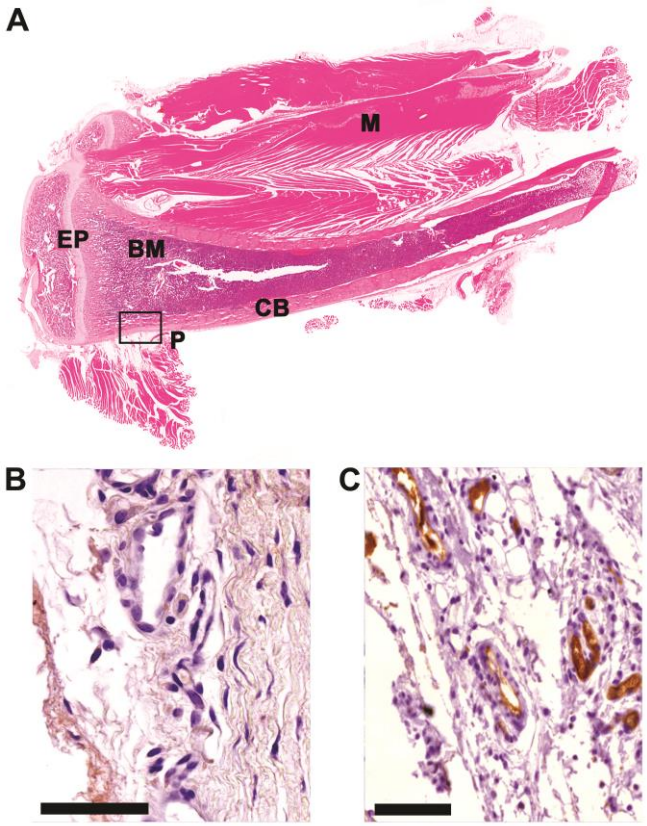

Figure 2. Representative longitudinal section of the rat tibia surrounded by soft tissues (stained with ICAM-1 plus hematoxylin) (**A**). Tibia epiphysis (EP), cortical bone (CB), bone marrow (BM), muscle (M) and periosteum (P) are indicated. Lower panels: weak ICAM-1 staining (primary antibody: a mouse monoclonal anti-rat ICAM-1; BD Pharmingen, BD Biosciences, San Jose, CA, USA), secondary antiobody: biotinylated goat anti-mouse antibody conjugated to HRP polymer (Envision®System; Dako, Glostrup, Denmark) in the periosteum of a sham-operated animal (**B**), positive staining in periosteal venules after 60 min of limb ischemia followed by a 180-min reperfusion period (**C**). The bar denotes 50 μm.

1.8. Microcirculatory conseqences of limb ischemic preconditioning (IPC)

Ischemic tolerance of the tissues can be increased via application of ischemic preconditioning (IPC), an approach when transient, brief periods of ischemia are followed by short intervals of reperfusion. The positive effect of IPC was first described in the heart (*Murry CE et al. 1986*), but was later demonstrated in many organs (*Gho BC et al. 1996; Cheung MM et al., 2006*), including the periosteum (*Hartmann P et al., 2011*). This protection is mediated by different endogenous mediators, including nitric oxide, carbon monoxide or adenosine (for reviews, see *Walsch SR et al., 2007; Tapuria N et al., 2008*), and it also exerts marked protection at the microcirculatory level (*Hartmann P et al., 2011*). The mechanism of protection provided by IPC includes modulation of the oxidative burst (*Quarrie R et al., 2012*) and downregulation of the expression of adhesion molecules responsible for PMN adhesion and transmigration, thus alleviating tissue damage (*Duda M et al., 2006*). Experimental results further show that IPC also affects the adenosine- and cAMP-dependent pathways and modulates intracellular redox homeostasis and mitochondrial functions (*Walsch SR et al., 2007; Tapuria N et al. 2008*). IPC performed on limbs or arms provides quite a robust protective signal against the subsequent IR insult via the relatively large affected tissue mass. The favorable local and remote microcirculatory effects of limb IPC have both been demonstrated in many organs (*Szabó A et al., 2009; Walsch SR et al., 2007; Tapuria N et al., 2008*). The amelioration of IR-induced inflammatory complications by limb IPC should offer a therapeutic benefit in elderly patients when the prevalence of skeletal injuries increases and osteoporotic bones are more prone to accidental fractures.

1.9. Periosteal manifestations of systemic diseases

Osteoporosis affects more than 75 million people worldwide (*Schuiling KD et al., 2011*), with every other woman and every fifth man over 50 years suffering an osteoporotic fracture of the extremities during her or his remaining lifetime (*Tarantino U et al., 2017*). As the prevalence of skeletal fractures and limb injuries increases with aging, restoration of the periosteal microperfusion is of particular importance in surgical interventions on elderly patients with or without tourniquet application. The microcirculatory patency is influenced by many functional and morphological changes in the microvasculature and, as a result of the osteogenic stem cell content of the periosteum and endosteum (*Brighton CT et al., 1992*), these membranes, and the periosteum in particular, play important roles in the pathogenesis of hormone-related (i.e. estrogen deficiency) and trauma-induced osteoporotic processes. Likewise, the periosteum can serve as a target of different anti-

osteoporotic approaches (*Allen MR et al., 2004*). In experimental settings, osteoporosis/osteopenia is often induced via bilateral ovariectomy (OVX) in animals.

Although relatively little information is available on the microcirculatory effects of chronic OVX itself, a considerable amount of evidence suggests that a disintegrated microcirculation is involved in the osteoporotic changes in the cortex. OVX-induced morphological alterations within the microvasculature have been reported in the heart (*Jesmin S et al., 2003*) and brain (*Jesmin S et al., 2002*), but our group did not observe significant changes in periosteal vessel density in a chronic OVX rat model (*Szabó A et al., 2011*). It is important to note that these changes were followed in the proximal tibial periosteum, where the predominant vessel type is the venule (see Fig. 1). The postcapillary venules are predilectory sites for PMN–endothelial interactions, and thus a relatively high venular density may predispose to local inflammatory complications. Again, this feature of the periosteal microarchitecture may account, at least in part, for a tissue-specific response (see Figs. 1–2).

On the other hand, there have been several experimental studies on the microcirculatory changes of sex hormone substitution. Since estradiol is protective in many forms of traumas and injuries, it is possible that 17beta-estradiol (E2) is protective independently of the presence of OVX. This view is supported by findings where a single dose of E2 administered hours or even minutes before reperfusion ameliorated PMN-related processes in different organs (e.g. the heart, liver and retina) (*Booth EA et al., 2003; Burkhardt S et al., 2008; Nonaka A et al., 2000*). Our group previously found that the postischemic periosteal microcirculatory complications were not aggravated as compared to the non-OVX, age-matched controls (*Szabó A et al., 2011*). The situation was similar as concerns TNF-alpha release, providing further evidence that OVX itself does not modify the inflammatory complications, whereas E2 supplementation greatly reduces this reaction. It has also been demonstrated that E2 inhibits TNF-alpha gene transcription via the beta-estrogen receptors (*Srivastava S et al., 1999*) and via the TNF-alpha-mediated increases in the expressions of adhesion molecules and chemoattractants (*Xing D et al., 2007*). A downregulation of nitric oxide synthesis by TNF-alpha may also contribute to the mechanisms of these microcirculatory reactions (*Yoshizumi M et al., 1993*). Data indicating the positive effect of prolonged E2 substitution on the PMN reactions and TNF-alpha release, however, point to another clinical implication: ovarial hormone deprivation supplemented with estrogen therapy (apart from the well-known positive effect in reducing the risk of osteoporotic fractures) affords marked protection against the release of inflammatory mediators. Based on the above findings, one of the present

studies was designed to ascertain whether IPC exerts its potentially positive anti-inflammatory effects on limb IR injury with chronic estrogen deficiency. In our study, we also sought to examine whether the periosteal microcirculatory reactions are modulated by exogenous estrogen supplementation. With these points in mind, we decided to characterize the effects of IPC with or without estrogen supplementation on local periosteal and systemic inflammatory changes in a rodent model of hindlimb IR injury with chronic estrogen deficiency.

1.10. Bisphosphonates (BIS) for the treatment of osteoporosis and oncological diseases

Bisphosphonates (BISs) are widely used for the treatment of osteoporosis and tumors with bone metastasis to inhibit osteoclast activity and bone resorption (*Rogers MJ et al., 2011; Ruggiero SL et al., 2014*) and ameliorate the osteoporosis-induced decrease of bone mineral density (*Tarantino U et al., 2017; Sanderson J et al., 2016*). Further, it has been shown that the risk of osteoporotic fractures can be reduced, in particular with the use of zoledronic acid (ZOL) (*Tarantino U et al., 2017; Byun JH et al., 2017*). The physicochemical and biological properties of BISs depend on the R2 side group, and the presence of nitrogen and its orientation influence their clinical effects (*Rogers MJ et al., 2011*). In the maxillofacial region, the regional BIS uptake reaches a much higher concentration compared to the appendicular and other axial bones (*Wen D et al., 2011*). The receptor activator of nuclear factor κB (RANK)/receptor activator of nuclear factor κB ligand (RANKL)/osteoprotegerin axis, a signaling pathway that regulates osteoclast differentiation, is also diversely affected by BISs, which cause a decrease in RANKL values in the mandible and the opposite effect in the tibia (*Çankaya M et al., 2013*). Furthermore, BIS treatment exerts site-specific, differential effects during the early healing processes of tibial and mandibular fractures by delaying callus, cartilage and bone remodeling specifically in the mandible (*Yu YY et al., 2012*). Defective angiogenesis of the mandibular mucoperiosteal tissues is also evoked by long-term treatment with BIS (*Wehrhan F et al., 2011*). Nevertheless, unwanted, necrotic reactions induced by BIS may also be present in the skeletal system. Specifically, chronic BIS treatment can effectively enhance the incorporation of bone implants in appendicular bones (*Stadelmann VA et al., 2008; Ying G et al., 2016*), but the likelihood of osteonecrotic complications also increases in parallel at the jaw bones (*Tarantino U et al., 2017; Ruggiero SL et al., 2014; Brozoski MA et al., 2012*). The incidence of necrosis is especially high in the mandible after oral surgical interventions, leading to a condition termed medication-related osteonecrosis of the jaws (MRONJ). An exact pathogenesis of this complication is still

unknown, but it seems to affect the appendicular and axial bones differently (*Senel FC et al., 2010*).

1.11. Microcirculatory consequences of BIS treatments

It has also been shown that BISs can induce significant inflammatory reactions in the mandibular periosteum after tooth extractions, while the microcirculation in the tibial region remained unaffected (*Janovszky Á et al., 2015*). The impact of periosteal microvascular reactions is also evident in the case of long-term systemic treatment with nitrogen-containing BISs. Treatment with osteoclast inhibitor BISs can cause MRONJ in the jaw after invasive dental procedures (*Kühl S et al., 2012; Marx RE et al., 2007; Wehrhan F et al., 2011; Yamashita J et al., 2010*). Such a reaction does not occur in the bones of the appendicular skeleton (*Blazsek J et al., 2009; Stadelmann VA et al., 2008*). Many theories have been put forward and several risk factors have been investigated in efforts to decipher the pathogenesis of MRONJ, but the cause is still unknown (*Mehrotra B and Ruggiero S, 2006*). The role of infection-induced inflammatory reactions has been suggested, among other factors, but since osteonecrosis usually develops several years after tooth extraction, this factor seems to be of only limited impact. Enhancement of PMN–endothelial cell interactions in the knee joint (*Zysk SP et al., 2003*) and the upregulation of pro-inflammatory cytokines such as IL-1 and TNF-alpha (*Anastasilakis AD et al., 2012; Norton JT et al., 2011*), however, have been clearly demonstrated in response to BIS administration. These effects also display spatial differences because certain inflammatory reactions were confined to the mandible and could not be detected in the femur (*Senel FC et al., 2010*). Moreover, in another sheep model with osteoporosis, the stability of implants in the femoral condyle was even enhanced after local BIS treatment (*Stadelmann VA et al., 2008*). Other data suggest that the production of vascular endothelial growth may be an important factor in the pathogenesis of MRONJ (*Vincenzi B et al., 2012*), a notion supported by the fact that BIS treatment combined with the anti-angiogenic drug bevacizumab increases the prevalence of MRONJ (*Aragon-Ching JB et al., 2009*). Furthermore, the critical concentration of BIS in the mandible (*Wen D et al., 2011*) and its direct toxic effect on the periosteal stem/osteoprogenitor cells and related inflammatory effects in the periosteum may also contribute to the development of MRONJ. High-dose BIS exacerbated the inflammatory response in a periodontitis model, where the bone lesions bear a striking resemblance to MRONJ (*Aguirre JI et al., 2012*). With prolonged use, BISs are known to accumulate in the skeleton, reaching the highest concentration in the mandible (*Reid IR et al., 2007; Wen D et al., 2011*), thus possibly explaining their potential toxic effects occurring predominantly in the jawbones.

Furthermore, osteoblasts have different proliferation properties at different locations in the appendicular or axial bones under physiological circumstances, and this phenomenon is also critically influenced by BIS treatment (*Marolt D et al., 2012*). The functional activity of osteocytes differs between the mandible and the tibia (*Çankaya M et al., 2013*), and the aggravating effects of BISs on bone healing are confined to the jaw (*Kuroshima S et al., 2014*). IVM data obtained in the proximity of the alveolar injury and from a contralateral, intact site on the mandibular periosteum were compared with those on the intact tibia (*Janovszky Á et al., 2015*). After chronic ZOL treatment, increased extents of PMN–endothelial interactions (rolling and firm adhesion) were observed in the mandibular periosteum, both at the site of the earlier tooth extraction and at the contralateral site, but the corresponding interactions in the tibia were significantly less pronounced.

Previously, our group also showed that BISs can induce significant inflammatory reactions in the mandibular periosteum after tooth extractions, while the microcirculation in the tibial region remained unaffected (*Janovszky Á et al., 2015*). The aim of the present study was to examine the effects of chronic BIS treatment on the postischemic periosteal microcirculatory changes in the lower extremities. To our knowledge, the possible modulator role of BIS on the inflammatory reactions of the appendicular bones disposed to IR injuries has not yet been evaluated elsewhere. Based on the relevant literature data and our previous results, our null hypothesis was that ZOL treatment does not influence the periosteal microcirculatory reactions of transient limb IR. We tested this hypothesis in a clinically relevant model of osteoporosis where anesthetized rats were challenged with standardized limb IR in the presence or absence of chronic ZOL treatment.

2. MAIN GOALS OF THE STUDIES

The major aim of our study was to examine and modulate the local (periosteal) microcirculatory and systemic inflammatory consequences of transient lower limb ischemia. We addressed the following clinically relevant problems, both of which affect the elderly population with osteoporosis:

- IPC has been shown to provide protection against the deleterious consequences of IR induced by limb ischemia. In the present study, we aimed to examine whether IPC exerts its potentially positive anti-inflammatory effects on limb IR injury with chronic estrogen deficiency. We also sought to examine whether the periosteal microcirculatory reactions are modulated by exogenous estrogen supplementation. Therefore, we characterized the effects of IPC with or without estrogen supplementation on local periosteal and systemic inflammatory changes in a rodent model of hindlimb IR injury with chronic estrogen deficiency.
- There is a wide range of clinical indications for BIS treatment including treatment for osteoporosis, but this treatment causes serious complications at axial bones (such as osteonecrosis of the jawbones). Our second aim was to assess the effects of chronic BIS treatment on the consequences of tourniquet ischemia (on the postischemic periosteal microcirculation and systemic inflammatory reactions) in a clinically relevant model of osteoporosis, where anesthetized rats were challenged with standardized limb IR in the presence or absence of chronic ZOL treatment.

3. MATERIALS AND METHODS

The experiments were performed in 2 studies. In the first study, the microcirculatory effects of chronic estrogen deprivation (elicited by OVX) and estrogen supplementation were examined on the efficacy of IPC in a tourniquet ischemia model. In the second study, the effects of chronic BIS treatment on the tibial periosteal microcirculatory consequences of limbs ischemia were examined in a shorter-term OVX model in rats.

3.1. Animals

All studies were carried out on Sprague–Dawley rats housed in an environmentally controlled room with a 12-h light-dark cycle, and kept on commercial rat chow (Charles River, Wilmington, MA, USA) and tap water *ad libitum*. The project was approved by the National Scientific Ethical Committee on Animal Experimentation (National Competent Authority) under license number V./144/2013. The studies were performed in adherence with EU Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and the National Institute of Health guidelines for the use of experimental animals. Animal welfare-related assessments and interventions were carried out prior to and during the experiments.

3.2. Surgical procedure for ovariectomy (OVX)

In both studies, 12-week-old female rats (weighing 180 to 200 g) were randomly allocated to ovariectomized or sham-operated groups. The animals were anesthetized with an intraperitoneal combination of ketamine and xylazine (25 mg kg^{-1} and 75 mg kg^{-1}, respectively), and a median laparotomy was performed under sterile conditions. The connection of the Fallopian tubes was cut between hemostats, the ovaries were removed, and the stumps were then ligated with a 3-0 non-absorbable thread (Ethibond Excel®, Ethicon, Somerville, NJ, USA). Thereafter, the abdomen was filled with warm sterile physiological saline and the abdominal wall was closed with a 4-0 absorbable suture and a 4-0 non-absorbable suture (Vicryl® and Prolene®, Ethicon, Somerville, NJ, USA) in two layers. Sham-operated animals underwent identical procedures, except of course that the Fallopian tubes and ovaries were not touched.

3.3. Experimental design and protocols

Study 1

Chronic estrogen (E2) treatment

Eight weeks after OVX (i.e. at 20 weeks of age) (see Fig. 3), chronic estrogen therapy was initiated in some of the OVX animals for 5 days/week with 20 μg kg^{-1} subcutaneous 17beta-estradiol (E2, Sigma, St. Louis, MO, USA), and it was continued for 5 weeks (i.e.

until the end of the experimental protocol in week 25) in the first study. The remaining OVX and sham animals received the vehicle for E2 (100% ethanol diluted in corn oil) in the same volume.

Experimental series

In the first study, *in vivo* experiments were performed in two major series 13 weeks after the OVX and sham operations (in week 25) (Fig. 3). In the first series of experiments, the tibial periosteal microcirculatory consequences of a 60-min complete hindlimb ischemia followed by a 180-min reperfusion (with or without limb IPC) were investigated with IVM. In a second series, identical protocols in the same groups were carried out to detect changes in various systemic inflammatory reactions (see below).

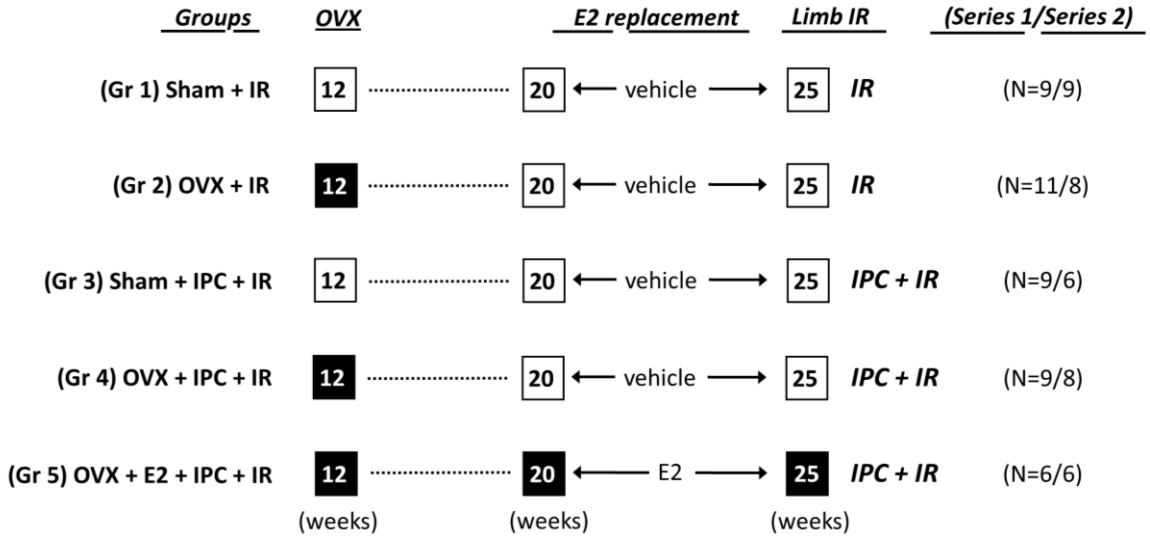

Figure 3. Groups and time sequence of surgical interventions, treatments and measurements in Study 1: Ovariectomy (OVX) or a sham operation (sham) was performed at 12 weeks of age; 17beta-estradiol treatment (E2) was performed for 5 weeks (5 days/week in a dose of 20 μg kg^{-1}); tourniquet ischemia of a hindlimb was performed at the end of the protocol followed by reperfusion (IR; 60'/180') with or without ischemic preconditioning of the hindlimb (IPC; 2x10'/10'). In Series 1, an assessment of local inflammatory reactions was carried out in the tibial periosteum using intravital microscopy, while in Series 2, evaluation of various systemic inflammatory parameters was performed. The number of animals used per group in each series is indicated in brackets.

In the final stage of Study 1, the animals were randomly allotted to one of the following 5 groups. These are shown in Fig. 3, week 25. Among the vehicle-treated animals, a 60-min complete hindlimb ischemia was induced by applying a tourniquet around the proximal femur and a miniclip on the femoral artery, which was followed by a 180-min reperfusion period in 9 sham-operated animals (sham+IR group) and 11 OVX animals (OVX+IR groups). Two other vehicle-treated groups were also subjected to 2 cycles of 10 min of limb IPC and 10 min of reperfusion (sham+IPC+IR group, N=9; OVX+IPC+IR group,

N=9). This IPC protocol has been shown to ameliorate local microcirculatory and systemic inflammatory complications caused by limb IR in male rats (*Szabó A et al., 2009*). In all of the E2-treated animals, limb IR was combined with IPC (OVX+E2+IPC+IR group, N=6) and the experiments were started 18–24 hrs after the last E2 injection. In this series, the periosteal microcirculation was observed with IVM at baseline and every 60 min during the 180-min reperfusion period (see below).

In a second series of Study 1, identical protocols for the same groups were applied to detect changes in the pro-inflammatory cytokine TNF-alpha concentrations in the plasma and in whole blood free radical productions, as well as in the expressions of a circulating PMN-derived adhesion molecule (see the groups above, N=6–9). It was necessary to separate the two series to avoid any interference between the fluorescent dyes used for IVM and the acquisition techniques used with flow cytometry and luminometry. In this series of experiments, measurements were made from blood samples taken at baseline and at every 60 min of the reperfusion phase. At the end of the protocol, periosteal specimens were harvested under RNase- and DNase-free conditions to detect periosteal estrogen receptor (ER) expressions, and then the samples were stored at –80°C until assay.

Study 2

In the second study, a chronic ZOL treatment was initiated in 16 animals (OVX+BIS group) 5 weeks after OVX (i.e. at 17 weeks of age) with 14 of the sham-operated animals serving as negative controls (sham+BIS group) (see Fig. 4). ZOL (80 μg kg^{-1} Zometa®, Novartis Europharm, Budapest, Hungary) was administered once a week intravenously into the tail vein under light ether anesthesia. The remaining OVX and sham-operated animals received physiological saline in the same volume (OVX+vehicle and sham+vehicle groups, N=16 each). These weekly injections were continued for 4 weeks. At the end of the experimental protocol (in week 21), all of the animals were subjected to a 60-min complete hindlimb ischemia followed by a 180-min reperfusion period. Limb ischemia was induced by applying a tourniquet around the thigh and placing a miniclip on the femoral artery. The experiments were performed in two experimental series. In series 1, the periosteal microcirculation was examined using IVM at baseline and every 60 min during the 180-min reperfusion period (N=7–9 per group) (see below). In the second experimental series, blood samples from the carotid artery were taken at baseline and during the reperfusion period to detect changes in the plasma concentrations of TNF-alpha and in the expression of the adhesion molecule CD11b (N=7 in each group). It was

again necessary to separate the two series to avoid any interference between the fluorescent dyes used for IVM and the acquisition techniques used with flow cytometry.

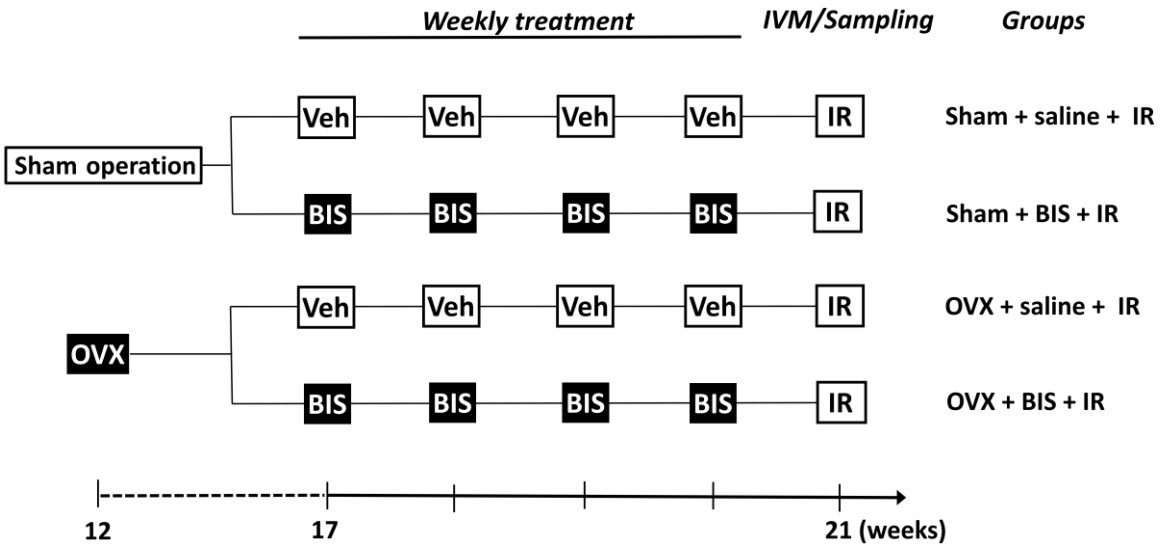

Figure 4. Groups and time sequence of interventions and treatments in Study 2. Rats were sham-operated or ovariectomized (OVX) at the age of 12 weeks. Five weeks later (week 17), chronic bisphosphonate (BIS, once a week with a dose of 80 μg kg^{-1} in the tail vein) or saline vehicle (Veh) treatment was initiated. At the end of the protocol (week 21), 60 min of limb ischemia was induced by 180 min reperfusion (IR), and periosteal microcirculatory measurements were conducted with fluorescence intravital microscopy (IVM; Series 1, N=7–9). Sampling for leukocyte adhesion molecule CD11b expression and serum TNF-alpha measurements were also carried out (Series 2, N=7 each).

3.4. Assessment of local inflammatory reactions using IVM

The experiments in both studies were performed under sodium pentobarbital (45 mg kg^{-1} ip) anesthesia and sustained with small supplementary intravenous doses when necessary. The right carotid artery and the jugular vein were cannulated to measure mean arterial pressure and to administer drugs and fluids, respectively. The animals were placed in a supine position on a heating pad to maintain their body temperature between 36 and 37°C. Here, Ringer's lactate was infused at a rate of 10 ml kg^{-1} h^{-1} during the experiments. The trachea was cannulated to facilitate respiration. The right femoral artery was isolated, and the periosteum of the medial surface of the right tibia was exposed under a Zeiss microscope with 6× magnification, using an atraumatic surgical technique (*Varga R et al., 2008*).

Microcirculatory measurements

3.4.1. Intravital microscopy (IVM)

The right hindlimb with the exposed tibial periosteum was positioned horizontally on an adjustable stage for examination of the microcirculation by IVM (Zeiss Axiotech Vario

100HD microscope, 100W HBO mercury lamp, Acroplan 20× water immersion objective, Carl Zeiss GmbH, Jena, Germany). Microcirculation was visualized with fluorescein isothiocyanate (Sigma, St. Louis, MO, USA)-labeled erythrocytes (0.2 ml iv), while PMNs were labeled with an iv injection of rhodamine 6G (Sigma, St. Louis, MO, USA, 0.2%, 0.1 ml iv). The microscopic images were recorded with a charge-coupled device video camera (Teli CS8320Bi, Toshiba Teli Corporation, Osaka, Japan) attached to an S-VHS video recorder (Panasonic AG-MD 830, Matsushita Electric Industrial Co., Tokyo, Japan) and a personal computer.

3.4.2. IVM - video analysis

A quantitative assessment of the microcirculatory parameters was performed off-line by a frame-to-frame analysis of the videotaped images, using image analysis software (IVM, Pictron Ltd., Budapest, Hungary) (Fig. 5). As for the periosteum, leukocyte–endothelial cell interactions were analyzed within 5 postcapillary venules (with diameters between 11 and 20 μm) per animal. IVM in the periosteum allows the observation of the primary and secondary PMN–endothelial interactions (rolling and adhesion, respectively). Rolling is a transient and reversible process, whereas adhesion represents a higher level of activation of leukocytes (when endothelial contact-dependent signals trigger the formation of the activation-dependent adhesion molecule expression of PMNs with accompanying NADPH oxidase activation and degranulation) (*Kolaczkowska E and Kubes P, 2013*). Based on their movements and contact with the endothelium of the postcapillary venules, 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 expressed here 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 were expressed as the number of cells/vessel circumference in millimeters.

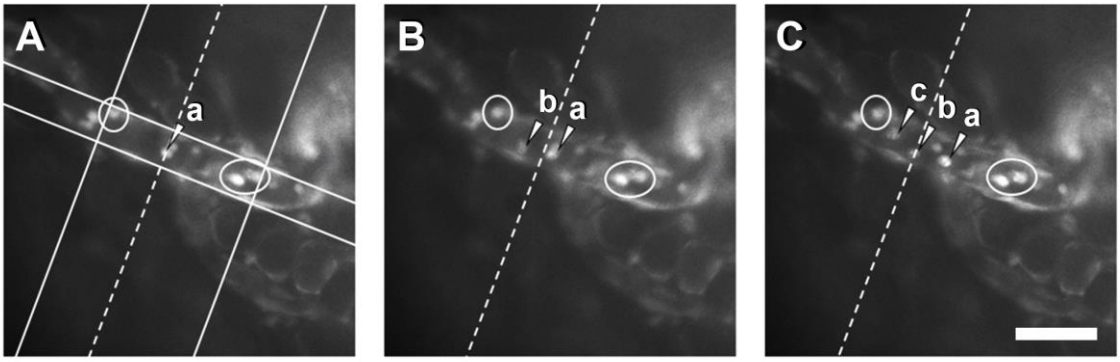

Figure 5. Representative micrographs showing the sequence of PMN−endothelial interactions on three consecutive images (Panels A–C) recorded using intravital

microscopy (recording rate: 20 frames/s). The segment of the examined tibial postcapillary vein is surrounded by lines in Panel A. Movement of rhodamine 6G-labeled PMN (marked a–c) is demonstrated frame by frame with reference to a dashed line. Stationary (adhesive) leukocytes are marked by ellipses. The bar in Panel C denotes a 50 μm scale, which applies to all photomicrographs.

3.5. Detection of systemic inflammatory reactions

3.5.1. Immune labeling and flow cytometric analysis of adhesion molecule CD11b expression of PMNs

The surface expression of CD11b on the peripheral blood PMNs was determined via a flow cytometric analysis of whole blood in duplicate. 100 μl of whole blood was incubated with 20 μl of (50 μg ml^{-1}) fluorescein isothiocyanate-conjugated mouse anti-rat monoclonal antibody (clone OX-42, AbD Serotec, Kidlington, UK) for 20 min. Negative controls were obtained by omitting the monoclonal antibody. The cells were then washed twice in Hanks' buffer and centrifuged (Heraeus Biofuge primoR, Thermo Scientific, Waltham, MA, rotor diameter: 65 mm) at 12,281 g for 5 min. The cells were again washed twice, and the erythrocytes were lysed with a lysis buffer (Erythrolyse Red Blood Cell Lysing Buffer (10x) Reagent, GenWay, San Diego, CA, USA) for 8 minutes, after which the cells were washed twice again (2,616 g, 5 min) and resuspended in 750 μl of Hanks' buffer. CyFlow ML (Partec GmbH, Münster, Germany) equipment was used for cytometry; the granulocytes were gated on the basis of their characteristic forward and sidescatter features. 10,000 events per sample were collected and recorded, and then the percentages of labeled (activated) granulocytes (relative to the overall marker-bearing cells) and the mean fluorescence intensity (average marker density) were calculated.

3.5.2. Determination of plasma TNF-alpha levels

Blood samples (0.5 ml) were taken from the carotid artery and placed into precooled EDTA-containing polypropylene tubes, centrifuged at 13,500 rpm for 5 min at 4°C and then stored at –70°C until assay. Proinflammatory cytokine TNF-alpha concentrations were determined in plasma samples by means of commercially available enzyme-linked immunosorbent assays (Quantikine Ultrasensitive ELISA kit for rat TNF-alpha; R&D Systems, Minneapolis, MN, USA).

3.5.3. Free radical-producing capacity of the blood

10 μl of blood dissolved in Hanks' buffer was incubated for 20 min at 37°C in lucigenin (5 mM; dissolved in Hanks' buffer) solution in the presence or absence of zymozan (190 μM, dissolved in Hanks' buffer). Superoxide production was estimated via the rate of the zymozan-induced increase in chemiluminescence (measured with an FB12 Single Tube

Luminometer (Berthold Detection Systems GmbH, Bad Wildbad, Germany) and normalized for leukocyte counts in the peripheral blood.

3.6. Determination of plasma E2 levels

Endogenous E2 levels were determined using the Elecsys Estradiol III kit (Roche Diagnostics GmbH, Mannheim, Germany) and the Roche Cobas e 601 immunology analyzer (Roche Diagnostics GmbH, Mannheim, Germany).

3.7. Determination of periosteal estrogen receptor-alpha (ER-alpha) and beta (ER-beta) mRNA expressions

Tissue Collection. Anteromedial tibial periosteal samples were harvested via sterile surgical exposure of the contralateral (non-ischemic) limbs under an operating microscope. The samples were washed in 0.3 ml of sterile DNase-, RNase- and protease-free water (Sigma, St. Louis, MO, USA) and placed in RNA stabilization solution (0.2 ml/sample; RNAlater, Ambion®, Thermo Fisher Scientific, Waltham, MA, USA). After overnight storage at 4°C, the RNA stabilization solution was removed, and tissue samples were stored at –80°C until RNA purification. Here, uterus samples were used as internal controls.

RNA purification. The total RNA taken from the tibial periosteum and the uterus in each animal was purified with the NucleoSpin® RNA XS kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the protocol provided by the manufacturer.

Real Time PCR for ER-alpha and ER-beta. 100 ng of RNA template in a 10 µl reaction mix were measured using a quantitative reverse transcriptase-mediated PCR kit (Verso 1-step RT-qPCR Mix, ROX kit; Thermo Fisher Scientific, Waltham, MA, USA). The amplification conditions were 50°C for 15 min, 95°C for 15 min, 40 cycles of 95°C for 15 s and 58°C for 15 s. RNA levels were calculated using the ΔΔCT method and were normalized to 18S mRNA. The Universal Probe Library (UPL) system (Roche, Basel, Switzerland) was used to design primers and probes for the experiments (see Table 1).

Table 1 Primers and probes for quantitative RT-PCR

Target	Forward primer	Reverse primer	Probe
ERα	TTCTTTAAGAGAAGCATTCAAGGAC	TCTTATCGATGGTGCATTGG	#130; 04693663001
ERβ	GGCTGGGCCAAGAAAATC	TCTAAGAGCCGGACTTGGTC	#111; 04693442001
18S	CTCAACACGGGAAACCTCAC	CGCTCCACCAACTAAGAACG	#77; 04689003001

3.8. Statistical analyses

The required number of animals (i.e. sample size) was assessed with the PS Power and Sample Size Calculations software package (version 3.1.2) prior to the experiments. Data analysis was performed with the SigmaStat statistical software package (Jandel Corporation, San Rafael, CA, USA).

Study 1:

The normality of the data sets was checked, and changes in variables within and between groups were analyzed in the case of normal distribution with the two-way repeated measures ANOVA test followed by the Holm–Sidak test. Data are expressed as means ± standard error of the mean (SEM). Due to the non-Gaussian distribution, PCR data were analyzed with the Kruskal–Wallis test, followed by the Dunnett test; the box plot figure shows the mean, the median, and the 25^{th} and 75^{th} percentile values. p values < 0.05 were considered statistically significant for all parameters.

Study 2:

Nonparametric methods were used: two-way RM ANOVA was used followed by the Holm–Sidak and Dunn's tests to assess differences within and between groups, respectively. Data are presented as mean value and SEM in all figures. p values < 0.05 were considered significant.

4. RESULTS

4.1. Effects of E2 on limb IPC-related periosteal microcirculatory changes

When compared with the baseline values, the values for the primary PMN–endothelial interactions (termed rolling) in the postcapillary venules of the tibial periosteum increased to a similar extent in the sham+IR and OVX+IR animals at all examined time points of reperfusion after limb IR (see Fig. 6A). When limb IR was combined with local IPC, moderately reduced rolling values were observed in non-ovariectomized rats (sham+IPC+IR group) at later stages of reperfusion (120 and 180 min), but no reduction was seen in OVX rats (OVX+IPC+IR group). At 60 min and 120 min of reperfusion, the lowest rolling values were detected in animals treated with chronic E2 (OVX+E2+IPC+IR group), but these differences were not statistically significant. Leukocyte adherence (sticking) revealed a similar pattern to that seen with PMN rolling. No ameliorating effect of IPC was seen in OVX animals (in the OVX+IPC+IR group), but some alleviating effect was observed after E2 treatment (in the OVX+E2+IPC+IR group) (see Fig. 6B).

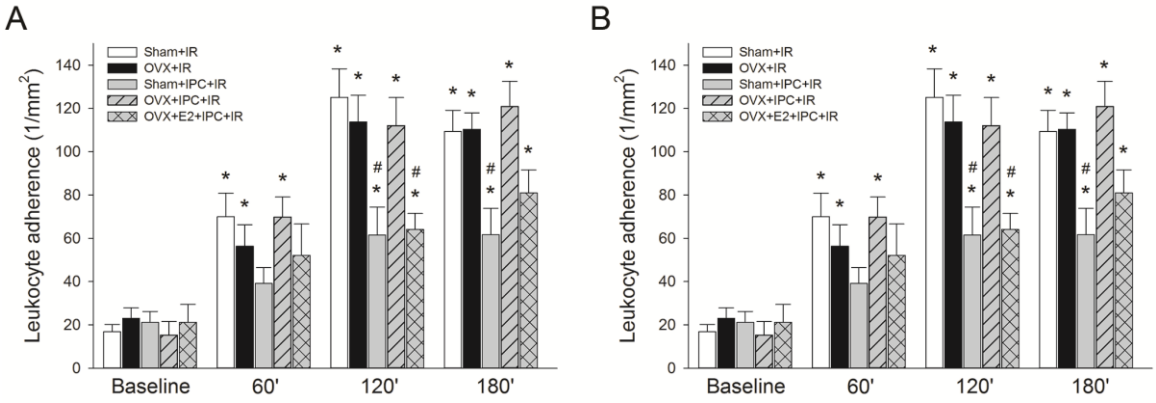

Figure 6. Changes in primary (rolling, panel A) and secondary leukocyte−endothelial cell interactions (adherence, panel B) in the postcapillary venules of the tibial periosteum at baseline, 60, 120 and 180 min after a 60-min limb ischemia in Study 1. Sham: sham operation; OVX: ovariectomy; IR: tourniquet ischemia of a hindlimb followed by reperfusion (60'/180'); IPC: hindlimb ischemic preconditioning (2x10'/10'); E2: 17beta-estradiol treatment. Two-way RM ANOVA was followed by the Holm–Sidak test. Here, data values are given as means±SEM, and *$p < 0.05$ vs baseline.

4.2. Effects of E2 on limb IPC-related systemic inflammatory changes

An increased expression of the adhesion molecule CD11b on the PMN surface was observed after 120 min and 180 min of reperfusion. Subsequently, no major differences could be seen between the values for the sham+IR and OVX+IR groups, but a slight decrease was observed after IPC in the sham-operated animals (sham+IPC+IR) (see Fig.

7). This amelioration, however, was not seen after OVX (in the OVX+IPC+IR group). It seems that chronic E2 treatment effectively prevented the IR-induced increase in CD11b expression (OVX+IPC+IR+E2).

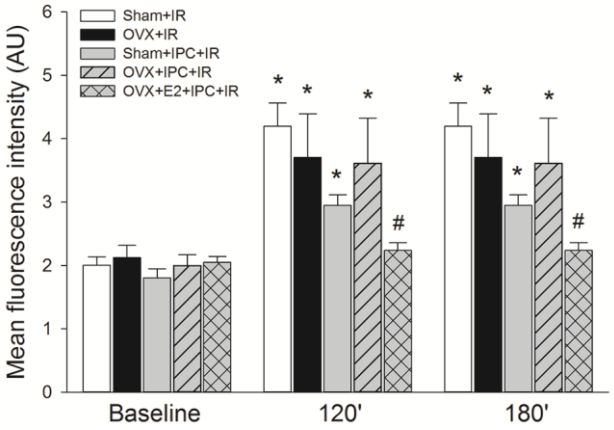

Figure 7. Changes in expression of the CD11b adhesion molecule on the surface of PMNs at baseline and in response to 60 min of limb ischemia followed by 120 min and 180 min of reperfusion in Study 1. Sham: sham operation; OVX: ovariectomy; IR: tourniquet ischemia of a hindlimb followed by reperfusion (60'/180'); IPC: hindlimb ischemic preconditioning (2x10'/10'); E2: 17beta-estradiol treatment. Two-way RM ANOVA was followed by the Holm–Sidak test. Data values are given here as means±SEM, and *$p < 0.05$ vs baseline and #$p < 0.05$ vs sham+IR.

The free radical-derived chemiluminescence of the whole blood (as determined by superoxide radical-dependent chemiluminescence measurements) accounted for the earliest increase (after 60 min of reperfusion) after IPC both in the sham-operated and OVX animals (sham+IPC+IR and OVX+IPC+IR), but it rose only slightly in the E2-treated OVX+IPC+IR animals (OVX+E2+IPC+IR) at this time point (see Fig. 8). Free radical production did not reveal any more differences between the different experimental groups at later time points.

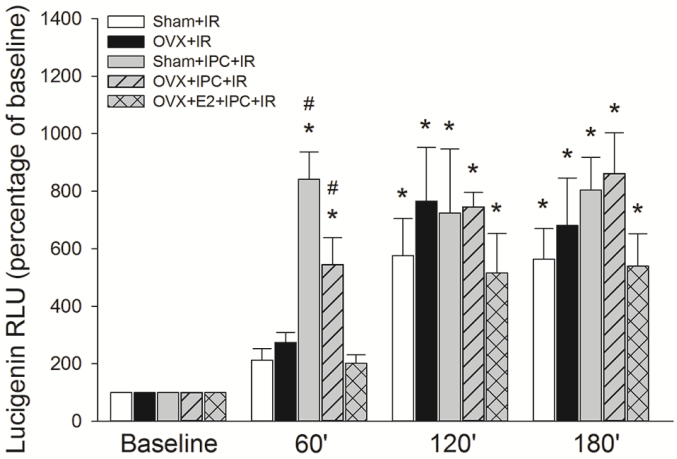

Figure 8. Whole blood superoxide production at baseline and in response to 60 min of limb ischemia followed by 60, 120 and 180 min of reperfusion in Study 1. Sham: sham operation; OVX: ovariectomy; IR: tourniquet ischemia of a hindlimb followed by

reperfusion (60'/180'); IPC: hindlimb ischemic preconditioning (2x10'/10'); E2: 17beta-estradiol treatment. Two-way RM ANOVA was followed by the Holm–Sidak test. Data values are given here as means±SEM, and *$p < 0.05$ vs baseline, #$p < 0.05$ vs sham+IR.

From the experiments, we found that IR brought about a significant increase in TNF-alpha levels in the plasma in all of the groups (see Fig. 9). Due to the high data dispersion, no statistically significant differences were seen between the groups at any time point, but the lowest increase was observed in the E2-treated animals.

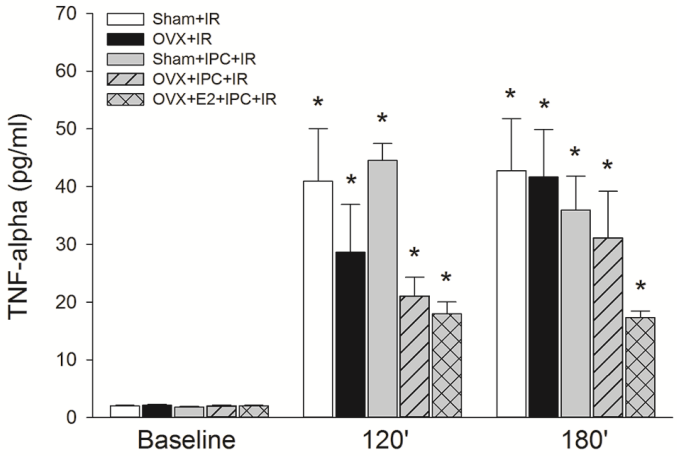

Figure 9. TNF-alpha levels in plasma samples at baseline and in response to 60 min of limb ischemia followed by 60, 120 and 180 min of reperfusion in Study 1. Sham: sham operation; OVX: ovariectomy; IR: tourniquet ischemia of a hindlimb followed by reperfusion (60'/180'); IPC: hindlimb ischemic preconditioning (2x10'/10'); E2: 17beta-estradiol treatment. Two-way RM ANOVA was followed by the Holm–Sidak test. Data values are given here as means±SEM, and *$p < 0.05$ vs baseline.

The protocol was not synchronized with the estrous cycles of the animals, and vaginal smear tests were not performed. The serum E2 concentrations ranged from 9.57 to 15.87 pg ml^{-1} in the sham-operated animals, while these levels were significantly lower in the OVX animals ($p < 0.001$), not even attaining the detection limit of the assay (>5 pg ml^{-1}). However, plasma E2 was restored by chronic E2 supplementation in the OVX animals, and the values were slightly higher than those in the sham group (20.06 median value pg ml^{-1}, $p < 0.05$).

4.3. Effects of estrogen supplementation on OVX-induced ER expression

In the periosteum, a similar level of ER-beta transcription was observed in the sham-operated and OVX animals, and the highest transcription level was noted after chronic E2 supplementation (see Fig. 10). Periosteal ER-alpha mRNA levels, however, remained below the detector threshold. We excluded any methodological issues related to the detection of ER-alpha by simultaneously examining uterus samples taken from the same

animals for an mRNA analysis of both receptors. Like Mohamed and Abdel-Rahman (*Mohamed MK and Abdel-Rahman AA, 2000*), we found higher mRNA levels (for both ER-alpha and beta) in the uterus in the OVX group than in the sham group (data not shown).

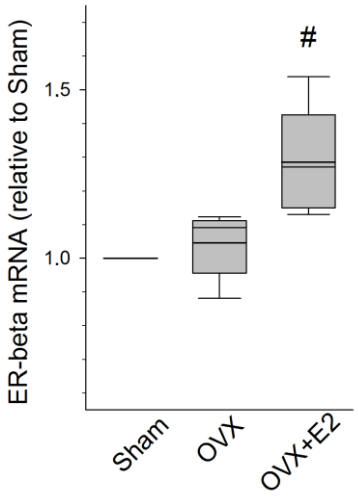

Figure 10. ER-beta mRNA expression levels in the tibial periosteum taken from sham-operated (Sham), ovariectomized (OVX) and OVX animals that were treated with 17beta-estradiol (OVX+E2) (Study 1). The Kruskal–Wallis test was followed by the Dunnett test. Data values are given here as mean, median, 25th and 75th percentiles, and #$p < 0.05$ vs sham.

4.4. Effects of chronic BIS treatment on limb IR-induced periosteal microcirculatory changes

Chronic ZOL treatment did not influence baseline values of leukocyte-endothelial interactions in the periosteal microcirculation (Figs. 11A–B). IR, however, induced significant increases in both PMN rolling and adhesion during the entire reperfusion period, and these changes reached a similar level in sham-operated and ovariectomized rats. BIS treatment caused a temporary increase in leukocyte rolling in OVX+IR animals and, similarly, an earlier rise in PMN adhesion in both sham+IR and OVX+IR animals at 60 min of reperfusion but did not influence PMN–endothelial interactions in later stages of reperfusion.

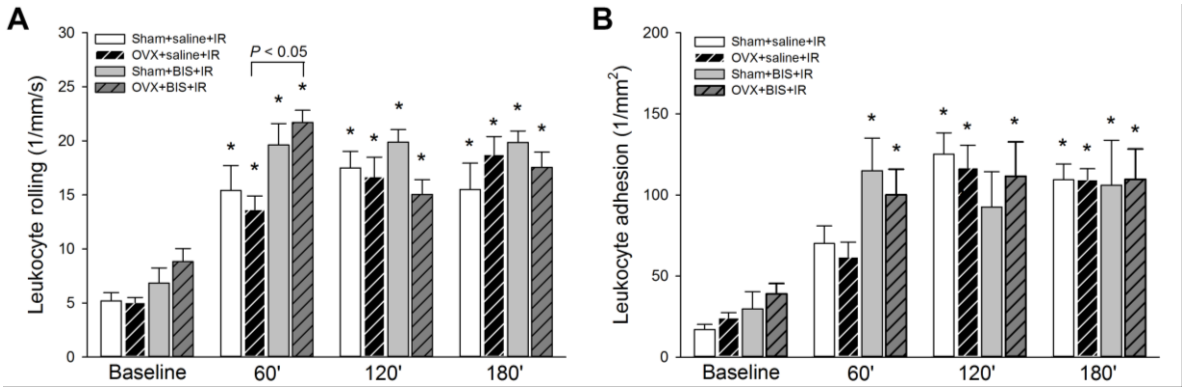

Figure 11. Changes in leukocyte rolling (**A**) and adhesion (**B**) in the tibial periosteal postcapillary venules in response to 60 min of tourniquet ischemia (IR) followed 60, 120 and 180 min of reperfusion in sham-operated (sham) and ovariectomized (OVX) rats treated with bisphosphonate (BIS) or a saline vehicle (Study 2). Data values are given here as means±SEM, and **p* < 0.05 vs baseline. Two-way RM ANOVA was followed by the Holm–Sidak and Dunn's *post hoc* tests.

4.5. Effects of chronic BIS treatment on limb IR-induced systemic inflammatory changes

As compared to baseline, TNF-alpha values showed marked increases during the reperfusion period under examination (Fig. 12). No differences could be traced among the different experimental groups.

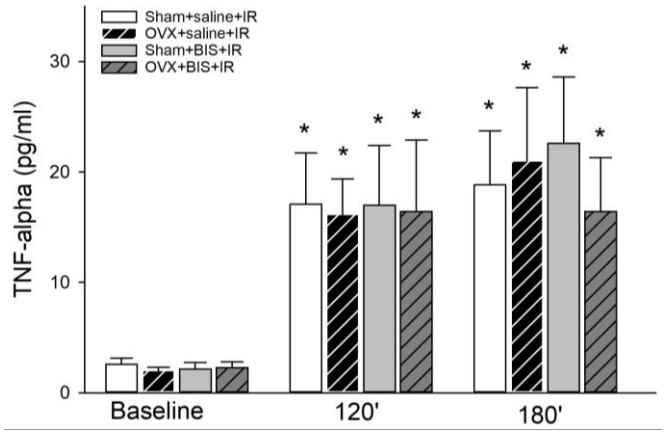

Figure 12. TNF-alpha levels in plasma samples at baseline and in response to 60 min of limb ischemia followed by 120 and 180 min of reperfusion (IR) in sham-operated (sham) and ovariectomized (OVX) rats treated with bisphosphonate (BIS) or a saline vehicle (Study 2). Data values are given here as means±SEM, and **p* < 0.05 vs baseline. Two-way RM ANOVA was followed by the Holm–Sidak *post hoc* test.

Compared to baseline values, the quantity of adhesion molecule CD11b on the PMN surface significantly increased in saline-treated sham-operated and OVX rats during reperfusion (Figs. 13A–B). In animals that received chronic BIS treatment, however, this elevation reached a significantly lower level.

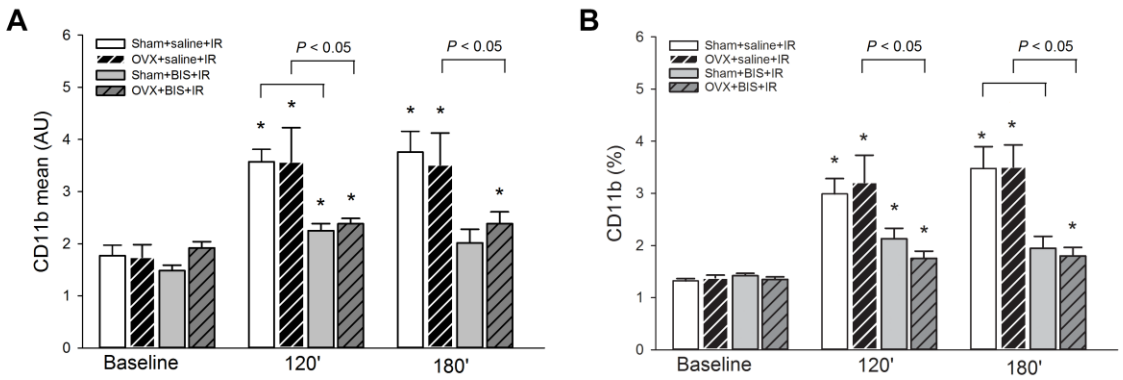

Figure 13. Changes in expression of the CD11b adhesion molecule on the surface of polymorphonuclear leukocytes (PMNs) expressed as mean fluorescence intensity values (**A**) and % of positive cells among the immune-labeled cells (**B**) within the gated PMN population in Study 2. Values are shown at baseline and at 120 and 180 min of reperfusion after 60 min of limb ischemia in sham-operated (sham) and ovariectomized (OVX) rats treated with bisphosphonate (BIS) or a saline vehicle. Data values are given here as means±SEM, and *$p < 0.05$ vs baseline. Two-way RM ANOVA was followed by the Holm–Sidak and Dunn's *post hoc* tests.

5. DISCUSSION

5.1. Effects of estrogen supplementation on the efficacy of IPC in reducing local postischemic periosteal microcirculatory injury

Previously, our group examined the periosteal microcirculatory consequences of tourniquet-induced ischemia in a clinically relevant, long-term follow-up study with osteoporotic rats (*Szabó A et al., 2011*). We showed that OVX did not enhance IR-induced periosteal microcirculation dysfunction, but chronic estrogen supplementation ameliorated local inflammatory complications. In the present protocol, we employed a shorter term of OVX, which does not cause osteopenia, but it is sufficient to evoke a chronic estrogen deficit in rats (*Iwaniec UT et al., 2001*). It appears that IPC mostly influences the second stage of IR-induced periosteal PMN–endothelial interactions (sticking) both here in females and in males (*Szabó A et al., 2009*), which might be explained by the effect of IPC on adhesion molecule expression responsible for leukocyte adhesion to the postischemic endothelium (*Szabó A et al., 2009*). This protection, however, disappeared in the OVX animals in this study, as both PMN rolling and adhesion increased. Hence, it appears that the IPC-induced periosteal protection against postischemic inflammatory complications is lost after estrogen depletion, and this observation has potential clinical implications. In a similar way, CD11b expression, a marker of activation of circulating PMNs (*Jones DH et al., 1988*), was lower in IPC animals only if OVX was not performed. It is therefore reasonable to suppose that endogenous estrogen in females plays a facilitating role in the anti-inflammatory mechanisms provided by IPC in the periosteum. This hypothesis is supported by the observation that E2 supplementation reverses the protection that was lost in OVX+IPC+IR animals. Similarly to our present results, the positive effects of IPC were shown to vanish in postischemic hearts harvested from OVX rats and reversed by E2 (*Shinmura K et al., 2008*). Prior to this, the microcirculatory benefits of E2 supplementation were examined after IR without IPC. The postischemic periosteal microcirculatory complications of tourniquet ischemia could be reversed by E2 supplementation (*Szabó A et al., 2011*), and E2 has also been shown to have beneficial microcirculatory effects in numerous other models of IR (*Booth EA et al., 2003; Burkhardt M et al., 2008*). Since the alleviating effects of E2 are present with or without IPC, it is difficult to differentiate between the beneficial effects of E2 treatment *per se* and its effect on IPC. Hence, one may suppose that the beneficial effects of E2 seen in this model might be independent of its effects on IPC.

In this study, the microcirculatory manifestations of reduced efficacy of IPC were demonstrated for the first time, but similar reactions were observed by others with other manifestations of postischemic tissue injury in other organs (i.e. cardiac dysfunction) (*Shinmura K et al., 2008; Kolodgie FD et al., 1997; Song X et al., 2003; Peng WJ et al., 2004*). The consequences of E2 supplementation in these scenarios, however, are not at all clear. As such, it was possible to restore the OVX-related loss of IPC-induced protection in cardiac functions with E2 in certain studies with rats (*Shinmura K et al., 2008; Kolodgie FD et al., 1997*). The results are somewhat controversial, as the protective effects of IPC were present in OVX rabbits (*Sbarouni E et al., 2006*). Also, E2 exerted no alleviating effects in other studies, where IPC was combined with OVX (*Song Z et al., 2003; Peng WJ et al., 2004*). Furthermore, long- and short-term estrogen administration produced different effects (*Kolodgie FD et al., 1997; Babiker FA et al., 2012*), and inter-species and inter-organ differences and dissimilarities cannot be ruled out either (*Sbarouni E et al., 2006; Kolodgie FD et al., 1997; Song Z et al., 2003; Doucet DR et al., 2010*). The reason for the differences between endogenous and exogenous estrogen effects in different experimental models is not well understood.

Some of these differences might be due to the number and function of estrogen receptors within the affected tissue as well as the effect of OVX and E2 on these receptor expressions. E2 is known to act as a transcription factor, as the binding of E2 to its ER-alpha or ER-beta receptors within the nucleus causes well-known genomic effects by inducing expression changes in different genes (e.g. nitric oxide synthase) (*Nuedling S et al., 1999*). In addition, the action of binding E2 to its (plasma and mitochondrial) membrane-associated receptors also mediates non-genomic events (*Stefano GB et al., 2000; Simoncini T et al., 2000*), including the prevention of injury/stress-induced apoptosis (*Stefano GB et al., 2000*) and cytochrome c release from myocardial mitochondria (*Hsieh YC et al., 2006a*). In our investigations, the ER-beta expression in the periosteum did not vary in response to OVX; instead, it displayed an elevation in response to chronic E2 treatment (whereas the ER-alpha expression remained below the detector threshold). The upregulation of the ER-beta receptor expression by E2 in the mitochondria and inhibition of apoptotic processes seems to be linked to the protective effect of E2 in trauma-hemorrhage *(Hsieh YC et al., 2006b*). Moreover, the cardioprotective effects of E2 were attributable to the ER-beta receptor-related changes in the transcription of metabolic genes in another study (*Gabel SA et al., 2005*). In all likelihood, ER-beta is involved in regulating the estrogen-related increase in nitric oxide synthase activation (*Nuedling S et al., 1999*), and others have demonstrated the impact of

ER-alpha as well (*Haynes PM et al., 2000*). PMN-related inflammatory processes were enhanced in OVX rats after trauma-induced hemorrhagic shock, which was prevented by the acute administration of E2 and an ER-beta agonist (*Doucet DR et al., 2010*). *In vivo* gene delivery of ER-beta to the endothelium greatly reduced the IR-induced formation of reactive oxygen species, increased nitric oxide formation and restored mitochondrial function in the adjacent cardiomyocytes (*Zhan Y et al., 2016*). In our study, some of the inflammatory processes (the CD11b expression of PMNs and free radical content in the blood) were ameliorated with chronic E2, and the possible role of the upregulation of ER-beta in these reactions cannot be ruled out. It should be noted, however, that estrogens also have a direct free radical scavenging effect via their phenolic A-ring (*Prokai L et al., 2013*), a glutathione-increasing effect (*Urata Y et al., 2006*) and a direct modulatory action on NADPH activity (*Dantas AP et al., 2002*). Antioxidant effects of E2 may also be related to its influence on NFκB signaling (*Xing D et al., 2012*) and the upregulation of Nrf2 (*Yu J et al., 2012*). As for the systemic effects, the involvement of ER-alpha-related actions of E2 also plays a role (in heart IR without IPC (*Favre J et al., 2010*), but a discussion of these reactions as well as those evoked by selective estrogen modulators lies outside the scope of the present study. As was suggested by Murphy and Steenbergen, the shorter-term effects of E2 may be caused by ER-alpha, whereas longer-term effects may be mediated mainly through ER-beta (*Murphy E and Steenbergen C, 2007*). Moreover, ER-independent effects of E2 in this study should not be ruled out either. It should be noted that the periosteal expression of ERs has yet to be examined in humans, but in the cortical and trabecular bone tissue, both ER proteins can be detected (via immunohistochemistry) with a different density during bone development (*Bord S et al., 2001*). It appears that only the ER-beta mRNA expression was examined in the tibial periosteum in the rat (*Petersen DN et al., 1998*), and here we were unable to detect any ER-alpha mRNA expression in the periosteum. This might mean that ER-alpha mRNA expression cannot be detected in the periosteum. However, the translation of our present findings (the absence of periosteal ER-alpha mRNA expression) to the human situation requires further in-depth investigation.

5.2. Effect of estrogen supplementation on the efficacy of IPC in reducing systemic inflammatory reactions

Systemic inflammatory parameters also displayed characteristic changes in Study 1. That is, the IR-induced increase in CD11b expression of circulating PMNs (a marker of their activation) was reduced by IPC only in sham-operated animals, but not in those with OVX. This reaction was also reversed by E2. The PMN-derived CD11b expression was

likewise reduced by E2 *in vitro* (*Nadkarni S et al., 2011*) and in trauma-hemorrhagic shock (*Deitch EA et al., 2006*) as well as in levels of other adhesion molecules, such as E-selectin (*Prestwood KM et al., 2004*). We are unaware of any studies that have investigated the effect of IPC in OVX animals from the viewpoint of adhesion molecule expressions. In the present study, whole blood free radical content was significantly increased in all groups. In the sham+IR and OVX+IR groups, local (periosteal) and systemic inflammatory reactions had a slightly different timeframe, since IVM data revealed increased PMN rolling and adhesion after 60 min of reperfusion (indicating an early activation of the affected endothelium and a simultaneous availability of primed leukocytes), but the superoxide levels displayed later changes (occurring after 120 min). The background of this phenomenon is not yet understood, but since increased CD11b expression in peripheral leukocytes also occurred at later stages of reperfusion (after 120 min), the contribution of other elements (e.g. activated macrophages) to the increased superoxide production may be assumed. Interestingly, IPC failed to induce any amelioration in whole blood free radical production; furthermore, it induced an earlier increase in this parameter in both sham-operated and OVX groups. It should also be noted that this increase was not present in the E2-treated group. Actually, free radicals are known to play a role in the pathomechanism of IPC because their accumulation could be detected *in vivo* and superoxide scavengers reversed the tissue protective effects of IPC (*Kevin LG et al., 2003; Baines CP et al., 1997*). ER-beta has been shown elsewhere to be involved in reducing neutrophil activation (*Doucet DR et al., 2010*) and the free radical-reducing effect of E2 was also highlighted (*Sovershaev MA et al., 2006*). Interestingly, levels of one of the central regulators of inflammation TNF-alpha were not influenced by IPC. Quite surprisingly, the phenomenon observed in humans (*Pfeilschifter J et al., 2002*) indicating increased serum TNF-alpha levels after OVX could not be confirmed in the present study (i.e. the baseline TNF-alpha values were not dissimilar after OVX), and even slightly lower values were found in all of the OVX animals (after 120 min of reperfusion). These differences might be the result of interspecies differences or changes in the immunological responses seen after OVX (which are outside the scope of the present study). TNF-alpha release has been shown to be reduced by E2 in numerous studies (with or without OVX) (*Babiker FA et al., 2012; Ma, 2001*) even in male patients (*Wei M et al., 2001*). In this respect, the changes induced by reperfusion or IPC+IR have yet to be compared in OVX studies elsewhere. Here, the lowest postischemic values were found after applying E2 (although not attaining any statistical significance due to the relatively high data dispersion). Together with reduced CD11b expression and the slower

postischemic increase in superoxide production, this parameter represents manifestations of the alleviated systemic inflammatory reactions after E2 supplementation.

Conclusions for Study 1

In our study, we found that the beneficial periosteal microcirculatory effects of local limb IPC vanished after OVX in rats. These observations suggest that during orthopedic trauma interventions in postmenopausal females, the efficacy of limb IPC in preventing the inflammatory complications of tourniquet ischemia might be limited. This conclusion is strengthened by our findings, which show that E2 supplementation reversed these changes by alleviating the local and systemic inflammatory reactions. Based on our previous and present findings in rats, some of the alleviating effects of E2 seen here might be independent of its effects on IPC and may be linked to those seen with periosteal ER-beta expression. The clinical significance of this finding, however, remains to be elucidated.

5.3. Effects of chronic BIS treatment on limb IR-induced periosteal inflammatory reactions

BISs are effective medications for bone metastases and osteoporosis and promising treatment modalities for complex regional pain syndrome upon fracture healing (*Adler RA et al., 2016; Wang L et al., 2016; Littlejohn G, 2013*). The use of ZOL has been shown to have a positive effect on spinal fusion (*Yasen M et al., 2015*) and to promote osseointegration and fixation of dental implants in autologous bone grafts in osteoporosis (*Qi M et al., 2012*). The periapical lesion-induced bone loss in the mandible was effectively ameliorated (*Wayama MT et al., 2015*), and osseointegration of titanium implants in postmenopausal osteoporosis was promoted by ZOL (*Ying G et al., 2016*). Furthermore, ZOL brought about periosteal bone formation after tooth extraction in osteopenic sheep (*Voss P et al., 2016*). ZOL treatment, however, also induced reactive periosteal hypertrophy and even BIS-related osteonecrosis of the jaw in the same osteopenic sheep model (*Voss PJ et al., 2016*). Nevertheless, the effect of BIS on IR-induced local and systemic inflammatory reactions has not been examined elsewhere in an osteopenic model.

It is noteworthy that both anti- and proinflammatory effects have been attributed to different BIS compounds. The anti-inflammatory aspects of BISs include upregulation of the number of inflammatory monocytes (*Ritz BW et al., 2011*), modulation of the proliferation and the viability and apoptosis of monocytes and macrophages (*Cecchini MG et al., 1990; Rogers MJ et al., 1996*) and downregulation of proinflammatory cytokines, such as TNF-alpha (*Pennanen N et al., 1995; Maksymowych WP, 2002*), as

well as other cytokines, such as IL-1, IL-6 and neurogenic growth factor (*Wang L et al., 2016*). Similarly, inhibitory effects of BIS against neurogenic inflammation have also been reported *(Wang* L *et al.,* 2016). On the other hand, an acute phase response (<3 days) was induced by different BISs including ZOL with increased TNF-alpha release in patients (*Pazianas M et al., 2013*), but tissue accumulation of PMNs, increased TNF-alpha release and marked oxidative stress were also demonstrated in other tissues, such as the gingiva (*de Barros Silva PG et al., 2017*) and the liver (*Karabulut AB et al., 2010*), in animal models. Furthermore, priming of immunological reactions was also attributed to ZOL (*Norton JT et al., 2011*). BISs cause ocular inflammatory complications in some clinical cases (*Pazianas M et al., 2013*) and healing complications of the jawbones after invasive dental interventions, even leading to osteonecrosis (*Ruggiero SL et al., 2014*). ZOL has been shown to aggravate kidney damage (by increasing cytokine production, metabolic acidosis and apoptosis) during IR injury in rats (*Sehitoglu I et al., 2015*).

Enhanced leukocyte-endothelial interactions have been demonstrated after BIS treatment in an arthritis model in mice, but little is known about ZOL-induced periosteal microcirculatory reactions (*Zysk SP et al., 2003*). Previously, we demonstrated that chronic BIS treatment induces some level of microcirculatory inflammation in the mandible, but such effects were not observed in the tibial periosteum (*Janovszky Á et al., 2015*). Therefore, in this study, we tested the effect of chronic ZOL treatment in a tourniquet-induced limb ischemia model, where the role of PMN–endothelial interactions in the development of postischemic microcirculatory inflammatory reactions is well established. We have shown here that the reduced endogenous estrogen levels evoked by OVX do not predispose to enhanced periosteal microcirculatory complications *per se* (*Szabó A et al., 2011*), with the results also demonstrating that, apart from temporary exacerbation of PMN–endothelial interactions at the early stages of reperfusion, no major microcirculatory inflammatory risk could be detected after chronic ZOL treatment.

5.4. Effect of chronic BIS treatment on limb IR-induced systemic inflammatory reactions

Estrogen withdrawal induces a release of TNF-alpha, which is involved in the pathomechanism of osteoporotic bone loss in women (*Cenci S et al., 2000*), but, in this study, we did not demonstrate between-group differences in TNF-alpha levels in the postischemic phase. Nevertheless, unlike humans, where increased serum TNF-alpha levels have been observed after OVX (*Pfeilschifter J et al., 2002*), we detected no differences in baseline TNF-alpha levels between the different experimental groups. It

should be noted that serum levels of TNF-alpha are rather low in rats and baseline values were close to the detection limit of the assay.

CD11b expression is a critical step for PMN adhesion to activated endothelial cells, and we detected a reduced IR-induced systemic PMN-derived CD11b expression after ZOL administration. BISs have been shown to influence PMN functions, which manifested in impaired PMN chemotaxis and reactive oxygen species production capacity *in vivo* (*Favot CL et al., 2013*) and reduced myeloperoxidase and NADPH oxidase activities *in vitro* (*Salvolini E et al., 2009; Kuiper JW et al., 2012*). The inhibitory effect of BIS was also demonstrated in other immune cells, such as macrophages (*Pennanen N et al., 1995*). In our study, ZOL reduced CD11b expression on the surface of circulating PMNs but did not influence the overall adhesion of PMNs in the periosteal postcapillary venules. This finding can only be explained by some degree of ZOL-induced endothelial activation and secondary endothelium-derived adhesion molecule expression. This possible ZOL-induced endothelial upregulation of adhesion molecules (the endothelial counterparts of CD11b), which might be responsible for the present results, should be investigated further.

Among other effects, BISs are known to inhibit vascular endothelial proliferation and to upregulate cellular apoptosis (*Lang M et al., 2016*). Furthermore, BISs (alendronate) have also been shown to inhibit nitric oxide synthase expression, which is an important endogenous modulator of PMN–endothelial interactions (*Silva RO et al., 2014*). These ZOL-induced acute postischemic reactions affecting the endothelium may also warrant further in-depth investigations.

Conclusions for Study 2

In summary, BIS treatment exerted only a minor influence on limb IR-induced PMN rolling and adhesion in the periosteum, and the PMN-derived adhesion molecule (CD11b) expression on circulating PMNs was even reduced. Further, no effect on postischemic TNF-alpha release was demonstrated in ZOL-treated rats. These results suggest that, although some level of local endothelial activation might be attributable to the treatment, chronic ZOL administration has no major influence on the risk of postischemic inflammatory microcirculatory complications in the tibial periosteum.

6. SUMMARY OF NEW FINDINGS

1. The beneficial periosteal microcirculatory effects of local limb IPC vanished after OVX in rats. Therefore, the efficacy of limb IPC in preventing the inflammatory complications of tourniquet ischemia might also be limited when orthopedic trauma interventions are performed on postmenopausal females.
2. Chronic extrogen supplementation reversed these local and systemic inflammatory reactions, but some of the alleviating effects of E2 might be independent of its effects on IPC and may be linked to those seen with periosteal ER-beta expression.
3. BIS treatment causes no systemic postischemic complications in leukocyte activation, but moderately enhances limb IR-induced periosteal microcirculatory reactions. This phenomemon might be explained by some level of local endothelial activation attributable to chronic ZOL treatment.
4. When administered in osteoporosis, chronic ZOL treatment causes no substantial acute postoperative complications in the periosteal microcirculation that develops during tourniquet ischemia as part of trauma orthopedic surgery.

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9. ANNEX

Paper I.

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The periosteal microcirculation in health and disease: An update on clinical significance

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ABSTRACT

Apart from its nutritive functions, the periosteum critically affects bone regeneration via its stem/osteoprogenitor cell content. Normal healing after bone fractures, trauma–orthopedic interventions and invasive dental procedures is critically linked to the reestablishment of the periosteal microcirculation, but the reconstruction, replacement or repair of lost tissues may also be performed with autologous periosteum. Besides the initiation of cell differentiation during bone repair and remodeling processes, the periosteum together with the endosteum plays significant roles in the pathogenesis of both hormone-related and trauma-induced osteoporotic alterations in the bone metabolism. Nevertheless, the axial bones, and in particular the jawbones, and the appendicular bones display differences not only in their blood supply and fracture healing characteristics, but also in respect of the development of osteoporosis and their reactions to treatment modalities (i.e. bisphosphonates). These reactions may also be linked to the differences in periosteal microcirculatory reactions. The present overview summarizes the relevant data of microcirculatory studies focusing on the periosteal reactions in different anatomical locations together with the optimal background methodologies, study models and the most significant observations.

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1. Introduction

The periosteal membranes separate the bones from the surrounding tissues and also bind to them the elements of the skeletal system, the tendons, septa and ligaments. Although it is well recognized that the periosteum is more than simply an envelope of the bone, it is a relatively infrequent site for microcirculatory studies. Despite several decades of research, the regenerative potential of the periosteum and the distinct role of the microcirculation in a range of important physiological and pathological events are only incompletely characterized, mainly due to methodological limitations. Functional changes within the periosteal microvasculature in different experimental settings can dynamically be assessed using intravital microscopy (IVM). In the present overview, the periosteal microcirculatory reactions are summarized based on IVM findings in clinically-relevant animal models of reconstructive surgery, orthopedic-trauma interventions and systemic diseases. Differences in

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the physiology and microcirculatory reactions between the axial (i.e. the skull, facial bones, vertebrae, ribs, sternum and the shoulder and pelvic girdles) and appendicular bones are also discussed.

2. Brief anatomy and physiology of the periosteum

The periosteum is composed of an outer fibrous and an inner osteogenic cellular layer (for reviews, see Augustin et al. 2007; Dwek, 2010; Lin et al., 2014). From a structural aspect, the superficial portion of the outer layer is the most vascularized part, supplying the deeper periosteal layers and the superficial layer of cortical bone. The endosteum has a similar histological structure to that of the periosteum with a rich blood supply and (albeit to a lesser extent) mesenchymal stem cells (Brighton et al., 1992). Providing a highly vascular connective tissue coverage, the endosteum also plays an active role in the regulation of the metabolism and regeneration of the bone.

The application of IVM methods has led to detailed descriptions of the morphological and functional characteristics of the periosteal microvasculature in different species (representative micrographs taken in rats are shown in Figs. 1, 2, see details later). The microvascular architecture has been most extensively examined in rats, in which certain differences in the organization of the microvessels can be observed. The microvascular network within the rat mandibular periosteum comprises mainly arterioles and venules (Varga et al., 2014). In rabbits, the

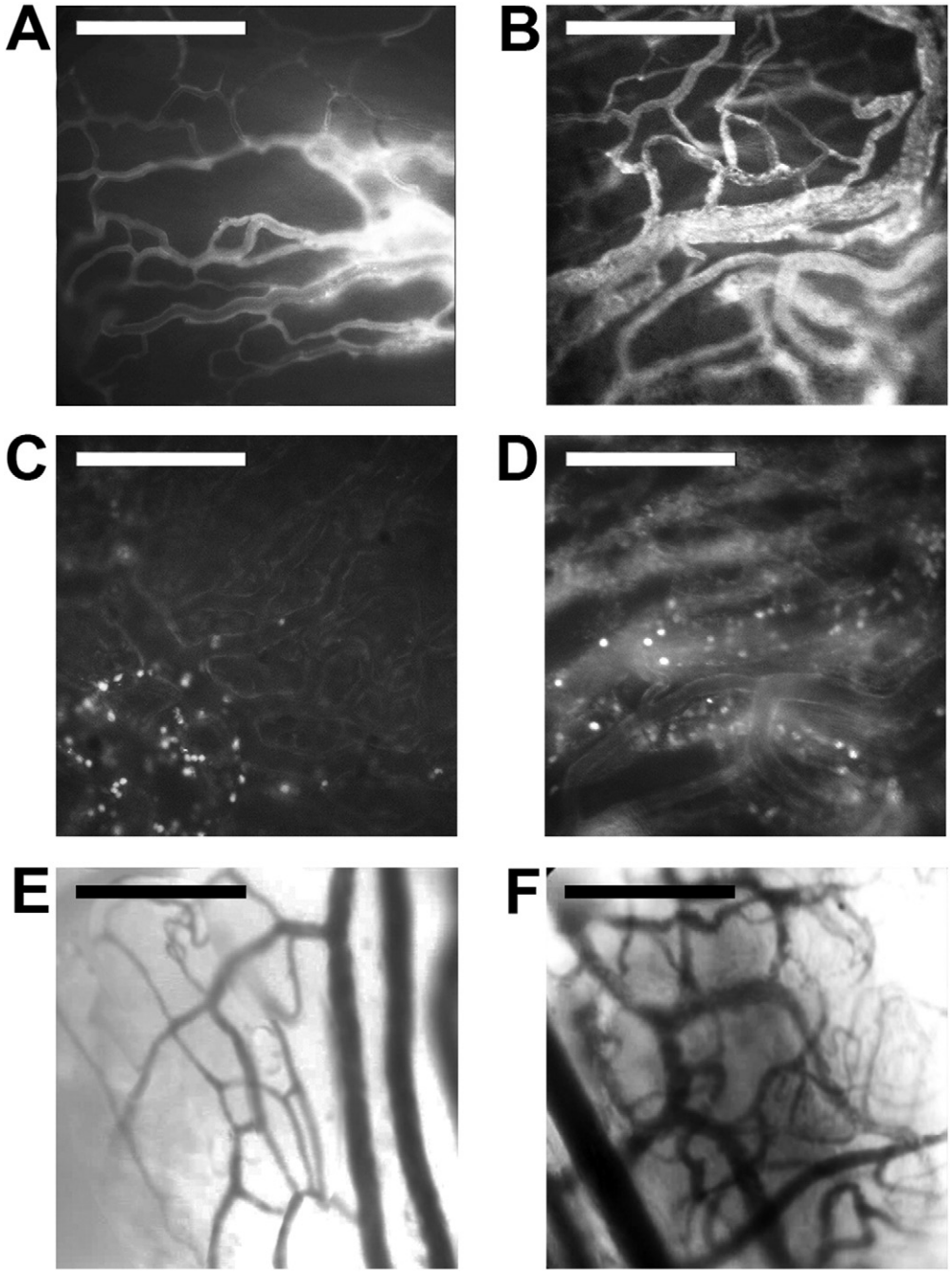

Fig. 1. Fluorescence intravital microscopic (IVM) and orthogonal polarization spectral (OPS) images of the anteromedial tibial (A, C, E) and mandibular periosteum (B, D, F) in Sprague-Dawley rats. Upper two panels: IVM images; plasma labeling with fluorescein isothiocyanate-dextran (150 KDa, Sigma, St. Louis, MO, USA) (A, B) and rhodamine 6G (Sigma, St. Louis, MO, USA)-labeled neutrophil leukocytes (C, D), respectively. The images were taken by using a Zeiss Axiotech Vario 100HD IVM microscope, 100W HBO mercury lamp, Acroplan 20× water immersion objective, Carl Zeiss GmbH, Jena, Germany) and a CCD camera (Teli CS8320Bi, Toshiba Teli Corporation, Osaka, Japan). On the lower panels, OPS images (taken by the Cytoscan A/R device; Cytometrics, Philadelphia, PA, USA) of the tibia (E) and the mandible (F) are shown. The bar denotes 200 μm (original recordings of the authors).

maxillary periosteum consists mostly of parallel capillaries (Rücker et al., 2005). Only a few capillaries and mostly venules are present in the tibial periosteum of the rat (Varga et al., 2008). The microvessel density in the mandibular periosteum is similar to that in the anteromedial tibia (180–220 mm/mm^2), whereas it is about 30% lower in the anterolateral tibia (Rücker et al., 2006; Varga et al., 2014). The calvarial periosteum has a somewhat lower microvessel density (120–130 mm/mm^2) than that of any of the above tissues (Rana et al., 2011).

Due to this rich nutritive vascular plexus, the periosteum is accurately described as the "umbilical cord of the bone" (Chanavaz, 1995). The inner layer, also called the cambium layer, contains many osteogenic progenitors, similar to mesenchymal stem cells (Soleymaninejadian et al., 2012; Tenenbaum and Heersche, 1985; Zohar et al., 1997) and osteoblasts situated directly on the outer surface of the bone cortex covered by fibroblasts. Besides the osteogenic stem cell properties, the cells of these membrane sheets have been shown to retain the ability to differentiate into fibroblasts, osteoblasts, chondrocytes, adipocytes and skeletal myocytes (Emans et al., 2005). Consequently, the reconstruction, replacement or repair of lost tissues may be performed appropriately with autogenous periosteum in experimental settings (Finley et al., 1978; Reynders et al., 1999). These differentiation processes are under hormonal control (e.g. estrogen, parathyroid hormone, calcitonin) being also influenced by alimentary factors (e.g. calcium and vitamin D3 uptake) and age (Geusens and Lems, 2011). Both regenerative properties of the periosteal progenitor cells and anabolic actions of parathyroid hormone are markedly reduced by age (Yukata et al., 2014).

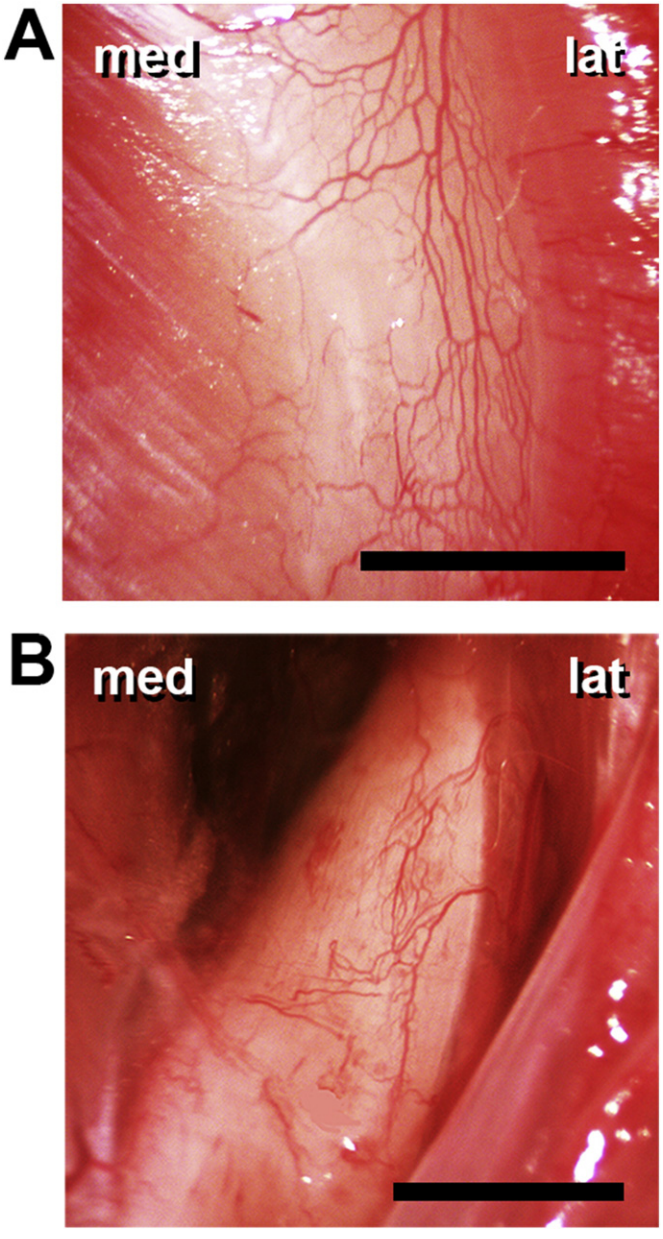

Fig. 2. Operating microscopic view of the left anteromedial tibial periosteum (A) and the right mandibular periosteum (B) in Sprague-Dawley rats. Medial and lateral sides are indicated (med, lat). The bar denotes 1000 μm (original recordings of the authors).

It is well known that successful healing after fractures requires the regeneration of the peri- and endosteal microcirculations (Macnab and Dehoas, 1974). Likewise, periosteal damage leads to perturbed bone healing with consequent delayed union or pseudoarthrosis formation (Esterhai and Gelb, 1991; Gustilo et al., 1990; Utvag et al., 1998). The importance of the periosteal microcirculation was hallmarked by early studies on dogs showing that autologous tibial grafts having only their periosteal blood supply intact survived transplantation (Berggren et al., 1982). In another study, free gingival autografts placed on the periosteum survived by receiving nutrients from the maxillary bone with an intact periosteum (Yanagihara, 1990). Further results demonstrated that the revascularization during wound healing is critically linked to the release of vascular endothelial growth factor by periosteal cells (Bourke et al., 2003). In the oral cavity, the tissues produced by cells with mesenchymal origin include cementum with periodontal ligament fibers and bone; under appropriate culture conditions, periosteal cells secrete an extracellular matrix and form a membranous structure in the periodontium (Mizuno et al., 2006). Apart from the initiation of cell differentiation during the bone repair and remodeling process after traumas and invasive dental procedures (Tran Van et al., 1982), the periosteal and endosteal membranes also play significant roles in the pathogenesis of metabolic bone alterations (Allen et al., 2004). In summary, it can be concluded that adequate microcirculation is a prerequisite of the bone metabolism and the regenerative potential of the periosteal and endosteal compartments.

3. Axial versus appendicular bones - regional differences in periosteal functions?

Although a continuous microcirculatory supply is necessary in order to ensure physiological remodeling, metabolism and regeneration, there are many differences within the skeletal system between axial (i.e. the skull, facial bones, vertebrae, ribs and sternum) and appendicular bones. While the appendicular long bones receive their vascular supply from the nutritive epiphyseal and metaphyseal vessels (Findlay, 2007; Johnson et al., 2004), the circulation of the maxillofacial bones, and especially the lower jaw, is provided by the mucoperiosteal tissue through the inferior alveolar and sublingual arteries (Huelke and Castelli, 1965; Shannon et al., 2011). It should be emphasized that the jaw region is endowed with additional, particular regeneration characteristics (Elshahat et al., 2004; Støre and Granström, 1999). As opposed to long bone fractures, which heal mainly through endochondral ossification, intramembranous ossification has a much higher impact in the mandible (Yu et al., 2012). In line with this, mandible periosteum-derived stem cells have been shown to possess the highest osteogenic potential among the different anatomical locations (Solheim et al., 1995; Ueno et al., 2002), while the tibial periosteum or the bone marrow stem cells are superior in terms of chondrogenesis (Park et al., 2012). Further, there are distinct differences in the expression pattern of bone development-related genes between the mandibular and tibial osteoblasts (Reichert et al., 2013). A point of interest is that systemic disorders such as osteoporosis affect the mandible to a significantly lesser extent (Liu et al., 2014; Mavropoulos et al., 2007; Yamashiro and Takano-Yamamoto, 1998). It has been proposed that the intense mechanical loading of the alveolar process during mastication may protect the alveolar bone from the osteoporosis-related bone loss observed at other skeletal sites (Mavropoulos et al., 2007).

Bisphosphonates (BISs) are widely used for the treatment of osteoporosis and tumors with bone metastasis to inhibit osteoclast activity and bone resorption (Rogers et al., 2011) also exerting distinctive effects on the bones in the maxillofacial region. Here the regional BIS uptake reaches a much higher concentration in comparison with the appendicular and other axial bones (Wen et al., 2011). The receptor activator of nuclear factor κB (RANK)/receptor activator of nuclear factor κB ligand (RANKL)/osteoprotegerin axis, a signaling pathway that regulates osteoclast differentiation, is also diversely affected by BISs, which cause a decrease in RANKL values in the mandible and the opposite effect in the tibia (Çankaya et al., 2013). Furthermore, BIS treatment exerts site-specific, differential effects during the early healing processes of tibial and mandibular fractures by delaying callus, cartilage and bone remodeling specifically in the mandible (Yu et al., 2012). Defective angiogenesis of the mandibular mucoperiosteal tissues is also evoked by long-term treatment with BIS (Wehrhan et al., 2011).

4. Examination of the periosteal microcirculation in axial and appendicular bones

Besides histology, various imaging methods, such as corrosion casting, microangiography, computer tomography and to some extent scintigraphy (Berggren et al., 1982; Bhatt et al., 2000; Fayad et al., 2005; Nobuto et al., 1989; Pazzaglia, 1996; Rhinelander et al., 1968; Trueta and Cavadias, 1955), can be used to visualize the architecture of the periosteal microcirculation in the jaw and long bones. Laser-Doppler flowmetry, laser speckle imaging (ElMaraghy et al., 1999; Swiontkowsky et al., 1986) and the radioactive microsphere technique (Barron et al., 1977) can characterize functional details of the periosteal perfusion, but only with limitations. If more accurate detection or improved spatial resolution of the microcirculation is needed, traditional fluorescence IVM can provide an opportunity for real-time examination of the microcirculation of superficial layers. Conventional fluorescence IVM visualizes not only changes in the efficacy of microvascular perfusion, but also cell-cell (e.g. polymorphonuclear leukocyte (PMN)–endothelial cell) interactions, such as rolling and adhesion, vascular diameter changes or signs of apoptosis (Abshagen et al., 2006; Horie et al., 1996) (see Fig. 1 A–D). Non-fluorescence techniques, including orthogonal polarization spectral imaging (OPS) (Groner et al., 1999) (Fig. 1 E–F) and sidestream dark-field imaging, have also been developed for the imaging of individual vessels and cells (Milstein et al., 2010). Nevertheless, observation of the microcirculation of the periosteal compartment would still necessitate surgical exposure (Fig. 2).

As concerns the appendicular bones, the anterior tibial periosteum appears to be one of the most easily accessible sites for microcirculatory examinations with IVM (see Table 1). Methods have been established for the examination of osteomyocutaneous flaps where microvascular perfusion characteristics and PMN-endothelial interactions can be dynamically assessed not only in the lateral tibial periosteum, but also in the adjacent muscle, subcutis and skin (Rücker et al., 1998). In the cases of the axial bones, the calvarian periosteum in rats can be visualized under experimental circumstances by means of different methods, either in acute settings (Stoetzer et al., 2014) or with chronic models, applying the periosteal window and chamber methods (Rana et al., 2011; Stuehmer et al., 2009; von See et al., 2010). The midfacial periosteum in rabbits (Rücker et al., 2005) and the mandibular periosteum in rats are also accessible for IVM, in the latter case also for OPS and confocal laser scanning microscopic assessments (Varga et al., 2014).

4.1. Changes in nutritive and angiogenetic functions in clinically relevant trauma-orthopedic models

The direct consequences of traumas or surgical interventions on the periosteal microcirculation have been examined in several studies (Table 1). Soft tissue damage was induced by periosteal elevation using conventional and piezoelectric devices and the short- and longer-term effects on the periosteal perfusion were compared (Stoetzer et al., 2014). The extents of immediate and long-term periosteal perfusion failure were demonstrated and quantified after closed soft tissue traumas and closed tibial fractures showing an interaction between the skeletal muscle damage and periosteal microvascular injury (Schaser et al., 2003; Zhang et al., 2003). In another study where functional capillary density changes were evaluated in the midfacial periosteum in rabbits, the risk of complications was higher after subperiosteal dissection in comparison to the supraperiosteal approach (Rücker et al., 2005). Periosteal and subperiosteal angioneogenesis could be induced by the self-inflating hydrogel expanders used for preliminary soft tissue augmentation before bone augmentation surgery (von See et al., 2010).

The message of these studies is that regeneration of the periosteal microvasculature is a prerequisite of its tissue-regenerative potential. Another point for consideration is the communication of the periosteal and endosteal vascular networks. Alterations in the anteromedial and anterolateral periosteal microcirculations (Fig. 3) were recently studied with an OPS technique following standardized surgical destruction of the endosteum (Greksa et al., 2012). The periosteal microvascular reorganization caused by reaming of the endomedullary cavity was not altered by implantation with titanium, a material with good osseointegrative properties, whereas the periosteal microcirculation was augmented by unstable endomedullary polyethylene nails. Though unstable implant materials can lead to inadequate restoration of the endosteal circulation, which remains insufficient to supply the cortical bone, this may be compensated by enhanced periosteal sources (Greksa et al., 2012).

The consequences of ischemia-reperfusion (IR) and perfusion deficiencies have been extensively examined in the periosteum of bones and composite flaps. Vasomotion has been described as a compensatory reaction of the microvasculature aiding tissue survival. Critical perfusion conditions induce capillary flow motion in muscle, but not in the periosteum (Rücker et al., 2000), and this potentially favorable reaction is preserved by heat shock priming (Rücker et al., 2005). Heat shock has been shown to stimulate periosteal angiogenesis in the intact calvaria and in the tibia in a chronic ischemia model of microthrombosis (Rana et al., 2011; Rücker et al., 2006). The positive effects of heat shock priming are manifested by improved periosteal microvascular recanalization, recovery of perfusion rate and blood flow in the capillaries, similarly to those observed in other components of the osteomycotanous flap (Rücker et al., 2006). In an acute composite flap ischemia model, heat shock reduced PMN adhesion in the postcapillary venules and intracellular adhesion molecule-1 (ICAM-1) expression in all examined tissues (Rücker et al., 2001).

Tourniquet-induced limb ischemia, a generally applied maneuver in orthopedic-trauma clinical practice, brings about marked local and systemic inflammatory effects. Since a tourniquet around the extremity causes IR of an appreciably high amount of tissue, the venous effluent or afferent neurogenic signals lead to further systemic inflammatory reactions (i.e. activation of circulatory PMNs). The affected tissues undergo the typical biochemical and microcirculatory changes of a local IR injury, the periosteal microcirculation primarily being affected in this process. The postischemic microcirculatory inflammatory reactions include perfusion failure and activation of the PMN–endothelial interactions and the upregulation of adhesion molecule expression (e.g. ICAM-1) (Szabó et al., 2009) (Fig. 4). Apart from activated PMNs (Vega et al., 1999), humoral factors (Goldman et al., 1990), mediators of oxidative stress (Koike et al., 1992, 1993), proinflammatory cytokines (e.g. TNF-α, PAF, IL-1β and IL-6) (Lawlor et al., 1999) and complement C5a (Fleming et al., 2003) have been implicated in remote organ dysfunctions and injuries (e.g. in the liver or lungs). These local processes also have relevant implications in remote organ preconditioning reactions. Limb ischemia has been proposed as an easily and relatively safely accessible preconditioning situation (Kanoria et al., 2006). Indeed, both periosteal and distant, systemic inflammatory reactions can be

Table 1
Dynamic measurements of periosteal microcirculatory changes using intravital videomicroscopy (IVM), orthogonal polarization spectral imaging (OPS) at different locations. The quantification methods include assessments of tissue perfusion, functional capillary density (FCD), red blood cell velocity (RBCV) and polymorphonuclear leukocyte (PMN)–endothelial interactions.

Target tissue	Reference	Animal	Method	Parameter	Disease model	Findings
Anterior tibial periosteum	Zhang et al. (2003)	Rat	IVM	Perfusion + PMN	Closed tibial fracture	Immediate microcirculatory derangements
Anterior tibial periosteum	Schaser et al. (2003)	Rat	IVM	Perfusion + PMN	Closed soft tissue trauma	Long-lasting disturbances
Midfacial maxillar periosteum	Rücker et al. (2005)	Rabbit	IVM	FCD	Sub/supraperiosteal dissection	Better periosteal perfusion after the subperiosteal approach
Calvarial periosteum	Stoetzer et al. (2014)	Rat	IVM	FCD	Periosteal elevator	New piezoelectric device reduces injury
Calvarial periosteum (chamber)	Rana et al. (2011)	Rat	IVM	FCD (angiogenesis)	Stress conditioning of intact periosteum by heat shock	Improvement by heat shock
Calvaria periosteum (window)	von See et al. (2010)	Rat	IVM	Vessel density (angiogenesis)	Self-inflating hydrogel expanders	Replacement of the periosteum by connective tissue (subcutaneous angiogenesis)
Muscle, skin, subcutis, lateral tibial periosteum	Rücker et al. (2001)	Rat	IVM	Perfusion + PMN	Local heat-shock + osteomyocutaneous flaps	Amelioration of inflammatory reaction by local heat-shock
Muscle, skin, subcutis, lateral tibial periosteum	Rücker et al. (2005)	Rat	IVM	RBCV, flowmotion, FCD	Critical perfusion of osteomyocutaneous flap	Loss of muscle capillary flowmotion prevented by heat shock-priming.
Muscle, skin, subcutis, lateral tibial periosteum	Rücker et al. (2006)	Rat	IVM	Thrombus formation (microvascular recanalization)	Thromboembolization of osteomyocutaneous flap	Heat shock promotes microvasculature recanalization
Anterior tibial periosteum	Wolfárd et al. (2002)	Rat	IVM	FCD, RBCV, PMN	Limb IR	Amelioration of inflammatory reaction by endothelin-A receptor antagonism
Anterior tibial periosteum Amelioration of inflammatory reaction by phosphatidyl choline	Gera et al. (2007)	Rat	IVM		Perfusion + RBCV + PMN	Limb IR
Anterior tibial periosteum	Varga et al. (2008)	Rat	IVM	Perfusion + PMN	Limb IR (infusion fluids)	Amelioration of inflammatory reaction by hydroxyethyl starch
Anterior tibial periosteum	Szabó et al. (2009)	Rat	IVM	Perfusion + PMN	Limb IR	Local and distal amelioration of inflammation by IPC
Anterior tibial periosteum	Hartmann et al. (2011)	Rat	IVM	Perfusion, RBCV, PMN	Limb IR	Involvement of chemo- (capsaicin-) sensitive afferent nerves in IPC
Anterior tibial periosteum	Szabó et al. (2011)	Rat	IVM	Perfusion + PMN	Limb IR + ovariectomy + exogen estrogen	Amelioration of inflammatory reactions by chronic estrogen supplementation
Anterior tibial periosteum + synovial membrane	Hartmann et al. (2012)	Rat	IVM	RBCV, perfusion + PMN, ICAM-1	Limb IR	Synovial microcirculation is less endangered to the consequences of short-term tourniquet IR than the periosteum
Mandibular and tibial periosteum	Varga et al. (2014)	Rat	IVM, OPS, laser scanning confocal microscopy	Perfusion + PMN	Intact	New method
Mandible periosteum	Janovszky et al. (2015)	Rat	IVM	Perfusion + PMN	MRONJ induced by chronic zoledronate treatment + previous tooth extraction	Microvascular inflammation

IR: ischemia/reperfusion, IPC: ischemic preconditioning, MRONJ: medication-related osteonecrosis of the jaw.

prevented by ischemic preconditioning of the limb (Szabó et al., 2009) and this protection appears to be mediated by the activation of chemosensitive afferent nerves (Hartmann et al., 2011). In other studies, antagonism of vasoconstriction-mediating endothelin-A receptors or pretreatment with anti-inflammatory agents (phosphatidylcholine and colloid solutions) likewise exerted some protection (Gera et al., 2007; Varga et al., 2008; Wolfárd et al., 2002). These studies further revealed that the microcirculation of the tibial periosteum is more prone to microcirculatory inflammatory complications than that of the synovial membrane (Hartmann et al., 2012).

4.2. Periosteal manifestations of systemic diseases

As the prevalence of skeletal fractures and limb injuries increases with aging, restoration of the periosteal microperfusion is of particular importance in surgical interventions on elderly patients with or without tourniquet application. The microcirculatory patency is influenced by many functional and morphological changes in the microvasculature and, as a result of the osteogenic stem cell content of the periosteum and endosteum (Brighton et al., 1992), these membranes, and the periosteum in particular, play important roles in the pathogenesis of hormone-related (i.e. estrogen deficiency) and trauma-induced osteoporotic processes. Likewise, the periosteum can serve as a target of different anti-osteoporotic approaches (Allen et al., 2004).

Although relatively little information is available on the microcirculatory effects of chronic ovariectomy (OVX) itself, a considerable amount of evidence suggests that a disintegrated microcirculation is involved in the osteoporotic changes in the cortex. OVX-induced morphological alterations within the microvasculature have been reported in the heart (Jesmin et al., 2003) and brain (Johnson et al., 2004), but we

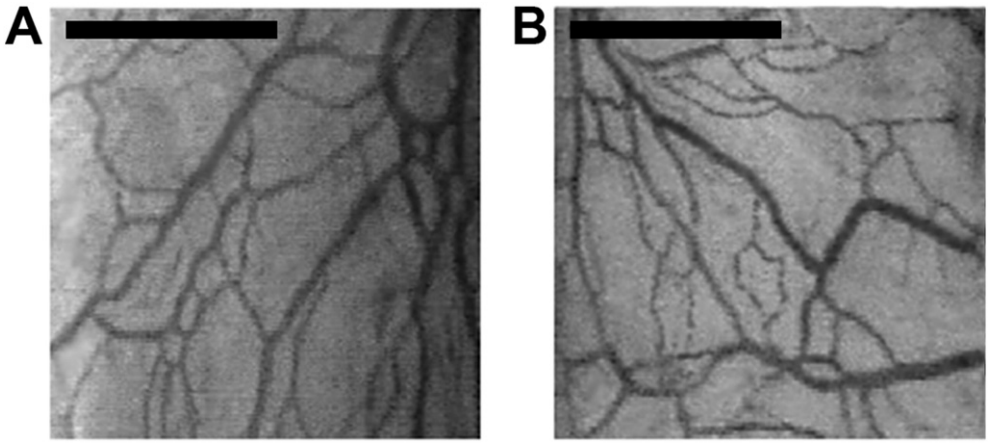

Fig. 3. Micrographs showing OPS (Cytoscan A/R; Cytometrics, Philadelphia, PA, USA) images of the microvascular architecture of the anteromedial (A) and anterolateral (B) surfaces of the tibial periosteum in Sprague-Dawley rats. The bar denotes 250 μm (original recordings of the authors).

did not observe significant changes in periosteal vessel density in a chronic OVX rat model (Szabó et al., 2011). It is important to note that these changes were followed in the proximal tibial periosteum, where the predominant vessel type is the venule (see Fig. 1). The postcapillary venules are predilectory sites for PMN–endothelial interactions, and thus a relatively higher venular density may predispose to local inflammatory complications. Again, this feature of the periosteal microarchitecture may account, at least in part, for a tissue-specific response.

On the other hand, there have been several experimental studies of the microcirculatory changes of sex hormone substitution. Since estradiol is protective in many forms of traumas and injuries, it is possible that 17-beta-estradiol (E2) is protective independently of the presence of OVX. This view is supported by findings where a single dose of E2 administered hours or even minutes before reperfusion ameliorated PMN-related processes in different organs (e.g. the heart, liver and retina) (Booth et al., 2003; Burkhardt et al., 2008; Nonaka et al., 2000). We found that the postischemic periosteal microcirculatory complications were not aggravated as compared with the non-OVX, age-matched controls (Szabó et al., 2011). The situation was similar as concerns TNF-α release, providing further evidence that OVX itself does not modify the inflammatory complications, whereas E2 supplementation greatly reduces this reaction. It has also been demonstrated that E2 inhibits TNF-α gene transcription via the beta-estrogen receptors (Srivastava et al., 1999) and via the TNF-α-mediated increases in the expressions of adhesion molecules and chemoattractants (Xing et al., 2007). A downregulation of nitric oxide synthesis by TNF-α may also contribute to the mechanisms of these microcirculatory reactions (Yoshizumi et al., 1993). Our data indicating the positive effect of prolonged E2 substitution on the PMN reactions and TNF-α release, however, point to another clinical implication: ovarial hormone deprivation supplemented with estrogen therapy (apart from the well-known positive effect in reducing the risk of osteoporotic fractures) affords marked protection against the release of inflammatory mediators.

The impact of periosteal microvascular reactions is also evident in the case of long-term systemic treatment with nitrogen-containing BISs. Treatment with osteoclast inhibitor BISs can cause local osteonecrosis, predilectorily in the jaw (also termed medication-related osteonecrosis of the jaw, MRONJ) after invasive dental procedures (Kühl et al., 2012; Marx et al., 2007; Wehrhan et al., 2011; Yamashita et al., 2010). Such a reaction does not occur in the bones of the appendicular skeleton (Blazsek et al., 2009; Stadelmann et al., 2008). Many theories have been put forward and several risk factors have been investigated in efforts to decipher the pathogenesis of MRONJ, but the cause is still unknown (Mehrotra and Ruggiero, 2006). Among others, the role of infection-induced inflammatory reactions has been suggested, but since osteonecrosis usually develops several years after tooth extraction, this factor seems to be of only limited impact. Enhancement of PMN–endothelial cell interactions in the knee joint (Zysk et al., 2003) and the upregulation of pro-inflammatory cytokines such as IL-1 and TNF-α (Anastasilakis et al., 2012; Norton et al., 2011; 104), however, have been clearly demonstrated in response to BIS administration. These effects also display spatial differences, because certain inflammatory reactions were confined to the mandible, and could not be detected in the femur (Senel et al., 2010). Moreover, in another sheep model with osteoporosis, the stability of implants in the femoral condyle was even enhanced after local BIS treatment (Stadelmann et al., 2008). Other data suggest that the production of vascular endothelial growth can be an important factor in the pathogenesis of MRONJ (Vincenzi et al., 2012), a notion supported by the fact that BIS treatment combined with the anti-angiogenic drug bevacizumab increases the prevalence of MRONJ (Aragon-Ching et al., 2009). Furthermore, the critical concentration of BIS in the mandible (Wen et al., 2011), and its direct toxic effect on the periosteal stem/osteoprogenitor cells and related inflammatory effects in the periosteum may also contribute to the development of MRONJ. High-dose BIS exacerbated the inflammatory response in a periodontitis model, where the bone lesions strikingly resemble MRONJ (Aguirre et al., 2012). On prolonged use, BISs are known to accumulate in the skeleton, reaching the highest concentration in the mandible (Reid et al., 2007; Wen et al., 2011), which may explain their potential toxic effects occurring predominantly in the jawbones. Furthermore, osteoblasts have different proliferation properties at different locations in the appendicular or axial bones under physiological circumstances, and this phenomenon is also critically influenced by BIS treatment (Marolt et al., 2012). The functional activity of osteocytes differs between the mandible and the tibia (Çankaya et al., 2013), and the aggravating effects of BISs on bone healing are confined to the jaw (Kuroshima et al., 2014). In our study, IVM data obtained in the proximity of the alveolar injury and from a contralateral, intact site on the mandibular periosteum were compared with those on the intact tibia (Janovszky et al., 2015). After chronic zoledronate treatment, increased extents of PMN-endothelial interactions (rolling and firm adhesion) were observed in the mandibular periosteum, both at the site of the earlier tooth extraction and at the contralateral site, but the corresponding interactions in the tibia were significantly less pronounced.

5. Concluding remarks

Reestablishment of the periosteal microvascular integrity after trauma and orthopedic surgery is essential for normal bony regeneration

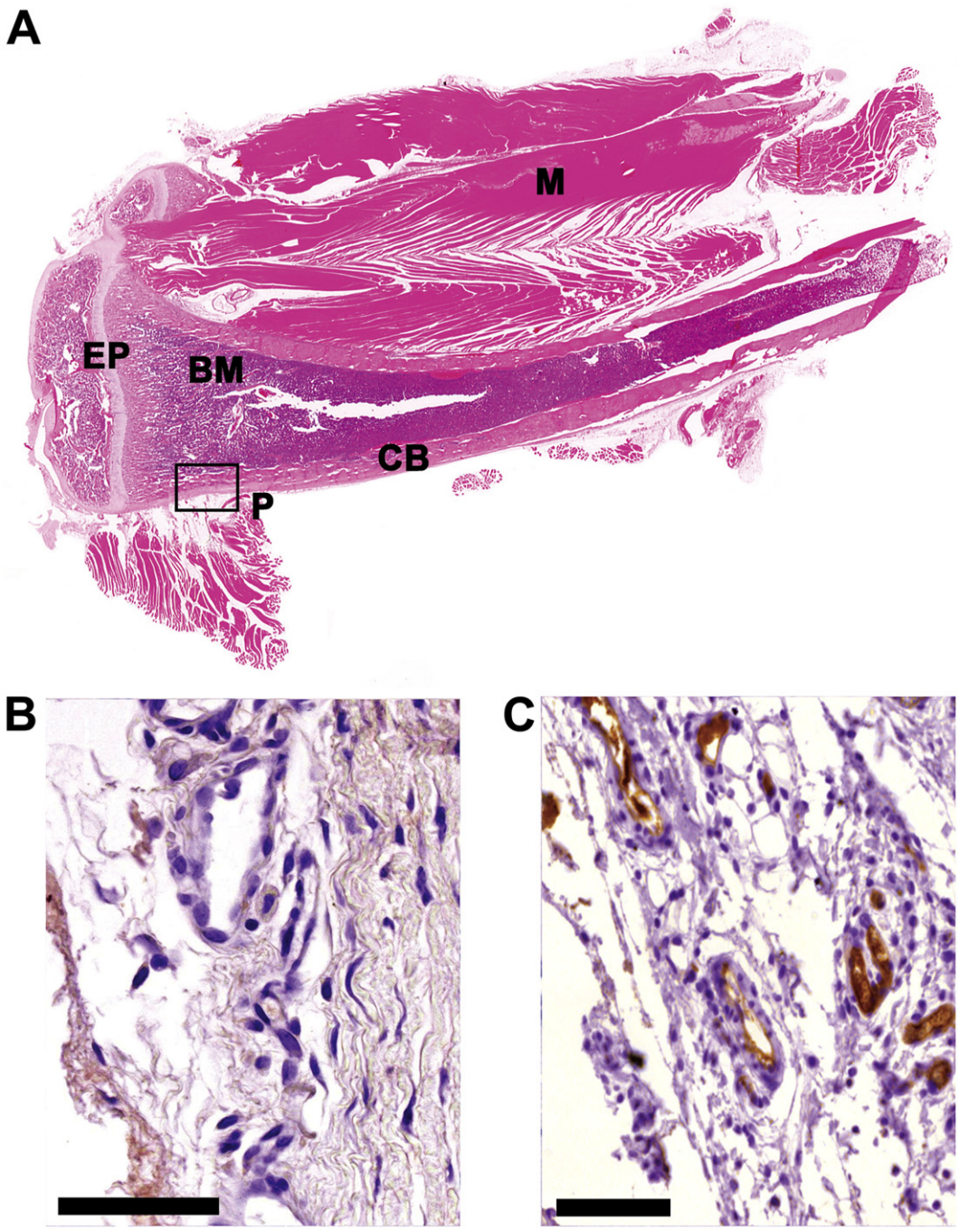

Fig. 4. Representative longitudinal section of the rat tibia surrounded by soft tissues (stained with ICAM-1 plus hematoxylin) (A). Tibia epiphysis (EP), cortical bone (CB), bone marrow (BM), muscle (M) and periosteum (P) are indicated. Lower panels: weak ICAM-1 staining (primary antibody: a mouse monoclonal anti-rat ICAM-1; BD Pharmingen, BD Biosciences, San Jose, CA, USA), secondary antiobody: biotinylated goat anti-mouse antibody conjugated to HRP polymer (Envision®System; Dako, Glostrup, Denmark) in the periosteum of a sham-operated animal (B), positive staining in periosteal venules after 60 min of limb ischemia followed by a 180-min reperfusion period (C). The bar denotes 50 μm (authors' own data).

processes. Observation of the periosteal microcirculation still necessitates surgical exposure, but many different methods have been established for the correct assessment of perfusion changes, and various imaging methods have been introduced that allow the visualization even of individual vessels, without disturbing their functional characteristics. The examined parameters include changes in vascular density, microvascular perfusion efficacy and cell-cell interactions. Through the use of these parameters, microcirculatory consequences of traumas and the related surgical interventions, IR injuries and the local periosteal manifestations of systemic diseases can be assessed and the efficacy of different treatment modalities can be quantified. The findings that systemic insults trigger different microcirculatory reactions in the periosteum at different anatomical locations warrant further studies.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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Paper II.

Estrogen-Dependent Efficacy of Limb Ischemic Preconditioning in Female Rats

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ABSTRACT: Our aim was to examine the effects of ischemic preconditioning (IPC) on the local periosteal and systemic inflammatory consequences of hindlimb ischemia-reperfusion (IR) in Sprague–Dawley rats with chronic estrogen deficiency (13 weeks after ovariectomy, OVX) in the presence and absence of chronic 17beta-estradiol supplementation (E2, 20 $\mu g\,kg^{-1}$, 5 days/week for 5 weeks); sham-operated (non-OVX) animals served as controls. As assessed by intravital fluorescence microscopy, rolling and the firm adhesion of polymorphonuclear neutrophil leukocytes (PMNs) gave similar results in the Sham + IR and OVX + IR groups in the tibial periosteal microcirculation during the 3-h reperfusion period after a 60-min tourniquet ischemia. Postischemic increases in periosteal PMN adhesion and PMN-derived adhesion molecule CD11b expressions, however, were significantly reduced by IPC (two cycles of 10′/10′) in Sham animals, but not in OVX animals; neither plasma free radical levels (as measured by chemiluminescence), nor TNF-alpha release was affected by IPC. E2 supplementation in OVX animals restored the IPC-related microcirculatory integrity and PMN-derived CD11b levels, and TNF-alpha and free radical levels were reduced by IPC only with E2. An enhanced estrogen receptor beta expression could also be demonstrated after E2 in the periosteum. Overall, the beneficial periosteal microcirculatory effects of limb IPC are lost in chronic estrogen deficiency, but they can be restored by E2 supplementation. This suggests that the presence of endogenous estrogen is a necessary facilitating factor of the anti-inflammatory protection provided by limb IPC in females. The IPC-independent effects of E2 on inflammatory reactions should also be taken into account in this model. © 2017 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res

Keywords: osteoporosis; ischemia-reperfusion; limb; ischemic preconditioning; estrogen; microcirculation

During elective orthopedic interventions or traumas, tourniquet application may lead to iatrogenic ischemia-reperfusion (IR) injury of the affected extremities, which may influence the healing of the bone and the surrounding soft tissues[1] including the periosteum.[2] The local IR injury may lead to systemic inflammatory activation as well,[3] and the injury in distant organs (e.g., the liver and lungs) is mediated by many factors, among others by circulating pro-inflammatory cytokines and activated polymorphonuclear leukocytes (PMNs).[1,4] Nevertheless, it has been shown that short, repeated local IR periods termed ischemic preconditioning (IPC) confer anti-inflammatory protection both in the periosteum,[5] and in remote organs through humoral and neurogenic signals.[6–9]

The amelioration of IR-induced inflammatory complications by limb IPC should offer a therapeutic benefit in elderly patients when the prevalence of skeletal injuries increases and osteoporotic bones are more prone to accidental fractures. However, the influence of osteoporosis on the efficacy of IPC against IR-induced injury remains unexplored and the results obtained concerning the estrogen status in this condition seem contradictory. Earlier it was demonstrated that endogenous estrogen does not play a role in the protective effect of IPC.[10] Other studies have shown that the positive cardiac effects of IPC are lost when the endogenous estrogen levels are reduced by ovariectomy (OVX), but cardioprotection could be re-established by estrogen supplementation.[11–13] So far, available data on the effects of estrogen replacement during IPC appear to conflict.[11–14] We showed earlier that OVX per se did not predispose female rats to more severe inflammatory reactions, but estrogen supplementation reduced the harmful consequences of limb IR.[15] Therefore the present study was designed to ascertain whether IPC exerts its potentially positive anti-inflammatory effects on limb IR injury with chronic estrogen deficiency. In our study, we also sought to examine whether the periosteal microcirculatory reactions are modulated by exogenous estrogen supplementation. With the above in mind, we decided to characterize the effects of IPC with or without estrogen supplementation on local periosteal and systemic inflammatory changes in a rodent model of hindlimb IR injury with chronic estrogen deficiency.

MATERIALS AND METHODS

Animals

All studies were carried out on Sprague–Dawley rats housed in an environmentally controlled room with a 12-h light-dark cycle, and kept on commercial rat chow (Charles River, Wilmington, MA) and tap water ad libitum.

Levente Pócs and Ágnes Janovszky contributed equally to this study.

Conflicts of interest: The authors declare that there were no conflicts of interest, financial or otherwise.

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The project was approved by the National Scientific Ethical Committee on Animal Experimentation (National Competent Authority), with the license number: V./144/2013. The study was performed in adherence with the EU Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and the National Institute of Health guidelines for the use of experimental animals. Animal welfare-related assessments and interventions were carried out prior to and during the experiments.

Experimental Protocol

Ovariectomy

Twelve-week-old female rats (weighing 180–200 g) were randomly allocated to ovariectomized ($N = 48$), or sham-operated ($N = 33$) groups. The animals were anesthetized with an intraperitoneal combination of ketamine and xylazine (25 mg kg^{-1} and 75 mg kg^{-1}, respectively), and a median laparotomy was performed under sterile conditions. The connection of the Fallopian tubes was cut between hemostats, the ovaries were removed, and the stumps were then ligated with a 3–0 non-absorbable thread (Ethibond Excel®, Ethicon, Somerville, NJ). Thereafter, the abdomen was filled with warm sterile physiological saline and the abdominal wall was closed with a 4–0 absorbable suture and a 4–0 non-absorbable suture (Vicryl® and Prolene®, Ethicon, Somerville, NJ) in two layers. Sham-operated animals underwent identical procedures, except of course that the Fallopian tubes and ovaries were not touched.

Chronic EstrogenTreatment

Eight weeks after OVX (i.e., at 20 weeks of age) (see Fig. 1), a chronic estrogen therapy was initiated in some of the OVX animals for 5 days/week with 20 μg kg^{-1} subcutaneous 17beta-estradiol[16] (E2, Sigma, St. Louis, MO) and it was continued for 5 weeks (i.e., until the end of the experimental protocol in week 25). The remaining OVX and Sham animals received the vehicle for E2 (100% ethanol diluted in corn oil) in the same volume.

Experimental Series

The later in vivo experiments were performed in two major series 13 weeks after the OVX and sham operations (in week 25) (Fig. 1). In the first series, the tibial periosteal microcirculatory consequences of a 60-min complete hindlimb ischemia followed by a 180-min reperfusion (with or without limb IPC) were investigated with intravital videomicroscopy (IVM). In a second series of experiments, identical protocols in the same groups were performed in order to detect changes in various systemic inflammatory reactions (see later on).

Series 1: Measurement of Local Inflammatory Reactions Using IVM

The experiments were performed under sodium pentobarbital (45 mg kg^{-1} ip) anesthesia and sustained with small supplementary intravenous doses when necessary. The right carotid artery and the jugular vein were cannulated for the measurement of mean arterial pressure and the administration of drugs and fluids, respectively. The animals were placed in a supine position on a heating pad to maintain their body temperature between 36 and 37°C. Here, Ringer's lactate was infused at a rate of 10 ml kg^{-1} h^{-1} during the experiments. The trachea was cannulated to facilitate respiration. The right femoral artery was isolated, and the periosteum of the medial surface of the right tibia was exposed under a Zeiss 6× magnification operating microscope, using an atraumatic surgical technique.[2]

In the final stage, the animals were randomly allotted to one of the following five groups. These are shown in Figure 1, week 25. Among vehicle-treated animals, a 60-min complete hind limb ischemia was induced by applying a tourniquet around the proximal femur and a miniclip on the femoral artery, which was followed by a 180-min reperfusion period in nine sham-operated animals (Sham + IR group) and 11 of the OVX animals (OVX + IR groups). Two other vehicle-treated groups were also subjected to two cycles of 10 min of limb IPC and 10 min of reperfusion (Sham + IPC + IR group, $N = 9$; OVX + IPC + IR group, $N = 9$). This IPC protocol has been shown to ameliorate local microcirculatory and systemic inflammatory complications caused by limb IR in male rats.[3] In all of the E2-treated animals, limb IR was combined with IPC (OVX + E2 + IPC + IR group, $N = 6$) and the experiments were started 18–24 h after the last E2 injection. In this series, the periosteal microcirculation was observed with IVM at baseline and every 60 min during the 180-min reperfusion period.

Microcirculatory Measurements

The right hindlimb with the exposed tibial periosteum was positioned horizontally on an adjustable stage for examination of the microcirculation by IVM (Zeiss Axiotech Vario 100HD microscope, 100W HBO mercury lamp, Acroplan 20× water immersion objective, Carl Zeiss GmbH, Jena, Germany). Microcirculation was visualized with fluorescein isothiocyanate (Sigma, St. Louis, MO)-labeled erythrocytes (0.2 ml iv), while PMNs were labeled with an iv injection of rhodamine 6G (Sigma, St. Louis, MO, 0.2%, 0.1 ml iv). The microscopic images were recorded with a charge-coupled device video camera (Teli CS8320Bi, Toshiba Teli Corporation, Osaka, Japan) attached to an S-VHS video recorder

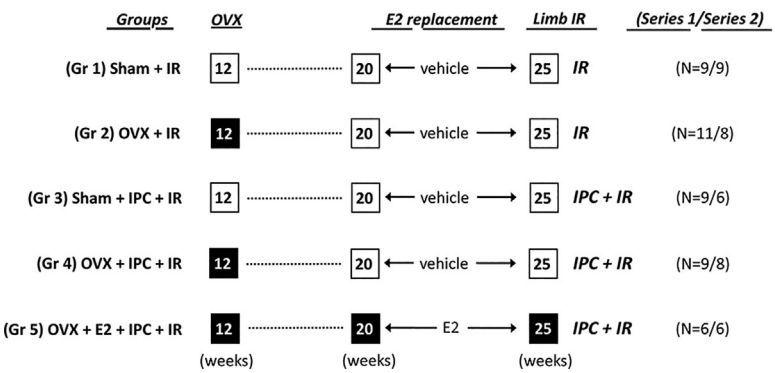

Figure 1. Groups and time sequence of surgical interventions, treatments and measurements: Ovariectomy (OVX) or a sham operation (Sham) was performed at 12 weeks of age; 17beta-estradiol treatment (E2) was performed for 5 weeks (5 days/week in a dose of 20 μg kg^{-1}); tourniquet-ischemia of a hindlimb followed by reperfusion (IR; 60′/180′) with or without ischemic preconditioning of the hindlimb (IPC; 2 × 10′/10′) was performed at the end of the protocol. In Series 1, the assessment of local inflammatory reactions in the tibial periosteum using intravital microscopy was carried out, while in Series 2, the detection of various systemic inflammatory parameters was performed. The number of animals used per group in each series is indicated in brackets.

(Panasonic AG-MD 830, Matsushita Electric Industrial Co., Tokyo, Japan) and a personal computer.

IVM—Video Analysis

A quantitative assessment of the microcirculatory parameters was performed off-line by a frame-to-frame analysis of the videotaped images, using image analysis software (IVM, Pictron Ltd., Budapest, Hungary) (Fig. 2). As for the periosteum, leukocyte–endothelial cell interactions were analyzed within five postcapillary venules (with diameters between 11 and 20 μm) per animal. IVM in the periosteum allows the observation of the primary and secondary PMN-endothelial interactions (rolling and adhesion, respectively). Rolling is a transient and reversible process, whereas adhesion represents a higher level of activation of leukocytes (when endothelial contact-dependent signals trigger the formation of the activation-dependent adhesion molecule expression of PMNs with accompanying NADPH oxidase activation and degranulation.[17] Based on their movements and contact with the endothelium of the postcapillary venules, 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 expressed here 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 expressed as the number of cells/vessel circumference in millimeters.

Series 2: Detection of Systemic Inflammatory Reactions

In a second series of experiments, identical protocols for the same groups were applied to detect changes in the proinflammatory cytokine TNF-alpha concentrations in the plasma and in whole blood free radical productions, as well as in the expressions of a circulating PMN-derived adhesion molecule (see the groups above, $N = 6$–9). The separation of the two series was necessary in order to avoid any interference between the fluorescent dyes used for IVM and acquisition techniques used with flow cytometry and luminometry. In this series of experiments, measurements were made from blood samples taken at baseline and at every 60 min of the reperfusion phase. And at the end of the protocol, periosteal specimens were harvested under RNase- and DNase-free circumstances to detect periosteal estrogen receptor (ER) expressions, then the samples were stored at −80°C until assay.

Immune Labeling and Flow Cytometric Analysis of Adhesion Molecule CD11b Expression of PMNs

The surface expression of CD11b on the peripheral blood PMNs was determined via a flow-cytometric analysis of whole blood in duplicate.[2] 100 μl of whole blood was incubated with 20 μl of (50 $\mu g\,ml^{-1}$) fluorescein isothiocyanate-conjugated mouse anti-rat monoclonal antibody (clone OX-42, AbD Serotec, Kidlington, UK) for 20 min. Negative controls were obtained by omitting the monoclonal antibody. The cells were then washed twice in Hanks buffer and centrifuged (Heraeus Biofuge primoR, Thermo Scientific, Waltham, MA, rotor diameter: 65 mm) at 12,281 g for 5 min. The cells were again washed twice, and the erythrocytes were lysed with a lysis puffer (Erythrolyse Red Blood Cell Lysing Buffer (10x) Reagent, GenWay, San Diego, CA) for 8 min, after which the cells were washed twice again (2,616 g, 5 min) and resuspended in 750 μl of Hanks buffer. CyFlow ML (Partec GmbH, Münster, Germany) equipment was used for cytometry; the granulocytes were gated on the basis of their characteristic forward and sidescatter features. Here, 10,000 events per sample were collected and recorded, then the percentages of labeled (activated) granulocytes (relative to the overall marker-bearing cells) and the mean fluorescence intensity (average marker density) were calculated.

Determination of Plasma TNF-Alpha Levels

Blood samples (0.5 ml) were taken from the carotid artery and placed into precooled EDTA-containing polypropylene tubes, centrifuged at 13,500 rpm for 5 min at 4°C, and then stored at −70°C until assay. Proinflammatory cytokine TNF-alpha concentrations were determined in plasma samples by means of commercially available enzyme-linked immunosorbent assays (Quantikine Ultrasensitive ELISA kit for rat TNF-alpha; R&D systems, Minneapolis).

Free Radical-Producing Capacity of the Blood

10 μl of blood dissolved in Hanks buffer was incubated for 20 min at 37°C in lucigenin (5 mM; dissolved in Hanks buffer) solution in the presence or absence of zymozan (190 μM, dissolved in Hanks buffer). Superoxide production was

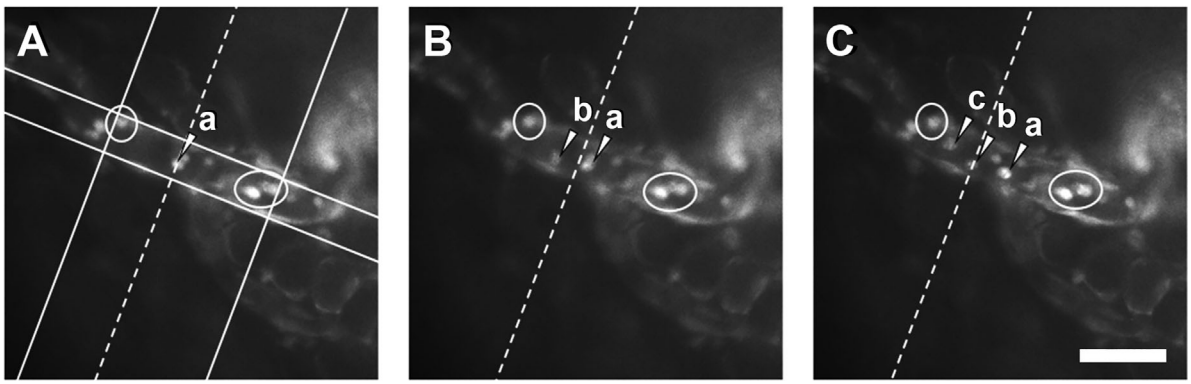

Figure 2. Representative micrographs showing the sequence of PMN–endothelial interactions on three consecutive images (Panels A–C) recorded by using intravital microscopy (recording rate: 20 frames/s). The segment of the examined tibial postcapillary vein is surrounded by lines in Panel A. Movement of rhodamine 6G-labeled PMN (marked by a–c) is demonstrated frame-by-frame referring to a dashed line. Stationary (adhesive) leukocytes are marked by ellipses. The bar in Panel C denotes 50 μm scale and this applies to all photomicrographs.

estimated via the rate of zymozan-induced increase in chemiluminescence (measured with an FB12 Single Tube Luminometer (Berthold Detection Systems GmbH, Bad Wildbad, Germany) and normalized for leukocyte counts in the peripheral blood.

Determination of Plasma E2 Levels

Endogenous E2 levels were determined using the Elecsys Estradiol III kit (Roche Diagnostics GmbH, Mannheim, Germany) and the Roche Cobas e 601 immunology analyzer (Roche Diagnostics GmbH, Mannheim, Germany).

Determination of Periosteal Estrogen Receptor-Alpha (ER-Alpha) and Beta (ER-Beta) mRNA Expressions

Tissue Collection

Anteromedial tibial periosteal samples were harvested via sterile surgical exposure of the contralateral (non-ischemic) limbs under an operating microscope. The samples were washed in 0.3 ml of sterile DNase, RNase and protease-free water (Sigma, St. Louis, MO) and placed in RNA stabilization solution (0.2 ml/each sample; RNAlater, Ambion®, Thermo Fisher Scientific, Waltham, MA). After overnight storage at 4°C, the RNA stabilization solution was removed, and tissue samples were stored at −80°C until RNA purification. Here, uterus samples were used as internal controls.

RNA Purification

The total RNA taken from the tibial periosteum and the uterus of each animal was purified with the NucleoSpin® RNA XS kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the protocol provided by the manufacturer.

Real Time PCR for ER-Alpha and ER-Beta

100 ng of RNA template in a 10 μl reaction mix were measured, using a quantitative reverse transcriptase-mediated PCR kit (Verso 1-step RT-qPCR Mix, ROX kit; Thermo Fisher Scientific, Waltham, MA). The amplification conditions were 50°C for 15 min, 95°C for 15 min, 40 cycles of 95°C for 15 s, and 58°C for 15 s. RNA levels were calculated using the ΔΔCT method and were normalized to 18S mRNA. The Universal Probe Library (UPL) system (Roche, Basel, Switzerland) was used to design primers and probes for the experiments (see Table 1).

Statistical Analysis

The required number of animals (i.e., sample size) was assessed by using the PS Power and Sample Size Calculations software package (version 3.1.2) prior to the experiments. Data analysis was performed with the SigmaStat statistical software package (Jandel Corporation, San Rafael, CA). The normality of data sets was checked, and in case of normal distribution, changes in variables within and between groups were analyzed by the two-way repeated measures ANOVA test, followed by the Holm–Sidak test. Data are expressed as means ± standard error of the mean (SEM). Due to the non-Gaussian distribution, PCR data were analyzed by the Kruskal–Wallis test, followed by the Dunnett test; the box plot figure shows the mean, the median, and the 25th and 75th percentile values. p values <0.05 were considered statistically significant at all parameters.

RESULTS

Effects of Local IPC on the Postischemic Tibial Periosteal Microcirculatory Inflammatory Reactions With Estrogen Depletion

When compared with the baseline values, the values of primary PMN–endothelial interactions (termed rolling) in the postcapillary venules of the tibial periosteum increased to a similar extent in the Sham + IR and OVX + IR animals at all examined time-points of reperfusion after limb IR (see Fig. 3). When limb IR was combined with local IPC, moderately reduced rolling values were observed in non-ovariectomized rats (Sham + IPC + IR group) at later stages of reperfusion (120 and 180 min), but no reduction was seen in OVX rats (OVX + IPC + IR group). At 60 and 120 min of reperfusion, the lowest rolling values were detected in animals treated with chronic E2 (OVX + E2 + IPC + IR group), but these differences were not statistically significant.

Leukocyte adherence (sticking) revealed a similar pattern to that seen with PMN rolling; no ameliorating effect of IPC was seen in OVX animals (in the OVX + IPC + IR group), but some alleviating effect was observed after E2 treatment (in OVX + E2 + IPC + IR group) (see Fig. 4).

Systemic Inflammatory Reactions

An increased expression of the adhesion molecule CD11b on the PMN surface was observed after 120 and 180 min of reperfusion. After, no major differences could be seen between the values for the Sham + IR and OVX + IR groups, but a slight decrease was observed after IPC in sham-operated animals (Sham + IPC + IR) (see Fig. 5). This amelioration, however, was not seen after OVX (in the OVX + IPC + IR group). It seems that chronic E2 treatment effectively prevented the IR-induced increase in CD11b expression (OVX + IPC + IR + E2).

The free radical-derived chemiluminescence of the whole blood (as determined by the superoxide radical-dependent chemiluminescence measurements) gave the earliest increase (after 60 min of reperfusion) after IPC both in the sham-operated and OVX animals (Sham + IPC + IR and OVX + IPC + IR), but it rose only slightly in the E2-treated OVX + IPC + IR animals (OVX + E2 + IPC + IR) at this time point (see Fig. 6). Free radical production did not reveal any

Table 1. Primers and Probes for Quantitative RT-PCR Used in This Study

Target	Forward Primer	Reverse Primer	Probe
ER-alpha	TTCTTTAAGAGAAGCATTCAAGGAC	TCTTATCGATGGTGCATTGG	# 130; 04693663001
ER-beta	GGCTGGGCCAAGAAAATC	TCTAAGAGCCGGACTTGGTC	# 111; 04693442001
18S	CTCAACACGGGAAACCTCAC	CGCTCCACCAACTAAGAACG	# 77; 04689003001

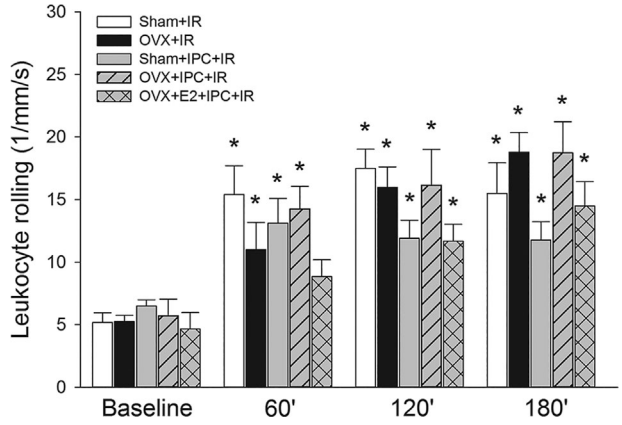

Figure 3. Changes in primary leukocyte–endothelial cell interactions (rolling) in the postcapillary venules of the tibial periosteum at baseline, 60, 120, and 180 min after a 60-min limb ischemia. Sham: Sham operation; OVX: ovariectomy; IR: tourniquet-ischemia of a hindlimb followed by reperfusion (60′/180′); IPC: hindlimb ischemic preconditioning ($2 \times 10'/10'$); E2: 17beta-estradiol treatment. Two-way RM ANOVA was followed by the Holm-Sidak test. Here, data values are given as means ± SEM, and $^{*}p < 0.05$ versus baseline.

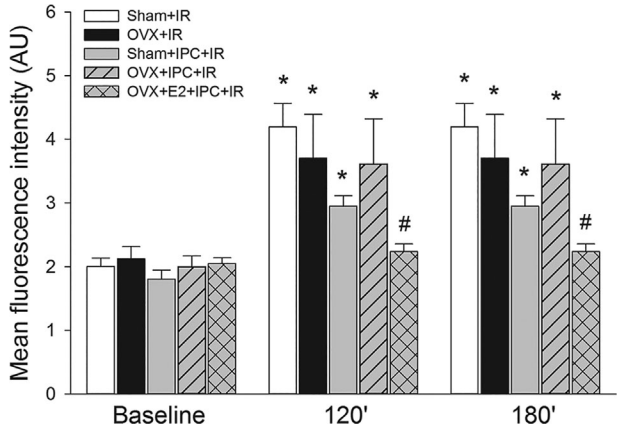

Figure 5. Changes in expression of the CD11b adhesion molecule on the surface of PMNs at baseline and in response to 60 min of limb ischemia followed by 120 and 180 min of reperfusion. Sham: Sham operation; OVX: ovariectomy; IR: tourniquet-ischemia of a hindlimb followed by reperfusion (60′/180′); IPC: hindlimb ischemic preconditioning ($2 \times 10'/10'$); E2: 17beta-estradiol treatment. Two-way RM ANOVA was followed by the Holm–Sidak test. Here, data values are given as means ± SEM, and $^{*}p < 0.05$ versus baseline and $^{\#}p < 0.05$ versus Sham + IR.

more differences between the different experimental groups at later time points.

From the experiments, we found that IR brought about a significant increase in TNF-alpha levels in the plasma in all of the groups (see Fig. 7). Due to the high data dispersion, no statistically significant differences were seen between the groups at any time point, but the lowest increase was observed in the E2-treated animals.

The protocol was not synchronized with the estrous cycles of the animals and vaginal smear tests were not performed. The serum E2 concentrations ranged from 9.57 to 15.87 pg/ml in the Sham-operated animals, while these levels were significantly lower in the OVX animals ($p < 0.001$), not even attaining the detection limit of the assay (>5 pg/ml). However, plasma E2 was restored by chronic E2 supplementation in the OVX animals and the values were slightly higher than those in the Sham group (20.06 median value pg/ml, $p < 0.05$).

Periosteal Estrogen Receptor Expression

In the periosteum, a similar level of ER-beta transcription was observed in the sham-operated and in the OVX animals; and the highest transcription level was noted after chronic E2 supplementation (see Fig. 8). Periosteal ER-alpha mRNA levels, however, remained below the detector threshold. We excluded any methodological issues related to the detection of ER-alpha by simultaneously examining uterus samples taken from the same animals for an mRNA analysis of both receptors. Similar to Mohamed and Abdel-Rahman,[18]

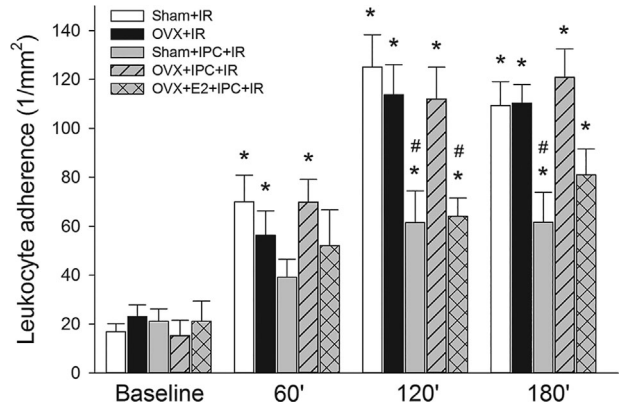

Figure 4. Changes in secondary leukocyte–endothelial cell interactions (adherence) in the postcapillary venules of the tibial periosteum at baseline, and 60, 120, and 180 min after a 60-min limb ischemia. Sham: Sham operation; OVX: ovariectomy; IR: tourniquet-ischemia of a hindlimb followed by reperfusion (60′/180′); IPC: hindlimb ischemic preconditioning ($2 \times 10'/10'$); E2: 17beta-estradiol treatment. Two-way RM ANOVA was followed by the Holm–Sidak test. Here, data values are given as means ± SEM, and $^{*}p < 0.05$ versus baseline and $^{\#}p < 0.05$ versus Sham + IR.

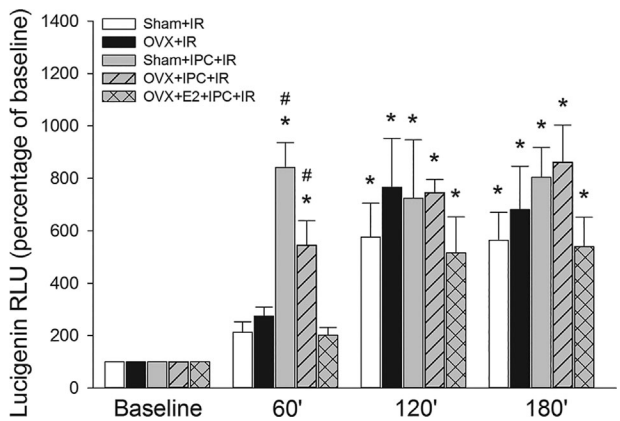

Figure 6. Whole blood superoxide production at baseline and in response to 60 min of limb ischemia followed by 60, 120, and 180 min of reperfusion. Sham: Sham operation; OVX: ovariectomy; IR: tourniquet-ischemia of a hindlimb followed by reperfusion (60′/180′); IPC: hindlimb ischemic preconditioning ($2 \times 10'/10'$); E2: 17beta-estradiol treatment. Two-way RM ANOVA was followed by the Holm–Sidak test. Here, data values are given as means ± SEM, and $^{*}p < 0.05$ versus baseline, $^{\#}p < 0.05$ versus Sham + IR.

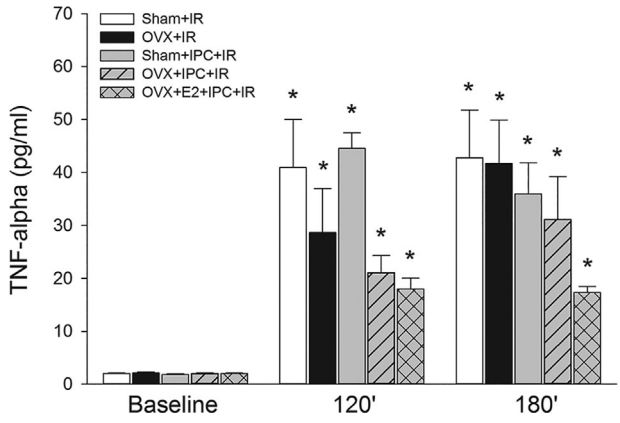

Figure 7. TNF-alpha levels in plasma samples at baseline and in response to 60 min of limb ischemia followed by 120, and 180 min of reperfusion. Sham: Sham operation; OVX: ovariectomy; IR: tourniquet-ischemia of a hindlimb followed by reperfusion (60′/180′); IPC: hindlimb ischemic preconditioning ($2 \times 10'/10'$); E2: 17beta-estradiol treatment. Two-way RM ANOVA was followed by the Holm–Sidak test. Here, data values are given as means ± SEM, and $^{*}p < 0.05$ versus baseline.

we found higher mRNA levels (for both ER-alpha and beta) in the uterus in OVX group than in the Sham group (data not shown).

DISCUSSION

Previously we examined the periosteal microcirculatory consequences of tourniquet-induced ischemia in a clinically relevant, long-term follow-up study with osteoporotic rats.[15] We showed that OVX did not enhance IR-induced periosteal microcirculation dysfunction, but chronic estrogen supplementation ameliorated the local inflammatory complications. In the present protocol we employed a shorter term of OVX, which does not cause osteopenia, but it is sufficient to evoke a chronic estrogen deficit in rats.[19] It appears that IPC mostly influences the second stage of IR-induced periosteal PMN-endothelial interactions (sticking) both here in females and in males,[3] which might be explained by the effect of IPC on adhesion molecule expression responsible for leukocyte adhesion to the postischemic endothelium.[3] This protection, however, disappeared in the OVX animals in this study, as both PMN rolling and adhesion increased. Hence, it appears that the IPC-induced periosteal protection against postischemic inflammatory complications is lost after estrogen depletion and this observation has potential clinical implications. In a similar way, CD11b expression (a marker of activation of circulating PMNs[20] was lower in IPC animals only if OVX was not performed. It is therefore reasonable to suppose that endogenous estrogen in females plays a facilitating role in the anti-inflammatory mechanisms provided by IPC in the periosteum. This hypothesis is supported by the observation that E2 supplementation reverses the protection that was lost in OVX + IPC + IR animals. Similarly to our present results, the positive effects of IPC were shown to vanish in postischemic hearts harvested from OVX rats, and reversed by E2.[11] Prior to this, the microcirculatory benefits of E2 supplementation were examined after IR, without IPC. The postischemic periosteal microcirculatory complications of tourniquet ischemia could be reversed by E2 supplementation[15] and E2 has also been shown to have beneficial microcirculatory effects in numerous other models of IR.[21–22] Since the alleviating effects of E2 are present with or without IPC, it is difficult to differentiate between the beneficial effects of E2 treatment per se and its effect on IPC. Hence, one may suppose that the beneficial effects of E2 seen in this model might be independent of its effects on IPC.

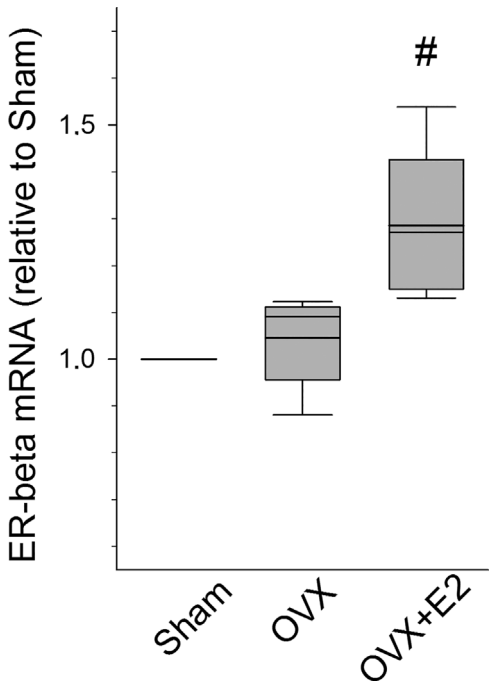

Figure 8. ER-beta mRNA expression levels in the tibial periosteum taken from sham-operated (Sham), ovariectomized (OVX) and OVX animals that were treated with 17beta-estradiol (OVX + E2). Kruskal-Wallis test was followed by the Dunnett test. Here, data values are given as mean, median, 25th and 75th percentiles, and $^{\#}p < 0.05$ versus Sham.

In our study, the microcirculatory manifestations of reduced efficacy of IPC after OVX were demonstrated for the first time, but similar reactions were observed with other manifestations of postischemic tissue injury in other organs by others (i.e., cardiac dysfunction).[11–14] The consequences of E2 supplementation in these scenarios, however, are not at all clear. As such, the OVX-related loss of IPC-induced protection in cardiac functions could be restored by E2 in certain studies with rats.[11–12] The results are somewhat controversial, as the protective effects of IPC were present in OVX rabbits.[10] Also, E2 did not exert any alleviating effects in other studies where IPC was combined with OVX.[13–14] Furthermore, long- and short-term estrogen administration produced different effects,[12,23] and inter-species and inter-organ differences and dissimilarities cannot be ruled out either.[10,12–13,24] The reason for the differences

between endogenous and exogenous estrogen effects in different experimental models is not well understood.

Some of the above differences might be due to the number and function of estrogen receptors within the affected tissue and also due to the effect of OVX and E2 on these receptor expressions. E2 is known to act as a transcription factor, as the binding of E2 to its ER-alpha or ER-beta receptors within the nucleus causes well-known genomic effects by inducing expression changes of different genes (e.g., nitric oxide synthase).[25] In addition, the action of binding E2 to its (plasma and mitochondrial) membrane-associated receptors also mediates non-genomic events[26–27] including the prevention of injury/stress-induced apoptosis[26] and cytochrome c release from myocardial mitochondria.[28] In our investigations, the ER-beta expression in the periosteum did not vary in response to OVX, but displayed an elevation in response to chronic E2 treatment (whereas the ER-alpha expression remained below the detector threshold). The up-regulation of the ER-beta receptor expression by E2 in the mitochondria and inhibition of apoptotic processes seems to be linked to the protective effect of E2 in trauma-hemorrhage.[29] Moreover, cardioprotective effects of E2 were attributable to the ER-beta receptor-related changes in the transcription on metabolic genes in another study.[30] In all likelihood, ER-beta is involved in regulating the estrogen-related increase in nitric oxide synthase activation[25] and others demonstrated the impact of ER-alpha as well.[31] PMN-related inflammatory processes were enhanced in OVX rats after trauma-induced hemorrhagic shock, which was prevented by the acute administration of E2 and an ER-beta agonist.[24] In vivo gene delivery of ER-beta to the endothelium greatly reduced the IR-induced formation of reactive oxygen species, increased nitric oxide formation and restored mitochondrial function in the adjacent cardiomyocytes.[32] In our study, some of the inflammatory processes (the CD11b expression of PMNs and free radical content in the blood) could be ameliorated by chronic E2; and the possible role of the up-regulation of ER-beta in these reactions cannot be ruled out. It should be noted, however, that estrogens also have a direct free radical scavenging effect via their phenolic A-ring,[33] a glutathione increasing effect,[34] and a direct modulatory action on NADPH activity.[35] Antioxidant effects of E2 may also be related to its influence on NFκB signaling[36] and the up-regulating of Nrf2.[37] As for the systemic effects, the involvement of ER-alpha-related actions of E2 also plays a role (in heart IR without IPC[38]), but discussion of these reactions as well as those evoked by selective estrogen modulators lies outside the scope of the present study. As was suggested by Murphy and Steenbergen, the shorter-term effects of E2 may be caused by ER-alpha, whereas longer-term effects may be mediated mainly through ER-beta.[39] Moreover, ER-independent effects of E2 in this study should not be ruled out either. It should be mentioned that the periosteal expression of ERs has not yet been examined in humans, but in the cortical and trabecular bone tissue, both ER proteins can be detected (via immunohistochemistry) with a different density during bone development.[40] It appears that only the ER-beta mRNA expression was examined in the tibial periosteum in the rat[41] and here we were unable to detect any ER-alpha mRNA expression of in the periosteum. This might mean that ER-alpha mRNA expression is not detectable in the periosteum. However, the translation of our present findings (the absence of periosteal ER-alpha mRNA expression) to the human situation requires further in-depth investigation.

Systemic inflammatory parameters also displayed characteristic changes. That is, the IR-induced increase in CD11b expression of circulating PMNs (a marker of their activation) was reduced by IPC only in sham-operated animals, but not in those with OVX. This reaction was also reversed by E2. The PMN-derived CD11b expression was likewise reduced by E2 in vitro[42] and in trauma-hemorrhagic shock[43] as well as in levels of some of other adhesion molecules such as the E-selectin.[44] We have not come across any studies that investigated the effect of IPC in OVX animals from the viewpoint of adhesion molecule expressions. In the present study, whole blood free radical content was significantly increased in all groups. In the Sham + IR and OVX + IR groups, local (periosteal) and systemic inflammatory reactions had a slightly different timeframe, since IVM data revealed increased PMN rolling and adhesion after 60 min of reperfusion (indicating an early activation of the affected endothelium and a simultaneous availability of primed leukocytes), but the superoxide levels displayed later changes (occurring after 120 min). The background of this phenomenon is not yet understood, but since increased CD11b expression in peripheral leukocytes also occurred at later stages of reperfusion (after 120 min), the contribution of other elements (e.g., activated macrophages) to the increased superoxide production may be assumed. Interestingly, IPC failed to induce any amelioration in whole blood free radical production, and furthermore, it induced an earlier increase in this parameter in both sham-operated and OVX groups. It should also be mentioned that this increase was not present in the E2-treated group. Actually, free radicals are known to play a role in the pathomechanism of IPC because their accumulation could be detected in vivo and superoxide scavengers reversed the tissue protective effects of IPC.[45–46] ER-beta has been shown elsewhere to be involved in reducing neutrophil activation[24] and the free radical reducing effect of E2 was also highlighted.[47] Interestingly, levels of one of the central regulators of inflammation TNF-alpha were not influenced by IPC. Quite surprisingly, the phenomenon observed in humans[48] indicating increased serum TNF-alpha levels after OVX could

not be confirmed in the present study (i.e., the baseline TNF-alpha values were not dissimilar after OVX), and even slightly lower values were found in all of the OVX animals (after 120 min of reperfusion). These differences might be the result of interspecies differences or changes in the immunological responses seen after OVX (which are outside the scope of the present study). TNF-alpha release has been shown to be reduced by E2 in numerous studies (with or without OVX),[23,49] even in male patients.[50] In this respect, the changes induced by reperfusion or IPC + IR have yet to be compared in OVX studies elsewhere. Here, the lowest postischemic values were found after applying E2 (although not attaining any statistical significance due to the relatively high data dispersion). This parameter together with reduced CD11b expression and the slower postischemic increase in superoxide production represent manifestations of the alleviated systemic inflammatory reactions after E2 supplementation.

CONCLUSIONS

In our study, we found that the beneficial periosteal microcirculatory effects of local limb IPC vanished after OVX in rats. These observations suggest that in postmenopausal females during orthopedic-trauma interventions, the efficacy of limb IPC in preventing the inflammatory complications of tourniquet ischemia might be limited. This conclusion is strengthened by our findings which show that E2 supplementation reversed these changes by alleviating the local and systemic inflammatory reactions. Based on our previous and present findings in rats, some of the alleviating effects of E2 seen here might be independent of its effects on IPC and may be linked to those seen with periosteal ER-beta expression. The clinical significance of this finding, however, remains to be elucidated.

AUTHORS' CONTRIBUTIONS

The experiments, the analysis of microcirculation data, the interpretation of data and drafting were carried out by LP, AJ, DG, and JK. The PCR analysis was supervised by GT and the FACS analysis was supervised by IO. The research design, drafting, and critical revision of the manuscript were performed by MB, JP, and AS. All the authors have read and approved the final submitted manuscript.

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SUPPORTING INFORMATION

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Paper III.

Journal of Orthopaedic Surgery and Research

RESEARCH ARTICLE Open Access

Microcirculatory consequences of limb ischemia/reperfusion in ovariectomized rats treated with zoledronic acid

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Abstract

Background: Nitrogen-containing bisphosphonates (BIS) are potent therapeutics in osteoporosis, but their use may result in osteonecrotic side-effects in the maxillofacial region. Periosteal microcirculatory reactions may contribute to the development of bone-healing complications, particularly in osteoporotic bones, where ischemia–reperfusion (IR) events often develop during orthopaedic/trauma interventions. The effect of BIS on the inflammatory reactions of appendicular long bones has not yet been evaluated; thus, we aimed to examine the influence of chronic zoledronate (ZOL) administration on the periosteal microcirculatory consequences of hindlimb IR in osteopenic rats.

Materials and methods: Twelve-week-old female Sprague–Dawley rats were ovariectomized (OVX) or sham-operated, and ZOL (80 μg/kg iv, weekly) or a vehicle was administered for 8 weeks, 4 weeks after the operation. At the end of the pre-treatment protocols, 60-min limb ischemia was induced, followed by 180-min reperfusion. Leukocyte-endothelial interactions were quantitated in tibial periosteal postcapillary venules by intravital fluorescence videomicroscopy. CD11b expression of circulating polymorphonuclear leukocytes (PMN, flow cytometry) and plasma TNF-alpha levels (ELISA) were also determined. Two-way RM ANOVA followed by the Holm–Sidak and Dunn tests was used to assess differences within and between groups, respectively.

Results: Limb IR induced significant increases in PMN rolling and firm adhesion in sham-operated and OVX rats, which were exacerbated temporarily in the first 60 min of reperfusion by a ZOL treatment regimen. Postischemic TNF-alpha values showed a similar level of postischemic elevations in all groups, whereas CD11b expression only increased in rats not treated with ZOL.

Conclusions: The present data do not show substantial postischemic periosteal microcirculatory complications after chronic ZOL treatment either in sham-operated or OVX rats. The unaltered extent of limb IR-induced local periosteal microcirculatory reactions in the presence of reduced CD11b adhesion molecule expression on circulating PMNs, however, may be attributable to local endothelial injury/activation caused by ZOL.

Keywords: Bisphosphonate, Periosteum, Inflammation, Intravital microscopy, Leukocytes

Introduction

Osteoporosis affects more than 75 million people worldwide [1], with every other woman and every fifth man over 50 years suffering an osteoporotic fracture of the extremities during her or his remaining lifetime [2]. Ischemia–reperfusion (IR) often takes place in the affected tissue, e.g. due to use of a tourniquet or other temporary occlusive devices and techniques during trauma surgeries. In these cases, IR can lead to unwanted complications through the development of an antigen-independent inflammatory process, which involves the activation and adhesion of polymorphonuclear (PMN) leukocytes in the periosteal endothelium [3] and the upregulation of several danger-associated molecular pattern-related pathways in the locally affected and contralateral limbs as well [4]. Pro-inflammatory cytokines (e.g. TNF-alpha, IL-1 and IL-6) typically reach peak values after fracture operations [5],

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and it is suggested that these proinflammatory reactions may critically influence the process of bone regeneration.

Bisphosphonates (BISs) are potent therapeutic agents that ameliorate the osteoporosis-induced decrease of bone mineral density [2, 6]. Further, it has been shown that the risk of osteoporotic fractures can be reduced, in particular with the use of zoledronic acid (ZOL) [2, 7]. Nevertheless, BIS-induced unwanted, necrotic reactions may also be present in the skeletal system. Specifically, chronic BIS treatment can effectively enhance the incorporation of bone implants in appendicular bones [8, 9], but the likelihood of osteonecrotic complications also increases in parallel at the jaw bones [2, 10, 11]. The incidence of necrosis is especially high in the mandible after oral surgical interventions, leading to a condition termed medication-related osteonecrosis of the jaws. An exact pathogenesis of this complication is still unknown, but it seems to affect the appendicular and axial bones differently [12, 13]. Previously, we have also shown that BISs can induce significant inflammatory reactions in the mandibular periosteum after tooth extractions, while the microcirculation in the tibial region remained unaffected [14].

The aim of the present study was to examine the effects of chronic BIS treatment on the postischemic periosteal microcirculatory changes of the lower extremities. To our knowledge, the possible modulator role of BIS on the inflammatory reactions of the appendicular bones disposed to IR injuries has not yet been evaluated. Based on the relevant literature data and our previous results, our null hypothesis was that ZOL treatment does not influence the periosteal microcirculatory reactions of transient limb IR. We tested this hypothesis in a clinically relevant model of osteoporosis where anaesthetized rats were challenged with standardised limb IR in the presence or absence of chronic ZOL treatment.

Materials and methods

All studies were carried out on Sprague–Dawley rats housed in an environmentally controlled room with a 12-h light–dark cycle. The animals were kept on commercial rat chow (Charles River, Wilmington, MA, USA) and tap water ad libitum.

The project was approved by the National Scientific Ethics Committee on Animal Experimentation (National Competent Authority) under licence number V./144/2013. The study was performed in compliance with EU Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and the National Institutes of Health guidelines on the use of experimental animals. Animal welfare-related assessments and interventions were carried out prior to and during the experiments.

Experimental protocol

Ovariectomy

Ovariectomy (OVX) is a well-established animal model of osteoporosis sharing many similarities with the human condition including increased rate of bone turnover, relatively rapid bone loss and most importantly, similar skeletal responses to treatments used in humans (e.g. oestrogen, calcitonin and BISs) [15, 16]. In our study, 12-week-old female rats (weighing 180 to 200 g) were randomly allocated to ovariectomized ($N = 32$) or sham-operated ($N = 30$) groups under anaesthesia administered intraperitoneally by a combination of ketamine and xylazine (25 mg kg^{-1} and 75 mg kg^{-1}, respectively). As conditions were sterile, a median laparotomy was performed and the connection of the Fallopian tubes cut between haemostats. The ovaries were then removed and the stumps ligated with a 3-0 non-absorbable thread (Ethibond Excel®, Ethicon, Somerville, NJ, USA). Thereafter, the abdomen was filled with warm sterile physiological saline, and the abdominal wall was closed with a 4-0 absorbable suture and a 4-0 non-absorbable suture (Vicryl® and Prolene®, Ethicon, Somerville, NJ, USA) in two layers. Sham-operated animals underwent identical procedures, except of course that the Fallopian tubes and ovaries were not touched.

Experimental protocol, experimental groups

Five weeks after OVX (i.e. at 17 weeks of age) (see Fig. 1), a chronic zoledronate treatment was initiated in 16 animals (OVX + BIS group) with 14 of the sham-operated animals serving as negative controls (Sham + BIS group). ZOL (80 μg kg^{-1} Zometa®, Novartis Europharm, Budapest, Hungary) was administered once a week intravenously into the tail vein under light aether anaesthesia. The remaining OVX and sham-operated animals received physiological saline in the same volume (OVX + vehicle and sham + vehicle groups, $n = 16$ each). These weekly injections were continued for 4 weeks. At the end of the experimental protocol (in week 21), all of the animals were subjected to a 60-min complete hindlimb ischemia followed by a 180-min reperfusion period. Limb ischemia was induced by applying a tourniquet around the thigh and placing a miniclip on the femoral artery. The experiments were performed in two experimental series. In series 1, the periosteal microcirculation was examined using fluorescence intravital microscopy (IVM) at baseline and every 60 min during the 180-min reperfusion period ($n = 7–9$ per group). In the second experimental series, blood samples from the carotid artery were taken at baseline and during the reperfusion period to detect changes in the plasma concentrations of TNF-alpha and in the expression of the adhesion molecule CD11b ($n = 7$ in each group). It was necessary to separate the two series to avoid any interference

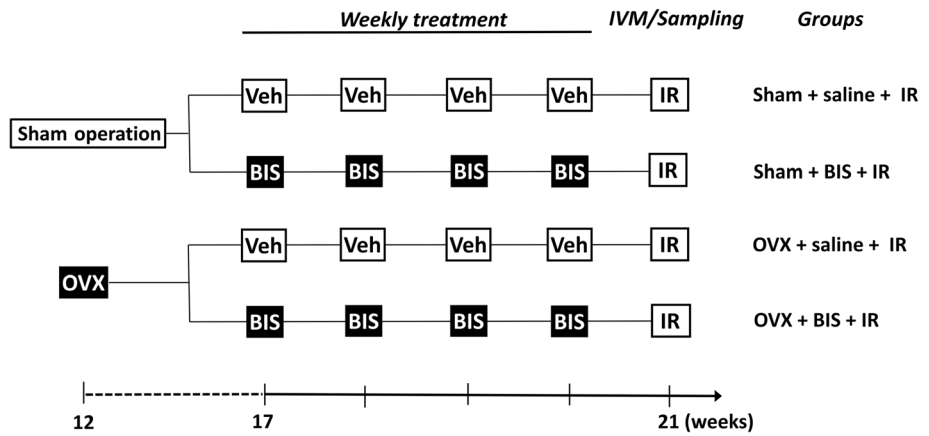

Fig. 1 Time sequence of interventions and treatments. Rats were sham-operated or ovariectomized (OVX) at the age of 12 weeks. Five weeks later (on week 17), chronic bisphosphonate (BIS, once a week in a dose of 80 μg kg^{-1} in the tail vein) or saline vehicle (Veh) treatment was initiated. At the end of the protocol (week 21), 60 min of limb ischemia followed was induced by 180 min reperfusion (IR), and periosteal microcirculatory measurements by fluorescence intravital microscopy (IVM; series 1, $n = 7–9$) were conducted as well as sampling for leukocyte adhesion molecule CD11b expression and serum TNF-alpha measurements (series 2, $n = 7$ each)

between the fluorescent dyes used for IVM and acquisition techniques used with flow cytometry.

Surgical procedure for intravital microscopic examination of the periosteal microcirculation

The animals were anaesthetized intraperitoneally with an initial dose of sodium pentobarbital (45 mg kg^{-1}). After tracheal intubation, one of the jugular veins was also cannulated to administer fluid and drugs (supplementary dose of sodium pentobarbital, 5 mg kg^{-1}). During the surgical procedures and investigation, the rats were placed in a supine position on a heating pad to maintain body temperature at 36–37 °C.

Under an operating microscope at × 4 magnification, the anteromedial surfaces of the tibial periosteum and the femoral artery on the same hindlimb were exposed with an atraumatic microsurgical technique [3]. The limbs were positioned horizontally on a special stage to expose periosteal vessels suitable for intravital fluorescence microscopy (IVM) in different phases of the experiment. At the end of the experiment, the animals were sacrificed with an overdose of sodium pentobarbital.

Intravital video microscopy

Leukocyte–endothelial cell interactions are decisive events among the complications of IR injury. After an initial low affinity interaction (i.e. rolling), a higher affinity binding (firm adhesion) takes place between the PMN and the endothelial surface. These dynamic cellular reactions can be detected in real time using IVM. In our model, the tibial periosteum was superfused with 37 °C saline, and the microcirculation was visualised by IVM (Zeiss Axiotech Vario 100HD microscope; Carl Zeiss GmbH, Jena, Germany, 100-W HBO mercury lamp, Acroplan 20x water immersion objective). Fluorescein isothiocyanate-labelled erythrocytes (0.2 ml intravenously; Sigma, St. Louis, MO, USA) were used to stain red blood cells, and rhodamine-6G (0.2%, 0.1 ml intravenously; Sigma, St. Louis, MO, USA) was used to label leukocytes [3]. The images from three to four fields of the tibial periosteum were recorded with a charge-coupled device video camera (Teli CS8320Bi, Toshiba Teli Corporation, Osaka, Japan), which is attached to an S-VHS video recorder (Panasonic AG-MD 830; Matsushita Electric Industrial Co., Tokyo, Japan) and a personal computer.

Video analysis

Quantitative evaluation of the microcirculatory parameters was performed offline by frame-to-frame analysis of the videotaped images (IVM; Pictron, Budapest, Hungary). Leukocyte–endothelial cell interactions were analysed in at least four postcapillary venules per rat. Rolling leukocytes were defined as cells moving with a velocity of less than 40% of that of the erythrocytes in the centreline of the microvessel passing through the observed vessel segment within 30 s and are given as the number of cells per millimetres per second. Adherent leukocytes were defined 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 square millimetre of endothelial surface, calculated from the diameter and length of the vessel segment [17].

Biochemical measurements

Immune labelling and flow cytometric analysis of CD11b expression of neutrophil leukocytes

Leukocyte–endothelial cell interactions are dependent upon simultaneously increased expression of adhesion

molecules on the surfaces of PMNs and those of the activated endothelia. The firm adhesion step is mediated by PMN-derived ß2 integrins (i.e. CD11a, b, c/CD18) which bind to various integrin receptors (intercellular adhesion molecules-1 and intercellular adhesion molecules-2 and vascular adhesion molecule-1) of the endothelium [18]. An increased expression of CD11b on PMNs in the systemic circulation can be detected after limb IR [3]. In the present study, these changes were determined from the peripheral blood by flow cytometry in duplicate samples. One hundred microlitres of whole blood was incubated with 20 μl of FITC-conjugated mouse anti-rat CD11b monoclonal antibody (BD Pharmingen, San Jose, CA, USA) for 20 min. Negative controls were obtained by omitting the antibody. The cells were then washed twice in Hanks' buffer and centrifuged for 5 min at 13,500 rpm, and the pellet was resuspended. The erythrocytes were lysed with a lysing kit (Biodesign, Saco, ME, USA), after which the cells were washed twice again (5 min, 6000 rpm) and resuspended in 750 μl of Hanks' buffer. 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. Generally, 10,000 events per sample were collected and recorded; the percentage of labelled (activated) granulocytes (relative to the overall marker-bearing cells) and the mean fluorescence intensity (average marker density) were calculated.

Detection of plasma level of TNF-alpha levels

The rest of the blood samples (0.5 ml) were centrifuged for 5 min at 4 °C at 13,500 rpm and then stored at 70 °C until assay. Plasma TNF-apha concentrations were determined in duplicate with a commercially available ELISA kit (Quantikine ultrasensitive ELISA kit for rat TNF-alpha; R&D Systems Inc., Minneapolis, MN, USA). The minimum detectable level was less than 5 pgml-1, and the inter-assay and intra-assay coefficients of variation were less than 10%.

Statistical analysis

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Corporation, San Rafael, CA, USA) using nonparametric methods. Two-way RM ANOVA followed by the Holm–Sidak and Dunn tests was used to assess differences within and between groups, respectively. Data are presented as mean value and SEM in all figures. P values < 0.05 were considered significant.

Results

Chronic ZOL treatment did not influence baseline values of leukocyte–endothelial interactions in the periosteal microcirculation (Fig. 2a, b). IR, however, induced significant increases in both PMN rolling and adhesion during the entire reperfusion period, and these changes reached a similar level in sham-operated and ovariectomized rats. BIS treatment caused a temporary increase in leukocyte rolling in OVX + IR animals and, similarly, an earlier rise in PMN adhesion in both sham + IR and OVX+IR animals at 60 min of reperfusion but did not influence PMN–endothelial interactions in later stages of reperfusion.

As compared to baseline, TNF-alpha values showed marked increases during the reperfusion period under examination (Fig. 3). No differences could be traced among the different experimental groups.

In comparison to baseline values, the amount of adhesion molecule CD11b on the PMN surface significantly increased in saline-treated sham-operated and OVX rats during reperfusion (Fig. 4a, b). In animals that received chronic BIS treatment, however, this elevation reached a significantly lower level.

Discussion

BISs are effective medications for bone metastases and osteoporosis and promising treatment modalities for complex regional pain syndrome upon fracture healing [19–21]. The use of ZOL has been shown to have a positive effect on spinal fusion [22] and to promote osseointegration and fixation of dental implants in autologous bone grafts in osteoporosis [23]. The periapical lesion-induced bone loss in the mandible was effectively ameliorated [24], and osseointegration of titanium implants in postmenopausal osteoporosis was promoted by ZOL [9]. Furthermore, ZOL brought about periosteal bone formation after tooth extraction in osteopenic sheep [25]. ZOL treatment, however, also induced reactive periosteal hypertrophy and even BIS-related osteonecrosis of the jaw in the same osteopenic sheep model [26]. Nevertheless, the effect of BIS on IR-induced local and systemic inflammatory reactions has not been examined elsewhere in an osteopenic model.

It is noteworthy that both anti- and proinflammatory effects have been attributed to different BIS compounds. The anti-inflammatory aspects of BISs include upregulation of the number of inflammatory monocytes [27], modulation of the proliferation and the viability and apoptosis of monocytes and macrophages [28, 29] and a downregulation of proinflammatory cytokines, such as TNF-alpha [30, 31], as well as other cytokines, such as IL-1, IL-6 and neurogenic growth factor [20]. Similarly, inhibitory effects of BIS against neurogenic inflammation have also been reported [20]. On the other hand, an acute phase response (< 3 days) was induced by different BISs including ZOL with increased TNF-alpha release in patients [32], but tissue accumulation of PMNs, increased TNF-alpha release and marked oxidative stress

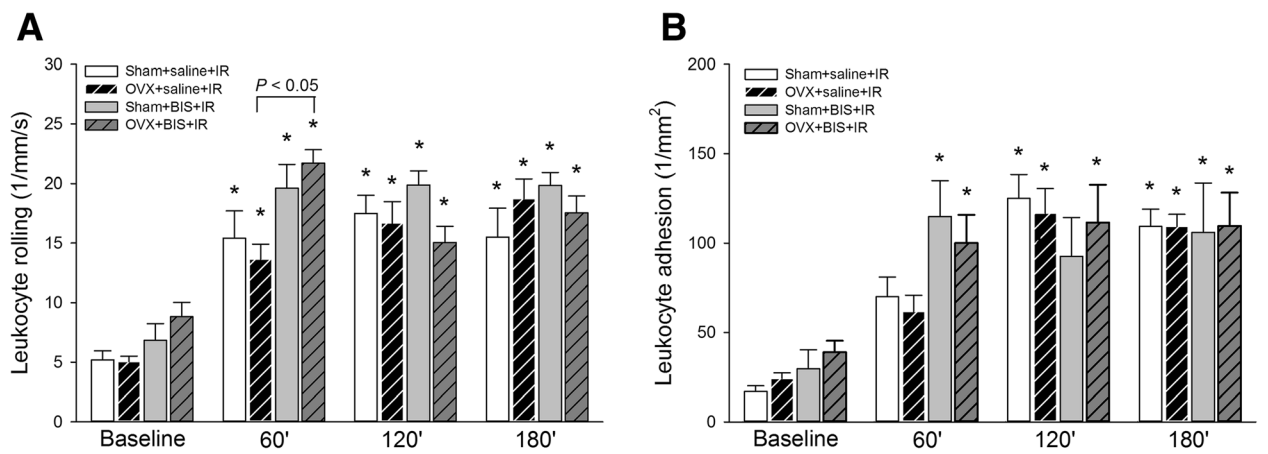

Fig. 2 Changes in leukocyte rolling (**a**) and adhesion (**b**) in the tibial periosteal postcapillary venules in response to 60 min of tourniquet ischemia (IR) followed 60, 120 and 180 min of reperfusion in sham-operated (sham) and ovariectomized (OVX) rats treated with bisphosphonate (BIS) or a saline vehicle. Here, data values are given as means ± SEM, and *$P < 0.05$ vs baseline. Two-way RM ANOVA was followed by the Holm–Sidak and Dunn post hoc tests

were also demonstrated in other tissues, such as the gingiva [33] and the liver [34] in animal models. Furthermore, priming of immunological reactions was also attributed to ZOL [35]. BISs cause ocular inflammatory complications in some clinical cases [36] and healing complications of the jawbones after invasive dental interventions even leading to osteonecrosis [10]. ZOL has been shown to aggravate kidney damage (by increasing cytokine production, metabolic acidosis and apoptosis) during IR injury in rats [37].

Enhanced leukocyte–endothelial interactions have been demonstrated after BIS treatment in an arthritis model in mice, but little is known on the ZOL-induced periosteal microcirculatory reactions [38]. Previously, we demonstrated that chronic BIS treatment induces some level of microcirculatory inflammation in the mandible, but such effects were not observed in the tibial periosteum [14]. Therefore, in this study, we tested the effect of chronic ZOL treatment in a tourniquet-induced limb ischemia model, where the role of PMN–endothelial interactions in the development of postischemic microcirculatory inflammatory reactions is well established. Here, we have shown that the reduced endogenous oestrogen levels evoked by OVX do not predispose to enhanced periosteal microcirculatory complications per se [39, 40], with the results also demonstrating that, apart from temporary exacerbation of PMN–endothelial interactions at the early stages of reperfusion, no major microcirculatory inflammatory risk could be detected after chronic ZOL treatment. Oestrogen withdrawal induces a release of TNF-alpha, which is involved in the pathomechanism of osteoporotic bone loss in women [41]; but here, we did not demonstrate between-group differences in TNF-alpha levels in the postischemic phase. Nevertheless, unlike humans, where increased serum TNF-alpha levels after OVX have been observed [42], we did not detect any differences between the baseline TNF-alpha levels between the different experimental groups. It should be noted that serum levels of TNF-alpha are rather low in rats and baseline values were close to the detection limit of the assay.

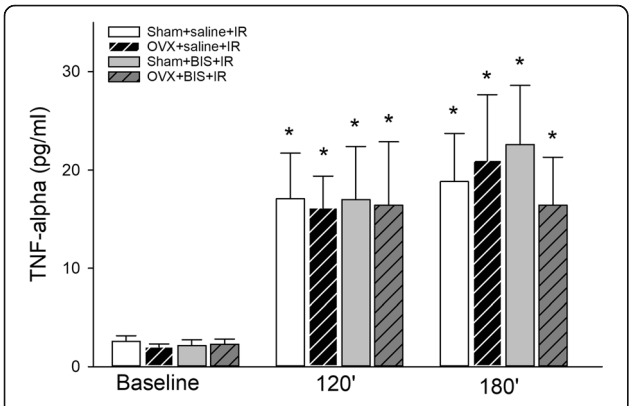

Fig. 3 TNF-alpha levels in plasma samples at baseline and in response to 60 min of limb ischemia followed by 120 and 180 min of reperfusion (IR) in sham-operated (sham) and ovariectomized (OVX) rats treated with bisphosphonate (BIS) or a saline vehicle. Here, data values are given as means ± SEM, and *$P < 0.05$ vs baseline. Two-way RM ANOVA was followed by the Holm–Sidak post hoc test

CD11b expression is a critical step for PMN adhesion to activated endothelial cells, and we detected a reduced IR-induced systemic PMN-derived CD11b expression after ZOL administration. BISs have been shown to influence PMN functions which manifested in impaired PMN chemotaxis and reactive oxygen species producing capacity in vivo [43] and reduced myeloperoxidase and NADPH oxidase activities in vitro [44, 45]. The inhibitory

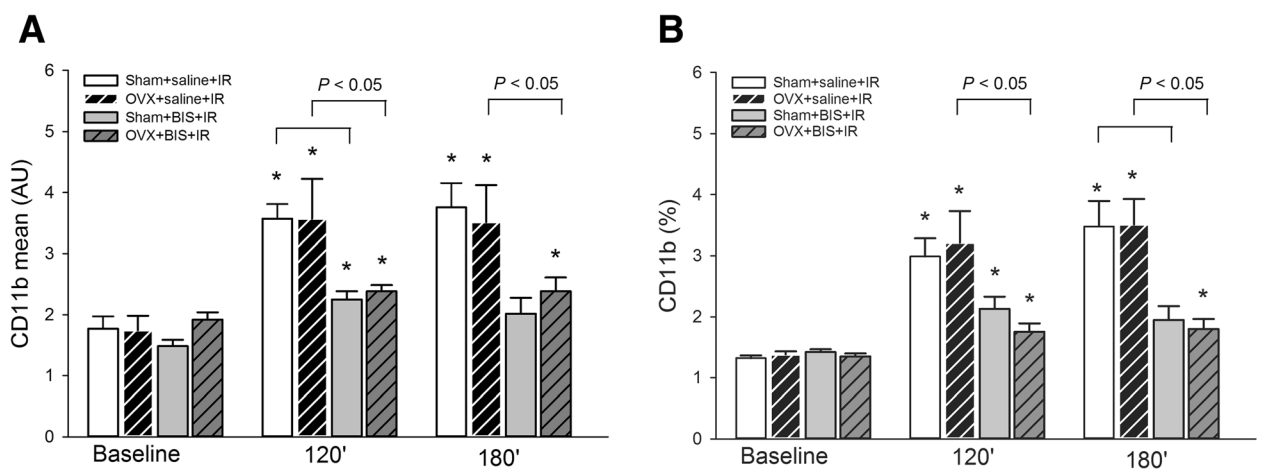

Fig. 4 Changes in expression of the CD11b adhesion molecule on the surface of polymorphonuclear leukocytes (PMNs) expressed as mean fluorescence intensity values (**a**) and percentage of positive cells of the immune-labelled cells (**b**) within the gated PMN population. Values are shown at baseline and at 120 and 180 min of reperfusion after 60 min of limb ischemia in sham-operated (sham) and ovariectomized (OVX) rats treated with bisphosphonate (BIS) or a saline vehicle. Here, data values are given as means ± SEM, and $*P < 0.05$ vs baseline. Two-way RM ANOVA was followed by the Holm–Sidak and Dunn post hoc tests

effect of BIS was also demonstrated in other immune cells such as macrophages [30]. In our study, ZOL reduced CD11b expression on the surface of circulating PMNs but did not influence the overall adhesion of PMNs in the periosteal postcapillary venules. This finding can only be explained by some degree of ZOL-induced endothelial activation and secondary endothelium-derived adhesion molecule expression. This possible ZOL-induced endothelial upregulation of adhesion molecules (the endothelial counterparts of CD11b) which might be responsible for the present results should be further investigated.

Among other effects, BISs are known to inhibit vascular endothelial proliferation and to upregulate cellular apoptosis [46]. Furthermore, BISs (alendronate) have also been shown to inhibit nitric oxide synthase expression, which is an important endogenous modulator of PMN–endothelial interactions [47]. These ZOL-induced acute postischemic reactions affecting the endothelium may also warrant further in-depth investigations.

In summary, BIS treatment exerted only a minor influence on limb IR-induced PMN rolling and adhesion in the periosteum, and the PMN-derived adhesion molecule (CD11b) expression on circulating PMNs was even reduced. Further, no effect on postischemic TNF-alpha release was demonstrated in ZOL-treated rats. These results suggest that although some level of local endothelial activation might be attributable to the treatment, chronic ZOL administration does not have a major influence on the risk of postischemic inflammatory microcirculatory complications in the tibial periosteum.

Abbreviations

BIS: Bisphosphonate; IR: Ischemia–reperfusion; IVM: Fluorescence intravital microscopy; OVX: Ovariectomy; PMN: Polymorphonuclear leukocytes; ZOL: Zoledronic acid

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LP and AJ performed the experiments and prepared the manuscript, IO performed the flow cytometry measurements, JK guided the ELISA measurements and JP and AS planned the experiments and corrected and proofread the manuscript. All the authors have read and approved the final manuscript. LP and AJ contributed equally to the study.

Ethics approval and consent to participate

The project was approved by the National Scientific Ethics Committee on Animal Experimentation (National Competent Authority) under licence number V./144/2013.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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