Microvascular reactions of normal and osteoporotic bones in response to surgical interventions

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II. Hartmann P., **Greksa F**., Garab D., Varga R., Széll M., Keresztes M., Boros M., Szabó A.: Microcirculatory consequences of osteoporosis. Timisoara Medical Journal. 2010; 60:100-106.

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Abstract relating to the subject of the thesis

I. **Greksa F.,** Szabó A., Wellinger K., Sohár G., Boros M., Tóth K. The effects of endomedullar implants on the periosteal vessel structures of the rat tibia. Osteoarthritis and Cartilage. 2009; 17:(S1) S93

List of abbreviations

IVM	intravital videomicroscopy
OPS	orthogonal polarization spectral imaging
OVX	ovariectomy
E2	17ß-estradiol
AD-SoS	amplitude-dependent speed of sound

Overview of the studies

The main purpose of our studies was to investigate the unexplored roles of the periosteum in bone biology, and to expand our knowledge on microvascular connections of bone pathologies such as osteoporosis or incomplete osseointegration after intramedullary implantations. The periosteum plays significant roles in the pathomechanisms of disorders affecting the bone metabolism, but the consequences of osteoporosis on the vascular organization of the periosteum have never been investigated. Hence, our primary aim was to determine the *in vivo* periosteal microvascular responses to oestrogen deprivation in a clinically relevant, long-term experimental osteoporosis study. Another aim was to examine the compensatory reorganization potential of the periosteal vascular network to stimuli leading to the integrity and regeneration of the endosteal vascular compartment. We wanted to clarify whether destruction of the endosteal microcirculation modifies the vascular characteristics of the periosteum after standardized intramedullary damage. To this end, we designed a chronic rat model of tibial reaming and nailing in order to study the periosteal microvascular consequences of a simulated orthopaedic operation by intravital videomicroscopy. We also hypothesized that the intramedullary healing conditions would significantly influence the outcome of reactive periosteal microcirculatory changes. For this reason, we used biomaterials with different osseointegration properties generally used in clinical practice, so as to compare and characterize the periosteal microvascular alterations caused by prosthesis materials expected to display higher or lower intramedullary stability. Specifically, titanium was chosen as it induces nearly complete osseointegration and relatively fast remodelling of the surrounding bone, and we hypothesized that the in-growth of vascular elements and regeneration of the endosteal vessels would be different after the use of polyethylene.

As a further aim, we set out to observe and characterize the *in vivo* human periosteal and endosteal microcirculatory reactions, as our most recent study was therefore designed to examine the microcirculatory disturbances of the periosteum and the endosteum simultaneously in a patient with pseudoarthrosis induced by long-term fixation. The tibial periosteal and endosteal circulatory changes were detected by laser-Doppler flowmetry in the clinical setting, in order to assess the concomitant microcirculatory perfusion deficit during surgical removal of the osteosynthesis materials.

1. INTRODUCTION

1.1. Blood supply of long bones

Constant circulation is essential for bone growth, mineral exchange and healing. The arterial supply is usually provided by three major sources (Johnson EO 2004; Findlay DM 2007). **1.** Nutrient arteries enter the bone at the proximal- and middle-third of the diaphysis. Intramedullary arteries divide into longitudinal branches running parallel with the axis and finally send branches supplying the endosteum. Their branches form anastomoses with metaphyseal arteries. These anastomoses supply the network of sinusoids, and branches enter the cortical bone. They have poor connections with periosteal vessels. The blood supply of long bones is therefore basically centrifugal in nature. **2.** Epiphyseal and metaphyseal arteries enter the bone from the periarticular anastomosis network supplying both ends of the bone. They form anastomoses with branches of the nutritive artery. These anastomoses are not of functional importance unless the diaphyseal circulation is interrupted. **3.** Periosteal arteries enter the periosteum at the attachments of the intermuscular septa and penetrate the bones in the same regions. The blood vessels of the periosteum run in a crosswise manner, supplying the superficial layer of cortical bone and usually not entering the deeper layers.

The venous drainage of bones is carried out by a well-defined, large vein, collecting blood from the intramedullary space and venous sinuses. Actually, a number of small veins in the periosteum collect blood from venules of the periosteum and the superficial layer of the cortex.

As mentioned above, the connection between the periosteal and endosteal circulations under normal circumstances is relatively poor (Danckwardt - Lilliestrom G 1970; Rhinelander FW 1987; ElMaraghy AW 1999; Mueller CA 2009). It is likely that structural regeneration of the periosteum can be initiated from epi- and metaphyseal vessels if the endosteal circulation is greatly interrupted, but the consequences of iatrogenic destruction of either vascular system on their reorganization potential has not yet been described.

1.2. Examination of the microcirculatory organization of the bone

Various static methods, e.g. microangiography (Trueta J 1955; Rhinelander FW 1968) or electron microscopy, have been employed to study the structural characteristics of

the bone microcirculation, while laser-Doppler flowmetry (Swiontkowsky MF 1986; ElMaraghy AW 1999) and radioactive microsphere techniques (Barron SE 1977)have been utilized to characterize functional details and dynamic aspects of the blood supply. Most of these techniques, however, have inherent limitations. The loading of capillaries during conventional microangiography can be incomplete, which limits the recognition of the periosteal vessel structures (Rhinelander FW 1968; Pazzaglia UE 1996). Electron microscopy or light microscopy can be used for qualitative studies, but quantitative analysis after serial sectioning of the tissues is particularly difficult. Laser-Doppler flowmetry is a semiquantitative method with which to monitor the perfusion characteristics at the surface of a tissue mass, including the bone cortex (Kowalski MJ 1996; Koo H 2010). The advantage of this method is that it does not require fluorescence labelling and the small sensors may fit into cavities, which is particularly advantageous if the measurements are targeting the endosteal vasculature.

An outstanding tool that provides information on dynamic changes in the perfusion conditions in a physiological context is intravital microscopy (IVM) imaging with or without contrast enhancement. Similarly to laser-Doppler flowmetry, IVM provides information on the surface of solid organs, but also allows the detection of spatial and temporal relationships of circulating cells and the acquisition of additional parameters, such as the cell velocity (Menger MD 1997; Ruecker M 1998; Wolfárd A 2002). Conventional fluorescence IVM yields visual information on structures after labelling with fluorescent markers (commonly used fluorochromes are rhodamine 6G and fluorescein isothiocyanate), while other, non-invasive, non-labelling-requiring techniques, such as orthogonal polarization spectral (OPS) imaging makes use of polarized reflected light to visualize haemoglobin-containing structures in vessels. After microsurgical exposure of the periosteum, IVM furnishes an opportunity for simultaneous visualization of the microvascular function and also allows computer-based quantification of morphological and functional characteristics.

1.3. Structural and functional characteristics of the periosteum and the endosteum

The periosteal membrane separates the bone from the surrounding tissues and binds elements of the skeletal system to it by means of tendons, septa and ligaments. The periosteum plays an active role in the regulation of the metabolism and regeneration of the bone. From a structural point of view, the periosteum is composed of an outer fibrous and an inner osteogenic cellular layer covering the whole length of the long bones up to the

articular cartilages (Dwek JR 2010).

The outer layer is composed of two distinct parts. The superficial sheet is less elastic and involves collagen fibres with some fibroblasts (Squier CA 1990). This most vascularized part of the periosteum supplies the deeper layers and the outer surface of the cortex with a distinct proprioceptive role owing to its dense innervations. The deeper part of the outer layer contains elastic fibres, and thus it has considerable elasticity, and it is poor in vessels and cellular components. Tendons of adhering muscles undergo branching in this layer.

The inner layer, also called the cambium layer, contains many mesenchymal stem cells, osteoblasts and fibroblasts in a loose collagen matrix. Osteoblasts are located directly on the outer surface of the cortex covered by fibroblasts. The cambium layer is thickest during foetal life and becomes thinner with the progression of age (the numbers of vessels and cellular components decrease) with a resultant decrease in osteogenic ability. Mechanical injuries of the periosteum, such as fractures, temporarily stimulate the osteogenic activity, but the efficacy of this process is reduced with age (Simon TM 2003; Srinivasan S 2003).

The endosteum is a soft and thin connective tissue that coats the inner surface of the long bones (Herget GW 2011). Its histological structure is similar to that of the periosteum, but it contains only a few connective fibres and nerves. It exhibits rich vascularization; branches of the nutritive artery run inside the endosteum before entering the cortical bone. Veins from the Volkmann channels are also connected to intramedullary vessels within the endosteum. Similarly to the periosteum (albeit to a lesser extent), the endosteum additionally hosts mesenchymal stem cells, which play roles during different regeneration processes of the bone (Brighton CT 1992). Due to the osteogenic stem cell content, these membranes, and in particular the periosteum, play important roles in the pathogenesis of both hormone-related and trauma-induced osteoporotic processes.

1.4. The roles of the periosteum and endosteum in the healing process of the bone after fractures

Successful bone healing requires the regeneration of both the peri- and endosteal circulations, the fixation of fractured bone endings and tissue immobilization (Macnab I 1974). The process starts with periosteal callus formation and the development of a new anastomosis network between the nutritive artery and the meta-epiphyseal vessels. In the first phase, the intramedullary space within the fracture gap under the elevated periosteum

is filled with haematoma. Fibrin bridges are formed between the fracture endings from the haematoma, mesenchymal stem cells migrate and newly-formed capillaries grow into the callus (Cruess RL 1975) from the periosteum at the edge of the fracture gap (Mindell ER 1971). Newly-formed woven bones appear in the granulation tissue in the callus bridging the fracture gap within 1-2 weeks (Einhorn TA 1998). Mesenchymal stem cells transform into chondrocytes which produce hyaline cartilage. The lower stability of fracture ends results in more intensive cartilage formation and proliferation. Enchondral ossification is the last step of the reparative phase, during which hyaline cartilage is replaced with lamellar bone. In the final, remodelling phase, primarily formed woven bone and trabecular bone are substituted with mature, compact bone.

In cases of non-dislocating fractures, the structure of the endomedullary vessels remains intact (Rhinelander FW 1962), but dislocating fractures are usually associated with destruction of the endosteal circulation. This is due to the longitudinal architecture of the endosteal vessels; periosteal blood vessels are relatively preserved because of their transverse segmental pattern (Macnab I 1974). The endosteum also plays an important role in the healing of bones, but it has been shown by means of microangiography that endosteal callus formation begins only 3-4 weeks after the fracture (Rhinelander FW 1968).

1.5. Consequences of gross destruction of the periosteum and endosteum

Clinical experience suggests that adequate healing requires a sufficient blood supply and appropriate, stable mechanical fixation of the broken ends. The extent of mechanical stability has an effect on tissue differentiation and the development of revascularization; instability results in delayed revascularization, thereby restraining or inhibiting healing.

The role of the periosteum and the importance of its preserved circulation in bone healing were first emphasized by Erich Lexer in 1925 (Lexer E 1925). Since that date, it has been well established that re-establishment of the integrity of the periosteum is critical for the healing process (Mindell ER 1971; Finley JM 1978; Dwek JR 2010). Major destruction of the periosteum results in the necrosis of fracture endings, delayed union or non-union due to ischaemic injury of the bone. Clinical observations support the notion that fractures with extensive soft tissue injury or/and periosteal damage result in delayed union or pseudoarthrosis formation (Gustillo RB 1990; Esterhai JL 1991; Utvag SE 1998).

As a result of fracture reconstruction surgery, the regeneration from the neighbouring periosteum can also be compromised due to the fixation plate itself. With certain osteosynthesis techniques, deterioration of the blood supply can lead to a nutritive

deficit and alterations in bone physiology, ranging from metabolic changes to pseudoarthrosis formation. As such, plate osteosynthesis has been shown to cause disruption of the periosteal blood supply, which leads to bone porosis persisting for several months after surgery (Perren SM 1988). In contrast with previous theories where unloading of the bone was the suspected cause of this form of osteoporosis, nutritive factors and avascular bone necrosis beneath the disrupted periosteal membrane have been found behind this phenomenon (Klaue K 2000). With regard to the importance of the periosteal integrity, careful periosteal stripping which results in relatively low alterations in blood supply is a widely accepted method for plate insertion (Whiteside LA 1978).

The integrity of the endosteal membrane is also affected during fractures or surgical interventions. Reamed intramedullary nailing has been shown to lead to cessation of the endosteal blood supply, and the insertion of the femoral component fitting tightly into the medullary cavity during hip joint endoprosthetic surgeries causes a major endosteal circulatory insufficiency. As a result of the endosteal circulation disturbances, the inner two-third of the cortical bone may be necrotized in the diaphysis of the long bones, where the endosteal vessels play the dominant roles in the blood supply (Rhinelander FW 1968; Barron SE 1977; Pazzaglia UE 1996; 1999; Mueller CA 2009).

Both experimental and clinical observations suggest that disruption of the continuity of the periosteum or the endosteum delays bone healing, but impairment of only one of these structures alone does not hinder overall fracture healing (Kregor PJ 1995; Seibold R 1995). The dependence of the healing capacity of the cortex after osteosynthesis upon the relative contributions of the endosteal and periosteal systems in supplying the cortex has been demonstrated in animal models (Lippuner K 1992), but little is known about the human situation and circulatory characteristics.

1.6. The possible role of the periosteum in osteoporosis

Osteoporosis is a progressive disease of bone loss associated with an increased risk of fractures. The structural bone deficit is generally attributed to enhanced bone resorption and simultaneously decreased bone formation. Several early studies provided convincing evidence that these changes can be suppressed or even prevented by restitution of the pre-menopausal oestrogen levels in females (Christiansen C 1982). The effector cells of these processes originate from mesenchymal osteoprogenitor cells found in the periosteum and endosteum, with the predominant role of the periosteum (Brighton CT 1992). Furthermore, the experimental and clinical evidence points to an important function for the periosteum

not only in the pathogenesis of osteoporosis, but also in the effects of different anti-osteoporotic approaches (Allen MR 2004). The periosteal region of the cortical bones exhibits a better anti-fracture efficacy than that of the endosteal region, and it is proposed that the major target of different bone-reinforcing therapies is at the periosteal site (Ferretti JL 1995).

Since the effector cells of bone remodelling originate mainly from the periosteum, this structure plays a decisive role in the pathogenesis of osteoporosis too. Moreover a considerable amount of clinical evidence suggests that the disrupted circulation of the periosteum leads to osteoporotic changes in the cortex. The consequences of senile osteoporosis on the periosteal vascular organization, however, have never been investigated.

1.7. Consequences of gross destruction of the endosteum

Fracture reduction and osteosynthesis techniques applied for the fixation of osteotomies, plate osteosynthesis and intramedullary nailing, may cause circulatory disturbances by damaging the periosteum or the endosteum (Barron SE 1977; Pazzaglia UE 1996; Mueller CA 2009). Selective disruption of the endosteum is caused by non-dislocating fractures and surgical interventions such as reaming of the medullary cavity. Reamed intramedullary nailing is a common procedure for the management of diaphyseal fractures. After reaming of the medullar cavity, the intramedullary vessel network is completely destroyed. A number of authors have described that the periosteal vessel structure is not capable of supplying the whole cortex after complete disruption of the endomedullary vessel network (Trueta J 1955; Macnab I 1958; Gustillo RB 1964; Danckwardt-Lillienström G 1970). According to other authors, the inner two-thirds of the cortex becomes avascular, and living cellular components necrotize as a result of ischaemic injury. The inorganic, calcareous structure of the bone, however, remains intact, apart from the drilling zone (Rhinelander FW 1968; Pazzaglia UE 1996; Mueller CA 2009). Necrosis is thought to be caused by the mechanical destruction of the nutritive endosteal vessels. Further damage is caused by the increased intramedullary pressure through blocking of the blood flow in the intact vessels (ElMaraghy AW 1999) and obstruction of the perforating arteries by fat embolism (Danckwardt-Lilliestrom G 1970; Barron SE 1977; Pazzaglia UE 1996; ElMaraghy AW 1999). These observations clearly indicate that the inner part of the cortical bone is mainly supplied by the endomedullary network.

Microangiographic studies of non-dislocated fractures have revealed that the

periosteal blood supply predominates during the early phase of fracture healing, which is later overtaken by the endosteal blood supply (Rhinelander FW 1968, 1979, 1987). There was an enhanced periosteal blood flow in the first 2 weeks after the reaming of long bones when no subsequent implantation was performed (Reichert IL 1995). This observation was supported by further data demonstrating neovascularization and an increase in periosteal blood flow lasting for only 10-14 days after surgery.

After reaming, the marrow space is filled with haematoma for 20-30 days, this being gradually followed by the accumulation of reactive newly-formed bone and loose connective tissue. Thus, the endosteal revascularization is initiated relatively late (60 days after reaming) from the metaphysis by the proliferation of metaphyseal and epiphyseal arteries. Reactive endosteal new bone formation and its gradual remodelling have been observed for 30-60 days following reaming (Pazzaglia UE 1996), an observation supported by the findings that the reamed medullary space is avascular for 30 to 60 days (Gustilo RB 1964; Danckwardt-Lilliestrom G 1970). Taken together, endosteal revascularization takes place relatively late, and is initiated from the metaphyses via the proliferation of epi-and metaphyseal vessels; this process occurring with a delay of 60-80 days. Periosteal vessels are not involved, or may have only a minor role in the process of regeneration of the endosteal circulation (Rhinelander FW 1968; Pazzaglia UE 1996; ElMaraghy AW 1999).

1.8. Effects of endomedullary implants on the bone circulation after drilling

The regeneration of the endosteum after medullary drilling and fracture fixation is a time-consuming process and depends greatly on the properties of the implant material. The major characteristics are the flexibility and intramedullary stability of the implant, the shaping of the surface, and the size of the interface between the bone and the implant. It has been shown that excessive drilling (without subsequent implantation) can significantly decrease the circulation of the diaphysis (Kline AJ 2006). When the drilled medullary cavity was completely filled with agar, 90% of the cortex underwent necrosis without any considerable degree of revascularization later (Mital M 1968). When drilling was followed by the application of bone cement with high pressure into the canine femur, marked decreases in the diaphyseal blood flow were observed by laser-Doppler flowmetry 6 weeks later (ElMaraghy AW 1999). Stable titanium implants placed firmly into the medullary cavity are incorporated by the bone with regular endomedullary vascularization around them. In contrast, granulation tissue with pathological vessel structures is formed around loose implants (Pazzaglia UE 1996). Clinical observations also reveal that a regular

endosteal circulation develops around intramedullary incorporated cemented or non-cemented hip prostheses. In contrast, loose implants induce the formation of granulation tissue with a pathological vessel structure.

1.9. Characteristics of implant materials applied in endoprostheses

Implant materials used in large joint prosthethetic reconstruction surgeries can be categorized according to their biological properties. Tolerable materials are steel alloys, cobalt-based alloys and plastics. Inert materials include titanium, aluminium oxide, ceramics, carbon compounds and tantalum, and bioactive substances include hydroxyl-apatite, bioglass and tricalcium phosphate.

1.9.1. Titanium and its alloys

Titanium, a bioinert metal, is one of the strongest materials in nature (Bjursten LM 1990; Hopp M 1996). It is widely used in implantology in orthopaedics, dentistry and traumatology (Joob-Fancsaly A 2001). It has a high affinity for bone tissue (Branemark PI 1977). The mechanical properties of titanium can be improved by alloying, but the pure metal is more resistant to corrosion and has a better bone integration capacity (Johansson CB 1998). In orthopaedics, the most frequent alloy (Ti Al6 V4; ISO 5832) contains 6% of aluminium and 4% of vanadium. Titanium has low density (4.51 kg dm^{-3}) and its good mechanical properties allow the production of precisely prepared implants. Titanium belongs in the group of transition metals, representing an intermediate state between noble and non-noble metals. On the surface of transition metals including titanium, a chemically stable oxide layer develops contributing to their excellent chemical and biological properties. This intermediate state provides extreme reactivity and good surface properties for titanium. It has a negatively charged surface and high surface tension, which provokes the bioadhesion of different molecules (Baier RE 1984). The process begins with hydration of the surface, water molecules, different ions and glycosaminoglycans adhering to the surface. Serum proteins adhere to this surface creating a 20-50 Å thick layer attached to the bone cells through collagen fibres. As a result of the above molecular interactions, a thin layer is formed between the implant and the bone cells; this is the interface zone. Bone integration develops faster on increased and porous surfaces that allow the ingrowth of bone tissue (Hulbert SF 1972). Titanium was used as an implant material for nailing in our studies as a model of excellent osseointegration and good stability (Pazzaglia UE 1998).

1.9.2. Polyethylene

The component of bipolar prosthesis that makes contact with the metal component (the acetabular component of a hip joint and the tibial component of a knee joint prosthesis) is polyethylene. Polyethylene belongs among the biotolerant materials and has been used in joint endoprosthetics for more than 40 years. During the polymerization of ethylene, depending on the method applied, a linear (low-pressure method) or a more or less branching (high-pressure process) polymer chain is formed. The density of flexible polyethylene, made by the high pressure process is 0.915 to 0.93 g cm^{-3} while that of the rigid polyethylene, made by low-pressure process, ranges from 0.94 to 0.965 g cm^{-3}. The properties of polyethylenes are largely determined by the material density, molecular weight and weight distribution within the molecule. In line with increased molecular weight, the impact resistance, tensile strength, toughness, wear resistance and resistance to burst formation are also increased. The Ultra High Molecular Weight Polyethylene used in endoprosthetics displayes a high level of polymerization and a high material density (ISO 5834 and ISO 7206-2). These polymers are firm and strong, with high wear resistance with favourable sliding and mechanical force-relieving properties. They are manufactured by milling and cutting and fixed to the bone by cement. A polyethylene oxide layer is formed at the free surface of implanted prosthesis where water, adhering prototeoglycans and fibrin fibres create a superficial biofilm. The material composition, structure, elasticity and surface of polyethylene do not allow osseointegration and we used this material as a model implant with weak stability.

2. MAIN GOALS

1. Our primary objective was to observe and characterize the consequences of osteoporosis on the microvascular organization of the periosteum. With this aim, we designed a bilateral ovariectomy model of osteoporosis in a clinically relevant time frame in rats. We also determined whether chronic treatment with oestrogen influences the possible ovariectomy-triggered periosteal changes in the structure of the microvasculature.

2. Fractures of long bones can cause destruction of the periosteal and endosteal circulations which will regenerate only if the healing process is ensured by appropriate fracture management. The reorganization potential of the periosteal and endosteal circulatory networks during iatrogenic destruction of these vascular systems has not yet been described. We hypothesized that compensatory microvascular reactions are induced in the periosteum in response to mechanical destruction of the endosteal microcirculation. Thus, our aim was to characterize the periosteal microcirculatory organization after standardized intramedullary damage in rats. For this purpose, we developed a chronic rat model of tibial reaming and nailing to study the periosteal microvascular consequences by means of IVM.

3. We hypothesized that the intramedullary healing conditions would significantly influence the outcome of reactive periosteal microcirculatory changes. For this reason, we used implant materials with different osseointegration properties. We compared and characterized the periosteal microvascular alterations caused by titanium and polyethylene nails, as prosthesis materials expected to display higher or lower intramedullary stability, respectively, 6 and 12 weeks after their implantation into the rat tibia.

4. Finally, we set out to examine the possible microcirculatory background of pseudoarthrosis in a clinical situation by using laser-Doppler flowmetry. We studied the clinical history of a patient who presented with tibial malunion, where simultaneous iatrogenic impairment of the periosteal and endomedullary circulations of the diaphysis was evoked by aggressive osteosynthesis techniques.

3. MATERIALS AND METHODS

The animal experiments were performed in accordance with the NIH Guidelines (Guide for the Care and Use of Laboratory Animals) and the study was approved by the Animal Welfare Committee of the University of Szeged.

3.1. Study I

In study I, the osteroporosis-related periosteal vascular reactions were characterized in rats subjected to bilateral overiectomy (OVX), and the development of osteopenia was assessed by using ultrasonographic densitometry. Five months after OVX, when signs of osteopenia could be demonstrated, chronic oestrogen treatment was initiated in a group of animals. Eleven months after OVX, periosteal microcirculatory reactions were assessed by using fluorescence IVM.

Surgical method of bilateral ovariectomy

In these studies, 3-month-old Wistar rats (weighing 200-210 g) were ovariectomized (n = 12) under anaesthesia with a combination of ketamine and xylazine (25 mg kg^{-1} and 75 mg kg^{-1} ip, respectively). Median laparotomy was performed under sterile conditions, the connection of the Fallopian tubes was cut between haemostats, the ovaries were removed, and the stumps were then ligated. Thereafter, the abdomen was filled with warm sterile physiological saline and the abdominal wall was closed in two layers. Sham-operated animals underwent identical procedures except that the Fallopian tubes and ovaries remained intact (Sham, n = 6).

Quantitative ultrasonographic bone densitometry

Under ketamine-xylazine anaesthesia (as above), bone density measurements were performed at the tibia and the tail with a DBM-Sonic 1200 (IGEA, Carpi, Italy) ultrasonographic bone densitometry device; the changes in the average of the amplitude-dependent speed of sound (AD-SoS) were determined (Joly J 1999). After calibration, the AD-SoS values of the soft tissues (muscle and skin above the thigh) were determined, and the system deducted this value from the bone density. The AD-SoS values were calculated via a computer program; the average of 5 measurements was used at each time point. Twenty-one weeks after OVX, statistically significant density alterations were observed in the proximal tibia, and this location was therefore used for further quantitative ultrasonographic (QUS) measurements at the ages of 8, 9, 12 and 14 months.

Experimental groups

Five months after OVX (at the age of 8 months), chronic oestrogen therapy (Sims N 1996) was initiated 5 days per week with 20 μg kg^{-1} sc 17ß-estradiol (E2; Sigma, St. Louis, MO, USA) in 6 animals of the ovariectomized group (OVX + E2, n = 6). The E2 substitution was continued weekly until the end of the experiments. The remaining OVX rats (OVX, n = 6) and the Sham animals received the vehicle for E2 (100% ethanol diluted in corn oil) in the same volume. Eleven months after OVX, the microcirculatory consequences of OVX and chronic E2 supplementation were investigated in the Sham, OVX and OVX + E2 animals by using IVM. The body weight changes and the development of osteoporosis were followed continuously in the proximal tibiae by means of ultrasonographic densitometry.

Examination of periosteal vessel structures by using fluorescence IVM

Intravital analysis of the periosteal microcirculation was performed at the end of the experimental protocol on 14-month-old animals. Anaesthesia was induced with sodium pentobarbital (45 mg kg^{-1} ip) and sustained with small supplementary iv 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 the body temperature between 36 and 37 ^{o}C. 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 dissected free, and the periosteum of the medial surface of the right tibia was exposed under a Zeiss 6x magnification operating microscope, using an atraumatic surgical technique (Varga R 2008). The microcirculation of the proximal tibia was visualized by IVM (Zeiss Axiotech Vario 100HD microscope, 100 W HBO mercury lamp, Acroplan 20x water immersion objective, Carl Zeiss GmbH, Jena, Germany), using fluorescein isothiocyanate (Sigma, St. Louis, MO, USA)-labelled erythrocytes (0.2 ml iv) for red blood cell staining.

Data analysis for IVM

Quantitative assessment of the microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images, using image analysis software (IVM, Pictron Ltd., Budapest, Hungary). At the examined anatomical fields of the tibia, the majority of the vessels were not capillaries, but venules; hence the total vascular density was calculated from the length of recognized vessels divided by the corresponding area (an average of 3 measurements per observation field was used).

3.2. Study II

In Study II, the microvascular reactions induced in the periosteum by mechanical destruction of the endosteal microcirculation and implantation with different implant materials were examined by using IVM with the OPS technique. The effect of drilling was examined 6 and 12 months after surgery (Series 1), while the effects of titanium and polyethylene implants on the periosteal density were examined after 12 weeks (Series 2). In the longer study, the implant stability and the endosteal histological reactions were also assessed.

Surgical method of reaming and nailing

Male Wistar rats (380±40 g) were anaesthetized with sodium pentobarbital (45 mg kg^{-1} ip). After a skin incision, both the left and right tibiae were reamed with a series of specially designed microdrills of increasing diameter after making a small drill hole over their proximal metaphyses.The diameter of the flexible drill ranged between 0.2 and 0.8 mm. During this procedure, the endosteum and the inner part of the cortical bone were destroyed by reaching the required drill diameter, and the skin incision was closed. The animals were not immobilized after this procedure. In some animals, the tibiae were drilled and a 13 mm long titanium or polyethylene nail with a diameter of 0.88 mm (Protetim Kft., Hódmezővásárhely, Hungary) was implanted into the right tibia. The left tibia was reamed, but not implanted. The optimum dimensions of the implants were chosen on the bases of a pilot study, where radiographs of tibiae were taken from rats of similar weight and age. We also used a special small impactor to ensure that the titanium nail fitted tightly in the medullar cavity.

Experimental series and groups

The experiments were performed in two series. In series 1, the rats were randomly allotted into two groups. The animals in the first group were sham-operated (n = 10); in the second, drilled group (n = 10), both the left and right tibiae were reamed. The periosteal vessel structure was examined at postoperative weeks 6 or 12 (n = 5 animals in each group).

In the series 2, rats were randomly allotted to one or other of the following 4 groups. In the sham-operated group, the bones were left intact (control group, n = 5). In the reamed groups, both tibiae were reamed and a 13 mm long, 0.88 mm wide titanium or polyethylene nail was implanted into the right tibia (see above). The periosteal vessel density was examined 12 weeks after the surgery (n = 6).

IVM investigations

Six or 12 weeks after first surgery, the animals were anaesthetized with sodium pentobarbital (45 mg kg^{-1}), their trachea intubated and the jugular vein was cannulated. The anteromedial and anterolateral surfaces of the tibial periosteum were exposed by microsurgical techniques with an operation microscopy of 4x magnification. The limbs were positioned horizontally on a special stage to expose the periosteal vessels suitable for IVM. The periosteal vessel structure and microcirculation were examined under a Cytoscan A/R (Cytometrics, USA) intravital microscope with employing the OPS imaging technique. The images were recorded with an S-VHS videorecorder (Panasonic AG-MD 830). Three fields of view were recorded on all bone surfaces: the upper, middle and lower third of the diaphysis.

Data analysis for IVM

The captured IVM images were analysed with the aid of a computer-assisted analysis system (IVM software; Pictron Ltd, Budapest, Hungary). The contours of capillaries, arterioles and venules were marked, and the vessel density (defined as the length of vessels per observation area) was recorded separately. Capillaries were defined as vessels in the diameter range 5-7 μm. The proportion of capillaries was calculated by the software and expressed as a percentage of the small vessel length relative to the total vessel length.

Examination of implant stability

At the end of the experiments, each implant was subjected to manual traction 5 times for 2 s under microscopic visual control by the same investigator (FG). The reaction was categorized on a qualitative scale according to previously defined criteria (Table 1).

Scale grading	Reaction upon traction	Characteristics
0	The implant can be removed	Unstable
1	The implant is loose, but can not be removed	Unstable
2	The implant is stable	Well fixed, anchored

Histological analysis of vascularization

Tissue samples taken from the periosteum were embedded in paraffin, 4 μm sections were placed on silanized slides and, after conventional methods of dewaxing and rehydration (initiated in xylene, followed by decreasing concentrations of ethanol and

methanol), the samples were stained with haematoxylin-eosin to characterize the inflammation by the number of infiltrating neutrophils.

3.3. Study III. Human observations, case history

Ethical permission

The intraoperative microcirculatory measurements were performed with the permission and signed consent of given by the patient, and the study protocol was approved by the Ethical Committee of the Medical Faculty of the University of Szeged.

Scope of the study

In this human study, the microcirculation of the tibial periosteum and endosteum was examined in an orthopaedic patient. Two years previoiusly, two long plates had been applied on both sides of the tibia and extension shaft of a cemented total knee endoprosthesis, and pseudoarthrosis had developed as a consequence of the long-term fixation. The roles of microcirculatory disturbances were assumed behind this phenomenon, thus microcirculatory measurements were performed to detect the concomitant microcirculatory perfusion deficit in the nearest proximity of the osteosynthesis materials during their surgical removal upon re-operation.

Previous medical history

Between 1999 and 2007, the patient underwent a sequence of prosthesis implantations in both knees to treat osteoarthrosis. Unicondylar knee arthroplasties were followed by total endoprostheses and finally two revision prostheses with extension shaft and homologous spongiosa grafts were implanted. In 2009, the left tibia diaphysis was fractured below the end of the prosthesis shaft; the treatment included non-weight-bearing plaster for 8 weeks, plus physiotherapy. In February 2011, left limb pain developed and tibial hypertrophic pseudoarthrosis was diagnosed by X-radiographic. The treatment was DC plate osteosynthesis plus a circular plaster. After 6 months, the plate was broken and pseudoarthrosis of the tibia was diagnosed. The treatment included refreshment of the fracture ends and the fracture gap was filled with homologous spongiosa and two long, angular stable plates were placed on the medial and lateral sides of the tibia. The patient was enrolled into our study 6 months thereafter, when the diagnosis of pseudoarthrosis was verified.

Measurement of the tibial periosteal and endosteal microcirculatory variables by using laser-Doppler flowmetry

The blood flow in the periosteum and endosteum was recorded with a laser-Doppler flowmetric device (supplied by a 780-nm laser diode; PeriFlux System 500, Perimed, Järfälla, Sweden) with a sterilized fibre-optic probe (# 416, "dental probe"; fibre separation: 0.25 mm, penetration depth ~ 1 mm). The tibial periosteum was explored via a conventional anterior incision on the re-operated limb and via a small (~ 2 cm long) skin incision on the contralateral limb. The flow probe was held perpendicularly to the surface of the periosteum by means of a plastic holder which reduced the contact pressure on the observed area and restricted the angular movements of the probe. The endoprosthesis was then removed and the endosteal membrane compartment was approached through the bone cavity. A small hole was drilled on the anterior cortex, providing access to the endosteum at the opposite (inner surface of the posterior) medullary wall (Figure 1). The size of the drilled hole allowed perfect fitting of the flow probe. Since an endoprosthesis was present on the contralateral (non-operated) side, the endosteal circulation could be approached distally to the local shaft, somewhat below the level of the measurement on the re-operated side; the difference was ~ 1 cm. Given the examination depth of the laser-Doppler device and the thickness of the periosteal and endosteal membranes, mostly the periosteum and the endosteum plus a portion of the underlying cortex could be examined.

Characteristic flow curves paralleling the heart circles were reproducibly detected in the $\tau = 0.2$ s mode, showing that pressure artifacts were avoided. After the required signal quality had been reached, recordings were made in 30 s periods and were repeated 3 times. Tissue perfusion was expressed in arbitrary Perfusion Units (PU); before the measurements , the probe was calibrated with a special Motility Standard supplied by the manufacturer. Data were collected and stored on a computer, and subsequently analysed with the computer software supplied together with the device.

Throughout the entire observation period, the room temperature (20±2 °C) and the core temperature of the patient were maintained constant; the stable macrohaemodynamic parameters were recorded continuously.

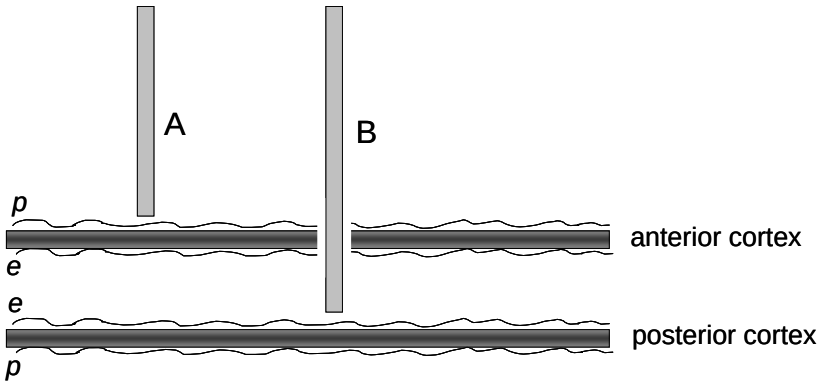

Figure 1. Scheme of the laser-Doppler flowmetry approach to observe the perfusion of the periosteal (**A**) and the endosteal regions **(B**) of the human tibia. The periosteum (*p*) was observed through a small incision made in the skin and the underlying tissues, while the endosteum (*e*) was reached through a small hole drilled in the anterior cortex, providing access to the endosteum at the opposite (inner surface of the posterior) medullary wall.

Brief description of the surgical intervention

The status at the time of the operation included pain fracture of both plates and hypertrophic pseudoarthrosis of the tibia. A special extension shaft with distal holes serving to hold the locking screws was designed and manufactured by the Johnson & Johnson Company. The plates previously placed on the pseudoarthrosis of the left tibia were removed together with the partially broken screws and the tibial component of the knee prosthesis. Subsequently, a new extension shaft was inserted into the tibial medullary cavity by bridging the fracture and this was compressed distally with two locking screws. The polyethylene plate in the metal backing of the new tibial component was replaced by a new one 15 mm in width. The bone ends of the pseudoarthrosis were refreshed and the fracture gap was tightly filled up with autologous spongiosa from the left iliac crest and platelet-rich plasma concentrate (Symphony^{TM}, DePuy) in order to induce osteogenesis (Sheth U 2012). The microcirculatory measurements were made during the removal of the old implants.

3.4. Statistical analysis methods

In all studies, the statistical software package SigmaStat version 2.03 (Jandel Corporation, San Rafael, CA, USA) was used. In Study I, changes in variables within and between groups were analysed by one-way ANOVA followed by the Bonferroni test. *P* values < 0.05 were considered statistically significant.

In Study II, differences between groups were analysed by two-way ANOVA, followed by the Holm-Sidak test or the ANOVA test (stability testing). All data are expressed as means ± standard error of the mean (SEM). *P* values < 0.05 were considered statistically significant.

Since the human study could be performed on only one patient and the measurements were conducted repeatedly at basically the same locations, no statistical comparisons were performed. Raw data (expressed in arbitrary units) are presented in Figure 9E to illustrate the potential differences.

4. RESULTS

4.1. Effects of ovariectomy and chronic oestrogen supplementation on the body weight, bone mineral density and periosteal vascular architecture

During the course of the experiments, the body weight of the animals increased in all groups (Figure 2), but OVX was followed by a significantly higher weight gain as compared with that in the Sham group. After the initiation of the E2 therapy (at the age of 8 months), the weight gain decreased to the level for the non-OVX animals.

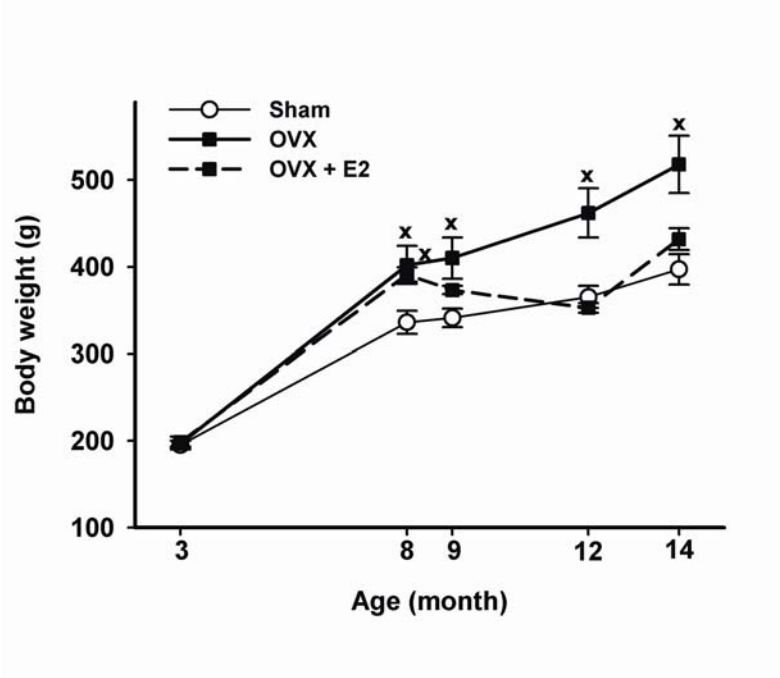

Figure 2. Body weight changes in sham-operated rats (Sham) and in ovariectomized animals treated with 17ß-oestradiol (OVX + E2) or vehicle (OVX). Data are presented as means ± SEM. ANOVA was followed by the Bonferroni test. x *P* < 0.05 *vs* Sham group; differences *vs* the baseline (3 months of age) are not shown.

As shown by bone densitometry on the proximal tibia, osteoporosis had developed by 21 weeks after bilateral OVX. The AD-SoS was significantly lower than that for the sham-operated animals (1674 ± 33 m s^{-1} *vs* 1850 ± 101 m s^{-1}, respectively). The OVX-induced osteopenia was completely restored by E2 therapy (Figure 3).

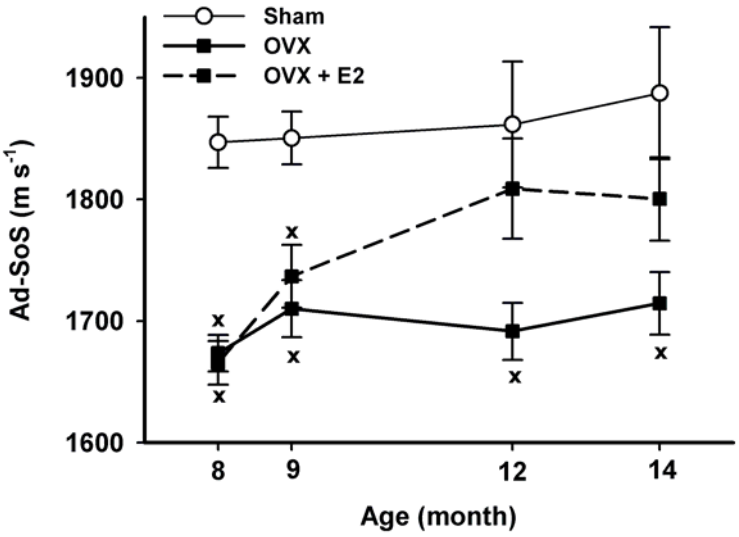

Figure 3. Bone density changes in the proximal part of the tibia in sham-operated rats (Sham) and in ovariectomized animals treated with 17ß-estradiol (OVX + E2) or vehicle (OVX). Data are expressed as Ad-SoS and presented as means ± SEM. ANOVA was followed by the Bonferroni test. x $P < 0.05$ *vs* Sham group.

In the examined anatomical fields of the tibia, the majority of the vessels were not capillaries, but venules (Figure 4). No significant differences in total vascular density were found between the 3-month-old controls (268.1 ± 10.1 cm^{-1}), the Sham group (262.6 ± 7.1 cm^{-1}), the OVX group (261.9 ± 7.6 cm^{-1}) or the OVX+E2 group (265.1 ± 1.1 cm^{-1}).

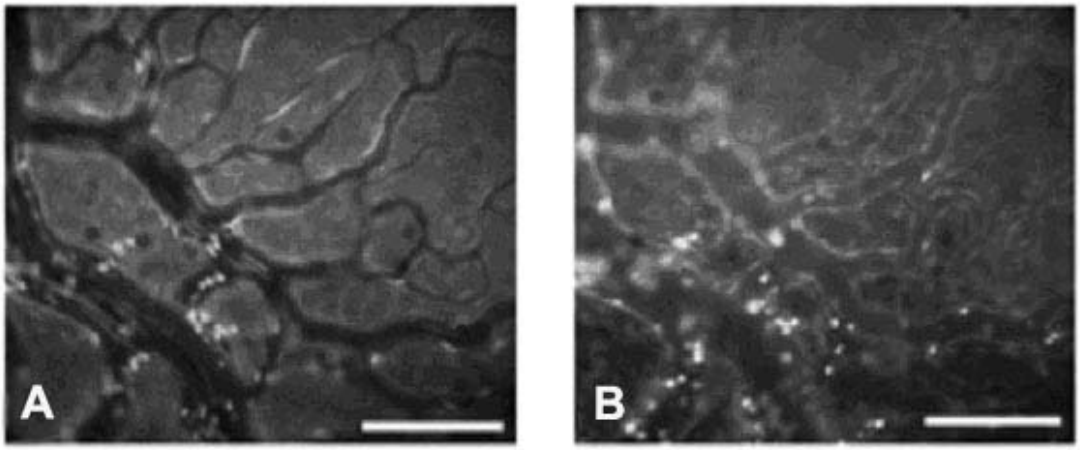

Figure 4. Representative micrographs of the venular network of the tibial periosteum, obtained by using conventional fluorescence IVM. Panel **A**: FITC-labelled erythrocytes; panel **B**: the same field with rhodamine 6G-labelled leukocytes. The bar denotes 100 μm.

4.2. Effects of drilling on the vascular and capillary density in the periosteum

Six weeks after the surgery, no significant alterations in vascular density or in the proportion of capillaries were found in the anteromedial and anterolateral periosteum of the tibia in the drilled animals as compared with the sham-operated group (Figure **5A-D**). In contrast, a significantly higher vascular density was observed on the anterolateral side (Figure **5B**) and a markedly higher proportion of capillaries on both the anteromedial and the anterolateral side in the drilled group than in the sham-operated group 12 weeks after surgery (Figure **5C,D**). Interestingly, both parameters were found to be lower on the anterolateral than on the anteriomedial side, even in the intact tibiae (Figure **5A-D**), which demonstrated the anatomical characteristic of the periosteal vascularization of the tibia in the rat. Destruction of the endosteal circulation was evidenced histologically in supplementary experiments (Figure **6**; n = 3).

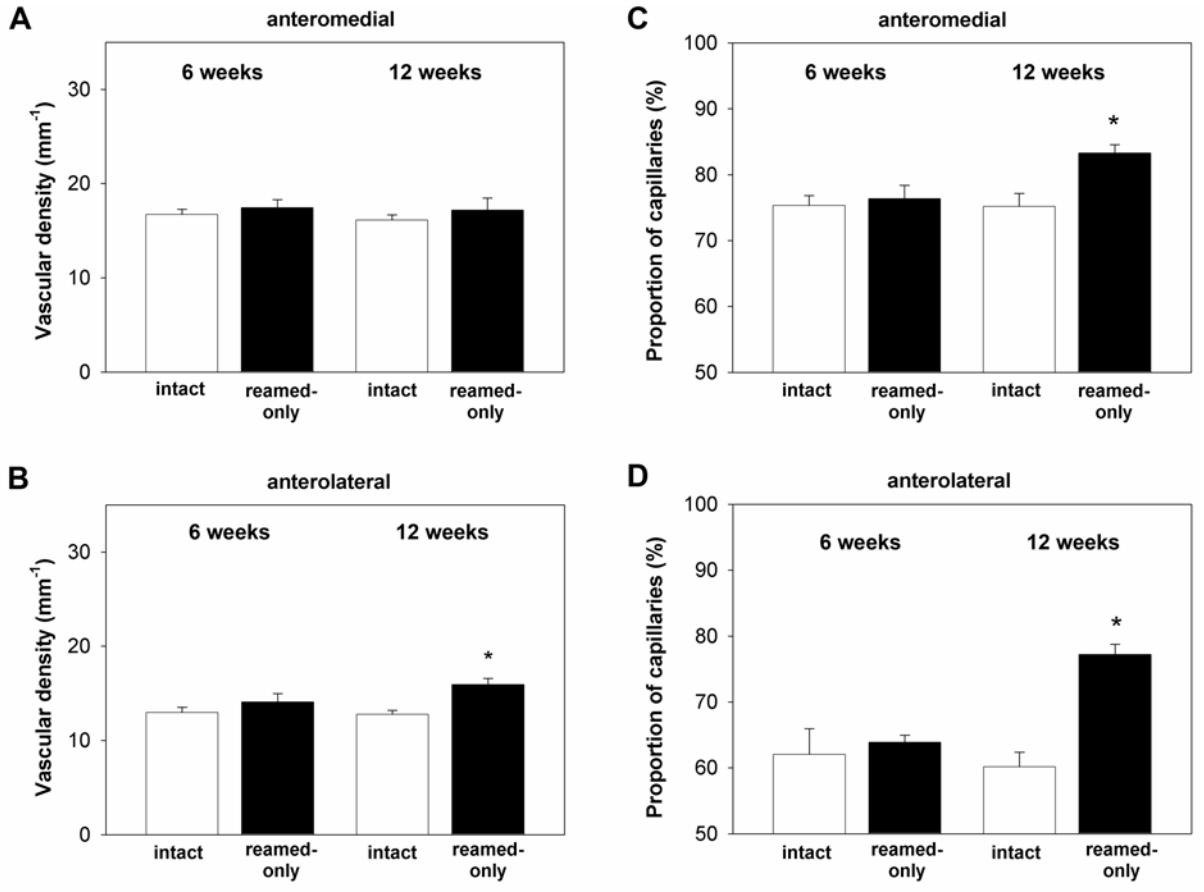

Figure 5. The effects of drilling on the vascular density (**A** and **B**) and the proportion of capillaries (**C** and **D**) in the anteromedial **(A** and **C)** and the anterolateral **(B** and **D)** periosteum of the rat tibia, at 6 and 12 weeks after the surgery. * $P < 0.05$ between the corresponding intact and drilled groups; # $P < 0.05$ *vs* between anteromedial and anterolateral data.

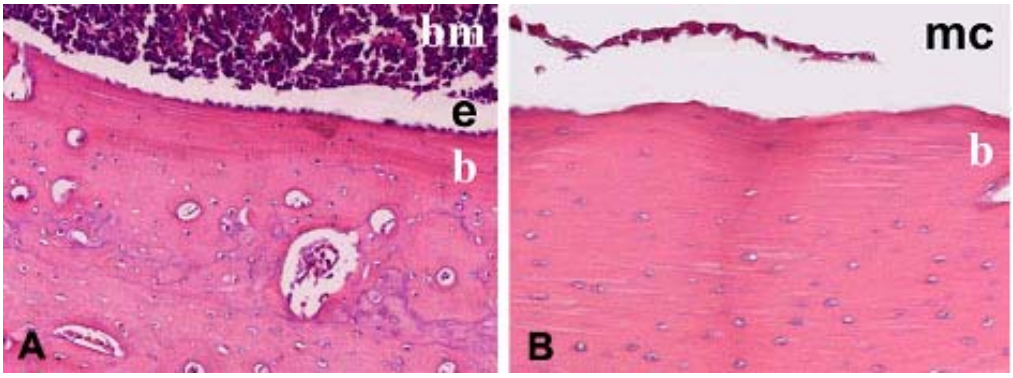

Figure 6. Representative micrograph demonstrating the tibial endosteum in sham-operated rats (**A**) (longitudinal section, H&E staining). The effect of reaming on the structure of the tibial endosteum after 1 week is shown in part **B. bm**: bone marrow, **e:** endosteum (with osteoblasts on the surface of the cortical bone), **b**: bone, **mc:** medullary cavity (with fragments of bone marrow).

4.3. Effects of titanium alloys and polyethylene implants on the periosteal vasculature

With both implant materials, nailing resulted in a significant higher vascular density on the anteromedial side of the tibia as compared with the contralateral, reamed-only side. (Figure **7A,C**; Figure **8)**. However, nailing with polyethylene resulted in significantly higher vascular densities on both the anteromedial and the anterolateral sides and an increased proportion of capillaries on the anterolateral side of the tibial periosteum (Figure **7A,C,D**). Although the vascular density was higher in the anteromedial periosteum in the animals implanted with a titanium nail, this material did not influence the examined parameters of vascular density at any of the other locations. The data on the control group subjected to bilateral drilling (without implantation) were similar to those measured in Series 1 (data not shown).

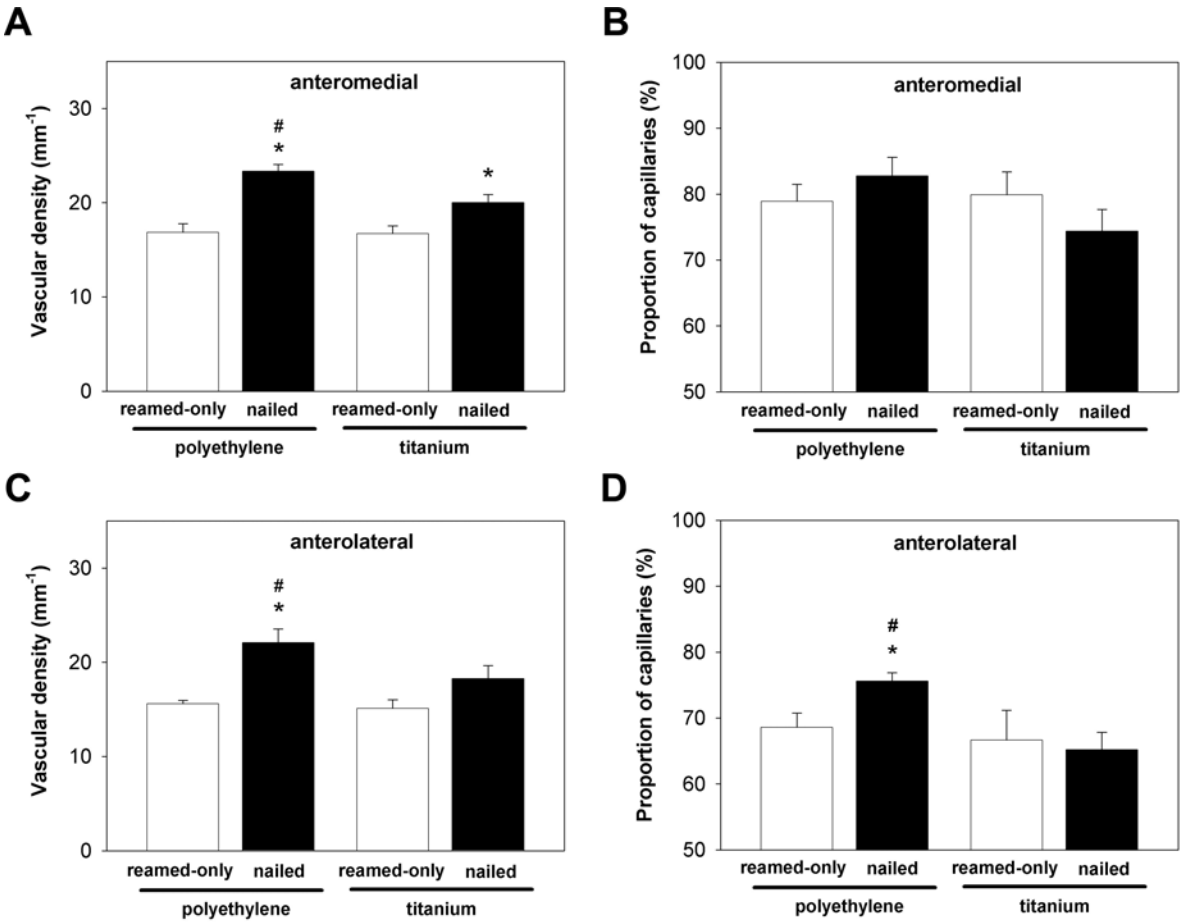

Figure 7. The effects of nailing with titanium or with polyethylene on the vascular density (**A** and **C**) and the proportion of capillaries **(B** and **D)** at the anteromedial **(A** and **B)** and anterolateral sides **(C** and **D)** on the tibial periosteum in rats. * $P < 0.05$ between corresponding reamed-only and nail-implanted groups, # $P < 0.05$ between corresponding groups implanted with a polyethylene or a titanium nail.

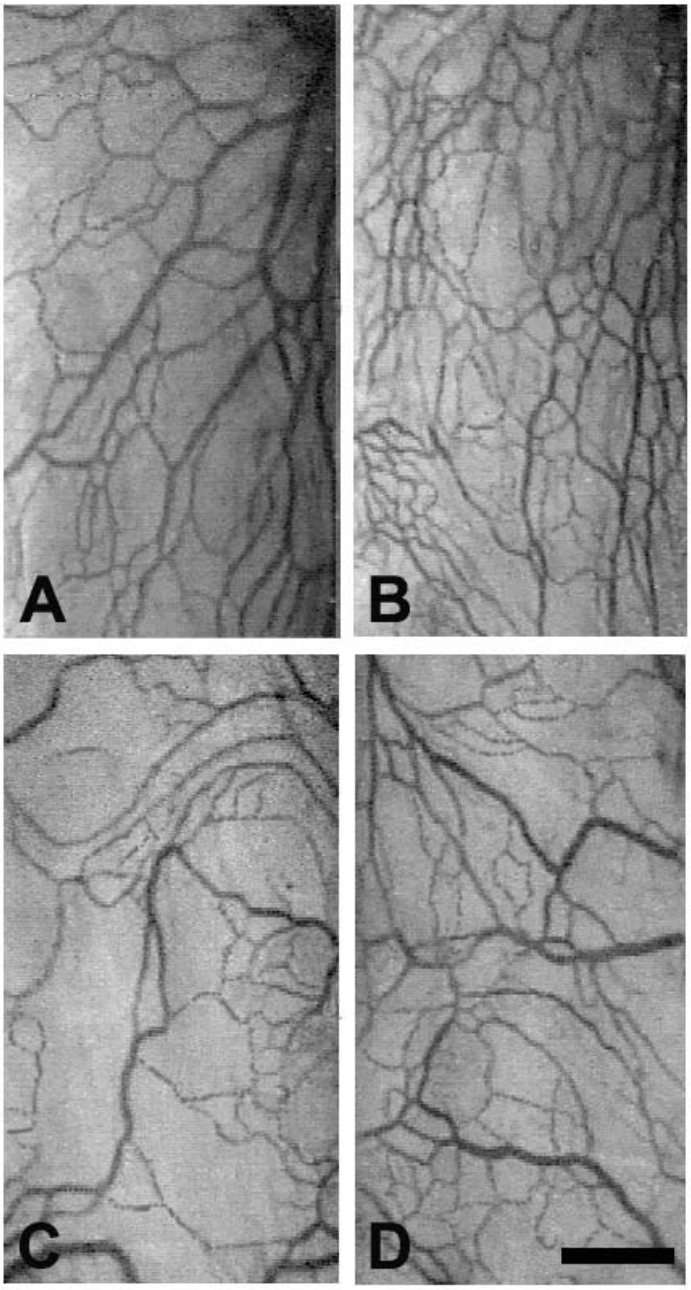

Figure 8. Representative micrographs of the microvascular architecture of the anteromedial (**A**) and anterolateral (**C**) surfaces of the tibial periosteum in animals after reaming and after nailing with polyethylene (**B** and **D**). The bar denotes 250 μm.

Stability of the implants

Macroscopically visible loosening of the implanted nails was not observed. Micro-movements could not be evoked in the titanium nail-implanted group, demonstrating the sufficient anchoring of the implant in the tibial medullary cavity (grade 2.0 ± 0). In the rats

implanted with a polyethylene nail, however, the stability was significantly lower (grade 0.33 ± 0.21), and 4 implants exhibited a high degree of instability.

4.4. Effects of excessive osteosynthesis on the microcirculation of the periosteal and endosteal membranes

Considerably lower periosteal blood flow values were measured in the re-operated (pseudoarthrotic) tibial periosteum (Figure **9B**) than in the contralateral non-operated limb (Figure **9A**) wiht average levels of PU was 76 and 106 PU, respectively (Figure **9E**). Much lower perfusion values were observed in the endosteum, however. Specifically, an average endosteal perfusion of 30 PU was found in the non-operated tibia (Figure **9C**). An even lower perfusion level was measured in the re-operated tibial endosteum (average 9 PU; Figure **9D**) even in the presence of a characteristically good signal quality (Figure **9E**).

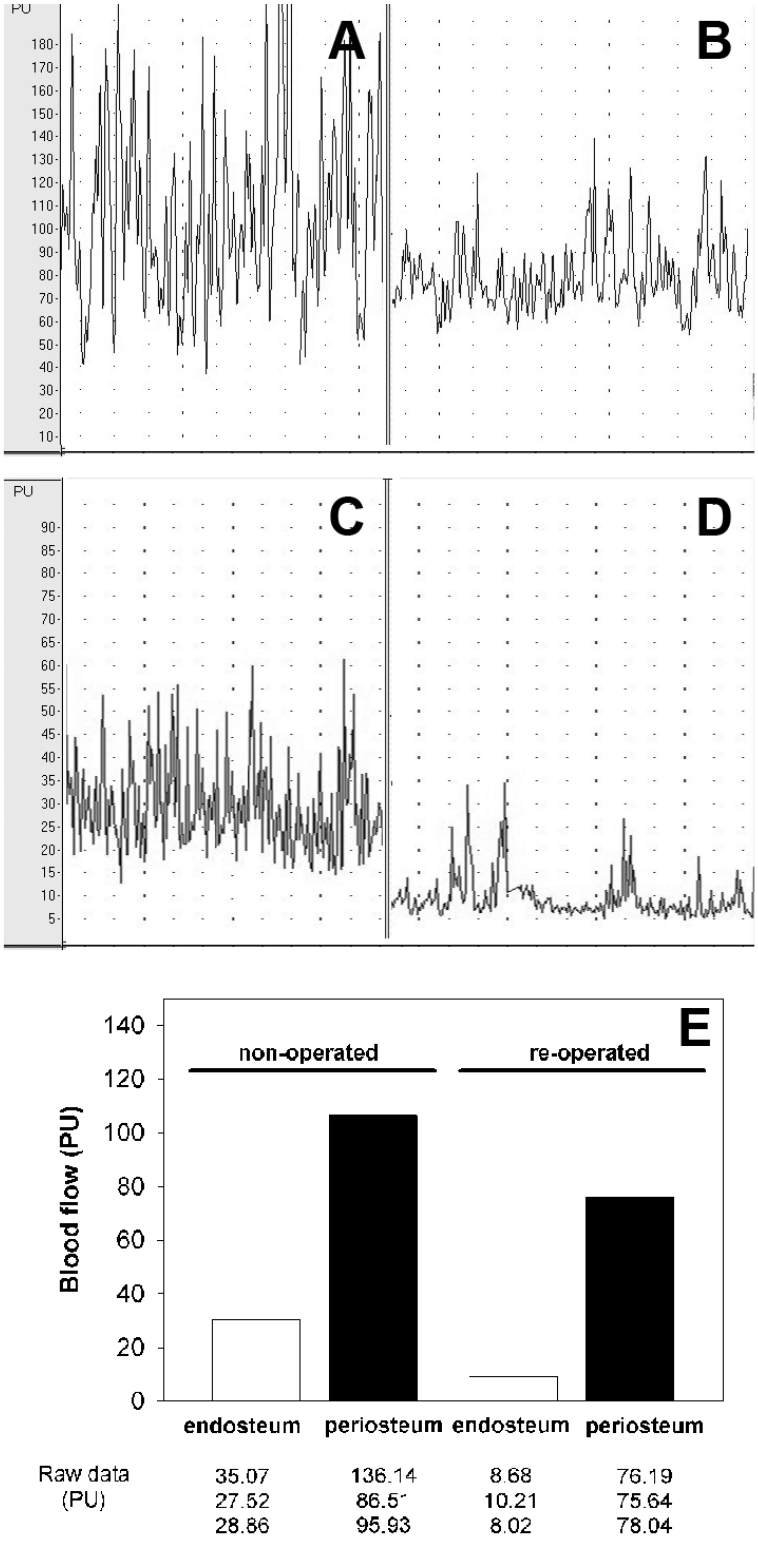

Figure 9. Original laser-Doppler flowmetry recordings of the periosteum (**A** and **B**) and the endosteum (**C** and **D**) in the non-operated (**A** and **C**) and operated (pseudoarthrotic; **B** and **D**) tibia. Data relating to the perfusion of the above compartments are presented in part **E**. Data are presented the means of three measurements.

Radiographic findings

Six weeks after the operation the radiographs demonstrated callus formation in the dorsal part of the fracture and good angulation of the fracture ends (Figure 10). The patient now walks with the aid of a knee-ankle-toe orthosis, without loading the osteosynthesis.

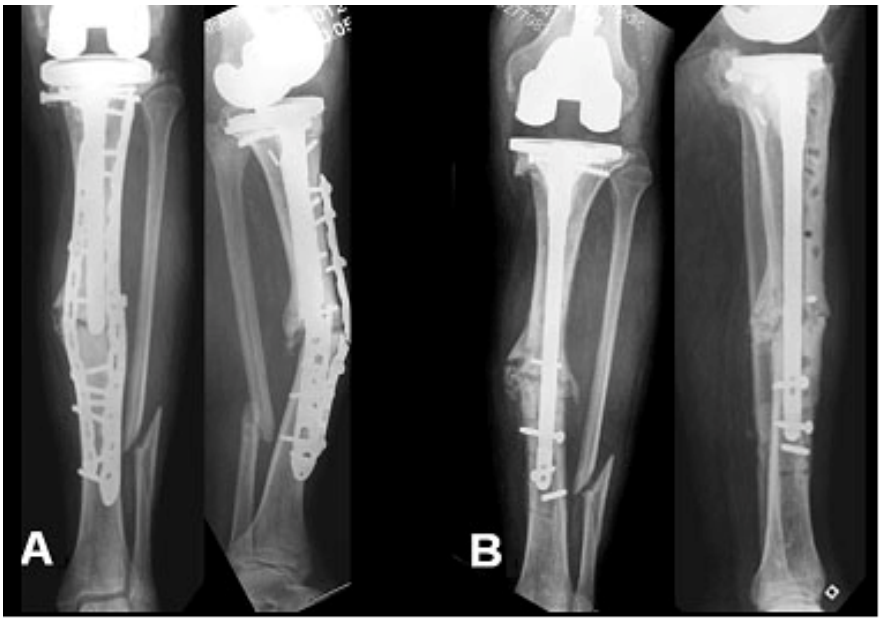

Figure 10. Anteroposterior and lateral views of the left tibia before the re-operation (**A**) and 6 weeks after the surgery (**B**). Callus formation was detected in the dorsal part of the fracture, with good angulation of the fracture ends.

5. DISCUSSION

Restoration of the blood supply is a prerequisite for proper bone healing, which points to an important role of the microcirculation in the pathogenesis of various bone diseases. Indeed, re-establishment of the periosteal microcirculation *per se* guarantees the survival of bone grafts, even in an environment with only a limited blood supply (Berggren A 1982). Early experimental studies revealed that alterations in the periosteum perfusion reflect perfusion changes of the whole bone; when the main nutrient artery was compromised, the periosteum integrity was still grossly preserved (Kolodny A 1923). The periosteum alone has also been shown to be capable of regenerating long bone defects even when the endosteum has been completely damaged (Knothe Tate ML 2007). The importance of endosteum in bone healing has also been recognized (Kojimoto H 1988). The endosteum, which covers all interior surfaces of bones, including the spongiosa, also contains osteoprogenitor cells which contribute to the healing of fractures (Brighton CT 1992). The above observations demonstrate that periosteal and endosteal microcirculatory changes are good indicators of the bone circulation; investigation of the microvasculature of these structures may therefore contribute to a better understanding of various bone-related pathological conditions.

Due to methodological constraints, the possibilities for studying the circulation of the endosteum and the periosteum, and particularly of the bone, are greatly limited. In the present studies, we utilized appropriate methods that provide dynamic information on the perfusion conditions of the endosteum or the periosteum or both. We made use of laser-Doppler flowmetry, which depicts flow conditions in arbitrary units; hence, the data yieled by this approach can be used only for spatial and time-wise comparisons (Swiontowsky MF 1986; ElMaraghy AW 1999). Nevertheless, with the application of small sensors and a method first introduced here to gain access to the endosteum, we were able to compare the perfusion conditions at different locations of a human tibia, thereby permitting comparisons of the flow characteristics of an asymptomatic and pseudoarthrotic limb. Methods for IVM examinations of the tibial periosteum were also introduced by our research group (Wolfárd A 2002; Gera L 2007). These latter IVM approaches provide data on well-defined structures such as microvessels, and all the results can be expressed numerically, together with direct visualization of the microvasculature (Menger MD 1997; Ruecker M 1998).

The periosteal membrane not only separates and binds elements of the skeletal system to the bone, but also plays regulatory roles in the regeneration and metabolism of the

bone, even in senile osteoporosis. The aim of our first study was to simulate a clinical situation where the magnitude of the local inflammatory-microcirculatory complications of bone traumas and tourniquet application can be determined. The rationale of this approach was that elective orthopaedic surgery and emergency conditions (such as fracture repairs) are frequently performed in the elderly, osteoporotic patient population and these interventions involve the use of tourniquet placement that causes an ischaemia-reperfusion injury. In the work presented, we set out to focus only on the potential microvascular re-organization of the periosteum elicited by an oestrogen deficit and E2 replacement therapy. OVX is a well-established animal model for osteoporosis research as it shares many similarities with the human disease. These include an increased rate of bone turnover (Wronski TJ 1986), and an initial rapid phase of bone loss, similarly to the skeletal responses to treatment with oestrogen, calcitonin, bisphosphonates and many other agents (Fleisch H 1993; Allen MR 2004). In our model, typical reactions after OVX, such as weight gain and osteopenia, reproducibly evolved, and the reversing efficacy of E2 therapy on osteopenia, together with the periosteal microcirculatory status of the tibia, could subsequently be evaluated in a clinically relevant, long-term period. OVX-related vascular alterations have already been demonstrated in certain organs (Santizo RA 2000; Watanabe Y 2001), but never in the periosteum. For instance, OVX has reduced the blood flow in the bone (Kapitola J 1995) and a lower total capillary density was observed in the heart (Jesmin S 2002) and brain (Jesmin S 2003), a phenomenon probably related to the well-known effect of oestrogen on angiogenesis. In our chronic rat model, however, the periosteal vessel density was not affected by OVX or E2 replacement therapy. The hypothesis that osteoporosis may cause reorganization of the periosteal microvasculature could therefore not be proven. It is important to note that these long-term changes were followed in the proximal tibial periosteum of the rat, where the venules are the predominant vessel type. The characteristically high venular density of the periosteal microarchitecture may account, at least in part, for the present finding. More studies would be needed to provide further structural details on other anatomical sites.

In consequence of the impairment caused in the osteogenic potential of the periosteal and endosteal compartments by different osteosynthesis techniques, plate- and implant-induced osteoporosis often occurs in clinical practice. This kind of osteoporosis is considered to be an intermediate stage in internal bone remodelling, and it is induced by avascular bone necrosis beneath the disrupted periosteal membrane (Klaue K 2000). The “plate osteporosis” that affects the cortical bone beneath the plate was earlier thought to be

a compensatory reaction of the bone to the implant-related mechanical stress and friction, but the impairment of the periosteal circulation was later revealed in the background (Perren SM 2005). Although osteoporosis is a transitory reaction in these cases, it causes marked weakening of the bone. A direct interrelationship has been revealed in animal experiments between the extent of plate osteoporosis in different species and variations in the relative importance of the endosteal and periosteal blood supplies (Perren SM 2005). It has also been postulated, that if direct pressure to the bone is avoided, the preserved vascularity will prevent plate osteoporosis (Lippuner K 1992). The same phenomenon was observed with intramedullary implants, where the development of osteoporosis was closely related to the disturbed endosteal circulation and the magnitude of contact surfaces between the implant and the bone (Perren SM 1988). Furthermore, the implant material was also a decisive factor, as plastic materials caused more severe osteoporosis than did steels (Perren SM 1988).

In our human study, the patient was subjected to a sequence of distinct osteosynthesis techniques simultaneously affecting the integrity of the periosteum and the endosteum, with excellent mechanical properties at the times of the operations, but pseudoarthrosis nevertheless developed. This debilitating condition can be regarded as a final step during the sequence of events along the malperfusion-osteoporosis-necrosis axis. To examine the potential microcirculatory background, laser-Doppler flowmetry was performed in the pseudoarthrotic limb; both the endosteal and the periosteal flow were found to be diminished as compared with the contralateral limb. These observations were made during a surgical intervention when the pseudoarthrotic limb was re-operated. In the course of the preoperative planning, preservation of the local biology was at the focus of surgical team. Relying on the regeneration capacity of the periosteum, including recovery of the blood supply of the cortex (Colnot C 2009), a decision was made to remove the long plates and a new prosthesis shaft was designed with distal holes destined to hold locking screws (serving as a long intramedullary nail bridging the fracture ends). In this way, the use of subperiosteal plate could be omitted completely so as to aid regeneration of the periosteal circulation. Furthermore, the fracture gap was tightly filled with a platelet concentrate produced from autologous blood in order to support new bone formation (Sheth U 2012). As a result, postoperative X-ray radiographs taken 6 weeks after the operation clearly inidicated new bone formation proving evidence of the efficacy of this approach.

In the above study, the modified endoprosthesis was applied to promote periosteal regeneration. The resultant step is the process of endochondral bone repair, which is the

primary form of bone healing when micromotion exists between the fractured parts in bones covered with periosteum (Shapiro F 2008). In these cases, periosteum-derived cells have the ability to proliferate and form periosteal callus in the initial phase of fracture healing (Knothe Tate ML 2007). While periosteal injuries heal by endochondral ossification, bone marrow injuries heal by intramembranous ossification (Colnot C 2009). As discussed above, the importance of the endosteum in bone repair should also be taken into account, because regions rich in spongiosa heal faster (Shapiro F 2008) and studies of bone lengthening have also indicated the presence of a considerable amount of endosteal callus formation between the fracture endings (Kawamura B 1968). Our present study demonstrated the simultaneous impairment of both circulatory systems. This may explain the negative clinical outcome, i.e. pseudoarthrosis formation after excessive prosthesis application in the long bones. In consequence of the ethical and technical limitations, the relationship and compensatory reactions between the periosteal and endosteal circulations can not be investigated in adequate numbers under clinical circumstances. A further potential flaw of this study is that no follow-up of the examinations was feasible. However, the present study provides a model for estimation of the circulatory consequences of long-term simultaneous iatrogenic cessation of both the interior and the exterior blood supply of a long bone.

Selective disruption of the endosteum also occurs in clinical practice, caused by dislocating fractures or reaming of the medullary cavity. As a result, avascularity and even partial necrosis of the cortex can be detected, suggesting that a significant proportion of the cortical blood flow is provided by the endomedullaryy vessels (Trueta J 1955; Gustilo RB 1964; Rhinelander FW 1987; Reichert IL 1995; Ruecker M 1998; Mueller CA 2009). During the bony healing process, recovery of the endosteal membrane is preceded by regeneration of the periosteum (resulting in periosteal new bone formation) (Rhinelander FW 1968, 1987; Aliabadi P 1989; Reichert IL 1995; Ruecker M 1998; Mueller CA 2009). Accordingly, signs of supranormal periosteal perfusion or increased vessel density have been found to persist for a period between 2 weeks (Reichert IL 1995; Pazzaglia UE 1996) and 2-3 months (Gustilo RB 1964). In our studies, we also set out to describe the microcirculatory alterations that occur in the periosteum following selective experimental destruction of the endosteum; we did not find significant alterations in the periosteal vasculature 6 weeks after intramedullary drilling. However, a significantly higher vascular density and a markedly higher proportion of capillaries were observed on both sides of the periosteum 12 weeks after drilling. Our results suggest that, although the vascular

connections between the periosteal and endosteal circulations under physiological circumstances are poor (Danckwardt- Lilliestrom G 1970; Rhinelander FW 1987; ElMaraghy AW 1999; Mueller CA 2009), the periosteum can respond to intramedullary reaming by compensatory neovascularization leading to an increased vessel density.

One important question that arises is how implantation affects the regeneration of the endosteal compartment and the periosteal reactions. Reaming and implantation with primary stable implants in long bones caused a similarly undisturbed endosteal regeneration process to that observed after reaming alone (Pazzaglia UE 1996). Revascularization occurring within 12-16 weeks after implantation is due to the proliferation of meta- and epiphyseal vessels (Danckwardt-Lilliestrom G 1970). To the best to our knowledge, our study is the first to reveal the periosteal microcirculatory reactions evoked by intramedullary polyethylene nail implantation. The results furnish evidence of the marked instability of the nail and the lack of osseointegration in the host bones. Implant loosening is well known to induce connective tissue formation (Rhinelander FW 1979; Pazzaglia UE 1996) and micromovements of the implant inhibit adequate revascularization around the endoprosthesis. However, we have detected a significant increase in blood vessel density and in particular in capillary density in the tibial periosteum in rats implanted with polyethylene nails. An explanation of this finding is that a polyethylene implant is not integrated into the medullary canal. The chemical composition and the smooth surface may contribute to the lack of stable fixation and to micromovements, thereby leading to inadequate restoration of the endosteal circulation (Pazzaglia UE 1996). The endosteal blood supply is probably insufficient to supply the cortical bone, and may be compensated by increased periosteal sources.

The results showed that IVM evaluations can be used to determine and compare the functional and morphology changes of the bone microvessels after implantations. The development of polyethylene implant wear is an important clinical problem (Linder L 1983; Yamada H 2009). Products of polyethylene wear after total hip arthroplasty cause granulation tissue formation and the loosening of an endomedullaryy implant. These observations indicate the necessity for the development of new or more enduring polyethylene implants in order to prevent the micromovements leading to the wear process and the formation of granulation tissue. Complete implant stability was achieved in our study through the use of a titanium nail implant, after which micromovements were not detected, and the proportion of small vessels and the blood vessel density did not differ significantly from those of the reamed-only tibiae. Stable implantation with a titanium nail

does not influence the periosteal blood vessel formation, and the lack of microcirculatory reorganization on the surface of bones may therefore be regarded as a sign of a preserved or restored endovascular microcirculation.

6. SUMMARY OF NEW FINDINGS

1. The tibial periosteal vascular density and the proportion of capillaries were quantified by IVM in a clinically relevant osteoporosis model with adequately long-term follow-up in rats. The results show that periosteal angiogenesis is not affected by ovariectomy-related oestrogen loss or E2 supplementation.

2. After microsurgical exposure of the periosteum, IVM provides an opportunity for simultaneous visualization of the microvascular function and also allows the computer-based quantification of morphological and functional characteristics. We have provided evidence that marked compensatory microvascular reactions, and increased vascular and capillary densities, are induced in the periosteum in response to mechanical destruction of the endosteal microcirculation. As evidenced by IVM, these reactions are manifest 12 weeks after surgery.

3. We have described the periosteal microcirculatory alterations following standardized surgical destruction of the endosteum and after the application of the most frequently used implant materials. The periosteal microvascular reorganization caused by reaming of the endomedullary cavity is not altered by implantation with titanium, a material with good osseointegrative properties. This reaction, however, is augmented by endomedullary polyethylene nails. Unstable implant materials probably lead to inadequate restoration of the endosteal circulation, which is insufficient to supply the cortical bone, and may be compensated by increased periosteal sources.
   .
4. A clinical case history is presented that demonstrates the importance of preserved endo- and periosteal microcirculations and the benefits of the application of primary stable osteosynthesis during orthopaedic surgical interventions. With the use of laser-Doppler flowmetry, simultaneous microcirculatory disturbances induced in the tibial periosteum and endosteum by long-term fixation modalities could be detected in the

operating theatre. This observation underlines the importance of preservation of the local circulation when placements of osteosyntheses are planned in clinical practice.

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9. ANNEX

PAPER I.

A Szegedi Tudományegyetem, Traumatológiai Klinika[1], a Sebészeti Műtéttani Intézet[2], és az Ortopédiai Klinika[3] közleménye

A csonthártya mikrokeringésének kísérletes vizsgálata intravitális fluoreszcens videó-mikroszkópiával*

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ÖSSZEFOGLALÁS

A csonthártya mikrokeringési zavara jelentősen befolyásolhatja a csontosodást és a traumás szövődmények kórtanát. Patkányokon végzett kísérleteinkben intravitális videó-mikroszkóppal megfigyeltük a 60 perces teljes hátsó végtag ischaemiát követő 180 perces reperfusio hatását a tibia periosteum mikrokeringésére. Célunk a csonthártya átmeneti, teljes vértelenségével járó klinikai állapotok (például tourniquet által kiváltott átmeneti végtag ischaemia, csont graftok sebészete) modellezése, valamint az ischaemia–reperfusio mikrokeringési következményeinek megismerése volt. Eredményeink szerint a szöveti perfusio jelentősen romlik a reperfusiós időszak végére; szignifikánsan csökken a csonthártya ereinek átjárhatóságát leíró funkcionális kapilláris denzitás és a vörösvértestek áramlási sebessége. Az ischaemia–reperfusio által kiváltott gyulladásos károsodás jeleként a posztkapilláris venulákban fokozódik a leukocita-endotélsejt interakciók számaránya (a leukociták gördülése és kitapadása). Eredményeinket összefoglalva elmondhatjuk, hogy az intravitális fluoreszcens videó-mikroszkópia alkalmas az ischaemia–reperfusiós csonthártya károsodás számszerűsítésére, a perfusio zavarának és a gyulladásos reakciók minőségi és időbeli változásainak jellemzésére. E technikával lehetőség nyílik a terápiás beavatkozások hatékonyságának számszerű elemzésére is.

Kulcsszavak: ***Állatkísérlet; Csonthártya – Vérkeringés; Izom, skeletalis – Vérkeringés; Mikrocirkuláció; Mikroszkóp, videó; Reperfusiós sérülés – Fiziopatológia;***

L. Török, P. Hartmann, A. Szabó, R. Varga, J. Kaszaki, F. Greksa, M. Boros: Intravital microscopic examination of the ischemia–reperfusion induced periosteal microcirculatory reactions

The periosteal microcirculation is a good indicator of perfusion changes of the whole bone induced by temporarily reduced blood flow conditions (such as tourniquet and bone graft surgery). The present study was conducted to examine the microvascular alterations in the rat tibial periosteum by means of intravital microscopy after 60-min complete hind limb ischemia and 180-min reperfusion. A significant impairment of tissue perfusion was evidenced by the decrease in functional capillary density (the ratio of perfused capillaries) and red blood cell velocity by the end of the reperfusion phase. The ischemia–reperfusion induced leukocyte-endothelial interactions (i.e. rolling and sticking), showed significant increases in the post-capillary venules. According to these data, intravital microscopic analysis of the microcirculation represents an appropriate, dynamic tool to quantify periosteal microcirculatory injury (marked by changes in perfusion and leukocyte-endothelial interactions) evoked by ischemia–reperfusion. By this means, the efficacy of therapeutic interventions can be compared and analyzed.

Key words: ***Animals; Microcirculation; Microscopy, video; Muscle, skeletal – Blood supply; Periosteum – Blood supply; Reperfusion Injury – Physiopathology;***

BEVEZETÉS

A végtagokat közvetlenül vagy közvetetten érő erők a csonttörések mellett a csonthártya sérüléséhez vezethetnek. A csontos váz zavartalan helyreállításhoz szükséges fizikai feltéte-

* *Kutatási támogatás: OTKA K60752*

lek mellett (stabilitás, kontrollált mikromozgások stb.) rendkívül fontos a csonthártya megfelelő biológiai válaszreakciója is. A késői csontgyógyulást tipikusan megelőzi a csonthártya leválása, és ez a folyamat nem egyesült csontosodást, vagy pseudoarthrosist is eredményezhet (4, 7, 18).

A végtagsérülések ellátása során alkalmazott művi érleszorítással (tourniquet) átmeneti vértelenség érhető el. A technika ma már pontos szabályokat követ, de az időkorlát túllépése esetén – sőt gyakran még azt megelőzően is – a primer ischaemiás sérülés mellett másodlagos, úgynevezett reperfusiós károsodásokkal kell számolni, amelyek kaszkádszerű aktiválódással helyi és távoli működési zavarokat, kóros biokémiai és mikrokeringési elváltozásokat okozhatnak (17, 19).

Az ischaemia–reperfusio (I–R) akár az életet is veszélyeztető káros következményei a technikailag sikeres végtagmegmentések, replantációk alkalmával is észlelhetők. I–R károsodás a rekonstrukciós sebészetben alkalmazott szabadlebeny átültetések legtöbbjében is előfordul. A helyi keringési zavarok mellett I–R károsodás felléphet az egész szervezetre ható kórállapotok esetén is, melyek szisztémás hatásaik mellett befolyásolhatják a csonthártya és a csontszövet keringését is. Sokk, vagy szepszis esetén olyan mikrocirkulációs zavarok jöhetnek létre, melyek jellegzetességeikkel és kórlefolyásukban sok hasonlóságot mutatnak a lokális sérülésekre adott I–R válaszreakciókkal (16).

Megfigyelések igazolják, hogy a csonthártya mikrokeringés jó indikátora a csont perfusiós változásainak, különösen az autotranszplantáció korai szakaszában. Kimutatták, hogy a csonthártya mikrokeringés helyreállítása már önmagában szavatolja a csontgraft túlélését, még mérsékelt vérellátású környezetben is (2).

Mindezek alapján egyértelműnek tűnik, hogy a csonthártya mikrokeringésének megismerése fontos a klinikai gyakorlat számára; a csonthártya postischaemiás változásainak kísérletes tanulmányozása pedig hasznos információt szolgáltathat az átmeneti vértelenséggel járó beavatkozások következményeiről.

Kísérleteinkben a csonthártya mikrokeringési változások in vivo megjelenítésére intravitális fluoreszcens videó-mikroszkópiát (IVM) alkalmaztunk. Más, dinamikus mikrokeringési vizsgálati módszerekkel szemben (például mikrogyöngyök, laser–Doppler véráramlás mérés), ez a technika alkalmas a változások jól meghatározott struktúrában történő vizsgálatára és számszerűsítésére.

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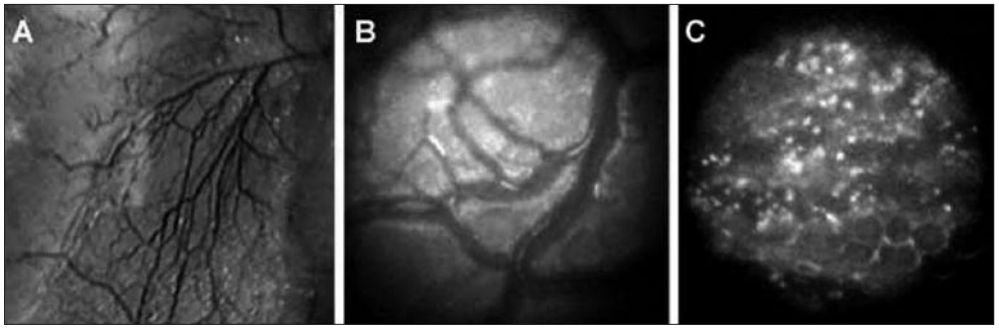
A kísérleteket a NIH irányelvei alapján (Guide for the Care and Use of Laboratory Animals) végeztük a Szegedi Tudományegyetem Állatvédő Etikai Bizottságának jóváhagyásával.

Sebészi beavatkozás

Hím Wistar patkányokat (átlagos súly: 300±20 g) random módon két kísérleti csoportra osztottunk (n=14). Intraperitonealis Na–pentobarbitál anesztéziát (45 mg kg^{-1}) alkalmaztunk, a vénás kanülön keresztül szükség esetén további kis, fenntartó dózisokat adtunk. A jobb oldali vena jugularisba és artéria carotisba kanülöket vezettünk infúzió és gyógyszerek adása, valamint artériás vérnyomásmérés (Statham P23Db transzducerrel) céljából. Az állatok testhőmérsékletét a kísérletek alatt 36–37°C között tartottuk, folyadékpótlásra 10 ml kg^{-1} h^{-1} Ringer laktát infúziót adtunk. A légzés biztosítása céljából a tracheába kanült vezettünk. A jobb femoralis artériát kipreparáltuk, és a jobb oldali gracilis izom átvágását követően a



1. ábra
A: A tibia csonthártya operációs mikroszkópos képe patkányon.
B: Fluoreszcein-izotiocianáttal jelzett vörösvértestek a csonthártya kapillárisaiban.
C: Rhodamin 6G-vel jelzett neutrofil leukociták a csonthártya posztkapilláris venuláiban.

tibia medialis felszínén lévő csonthártyát atraumatikus sebészi technika alkalmazásával feltártuk (19) *(1. ábra).*

Kísérleti protokoll

30 perces stabilizáció után kontroll cardiovascularis és mikrohemodinamikai méréseket végeztünk. Ezt követően az állatok első (I–R) csoportjánál (n=7) 60 perc időtartamú teljes végtag ischaemiát hoztunk létre a femoralis artériára helyezett miniklip, és a proximális femur köré feltett tourniquet segítségével. Az occlusio felengedését követően (t=0 időpont), a reperfusio 30, 60, 120, és 180. percében intravitális mikroszkóp alatt vizsgáltuk a csonthártya mikrokeringését, és video felvételeket készítettünk. A második (álműtött) csoport protokollja (n=7) az ischaemia kiváltásán kívül minden másban megegyezett az 1. csoporttal, és álműtött kontrollként arra szolgált, hogy az anesztézia és sebészi beavatkozás következtében létrejövő eltéréseket az I–R által kiváltott változásoktól elkülöníthessük.

Intravitális videó-mikroszkópia

A jobb hátsó végtagot vízszintes helyzetben rögzítettük, hogy a tibia csonthártyája mikroszkóp alatt jól vizsgálható legyen. A distalis tibia mikrokeringését Zeiss Axiotech Vario 100 HD intravitális videó-mikroszkóppal vizsgáltuk (100 W HBO higanygőz lámpa, Acroplan 20 víz immerziós objektív). Fluoreszcein isothiocianáttal (Sigma Chemicals, St. Louis, MO) jelölt vörösvértestek *(1. B ábra)* (0,2 ml i.v.) szolgáltak fluoreszcens perfusiós markerként (15); a leukociták festésére *(1. C ábra)* pedig rhodamine–6G-t (Sigma, 0,2%, 0,1 ml i.v.) használtunk. A mikroszkópos képeket CCD videokamera (AVT HORN–BC 12) segítségével S-VHS videó rekorderrel (Panasonic AG–MD 830) rögzítettük, amit egy személyi számítógéphez csatlakoztattunk az adatok analízise céljából.

Videó analízis

A mikrokeringési paraméterek mérése a kísérleteket követően számítógépes szoftverrel (IVM, Pictron Ltd., Budapest) történt. A *funkcionális kapilláris denzitást* (FCD; a perfundált kapillárisok hossza, és a megfigyelt terület aránya; cm^{-1}) és a *vörösvértestek áramlási sebességét* (RBCV; μm^{-1}) 5 különböző látótérben és 5 kapillárisban mértük. A kísérlet minden mérési időpontjában 25 mérés átlagát számoltuk. A leukocita-endotélsejt interakciókat állatonként 5 (10–20 μm átmérőjű) posztkapilláris venulában vizsgáltuk. Kitapadó leukocitáknak azokat a sejteket definiáltuk, melyek nem mozdultak, vagy legalább 30 másodpercig rögzültek az

endotélsejt felszínéhez a megfigyelési periódus alatt. Gördülő leukocitáknak tekintettük azokat a sejteket, melyek sebessége nem érte el a mikroerek középvonalában mért vörösvértest áramlási sebesség 40%-t. Számukat az átáramló leukociták százalékos arányában határoztuk meg a megfigyelési periódus 30 másodperce alatt.

Statisztikai analízis

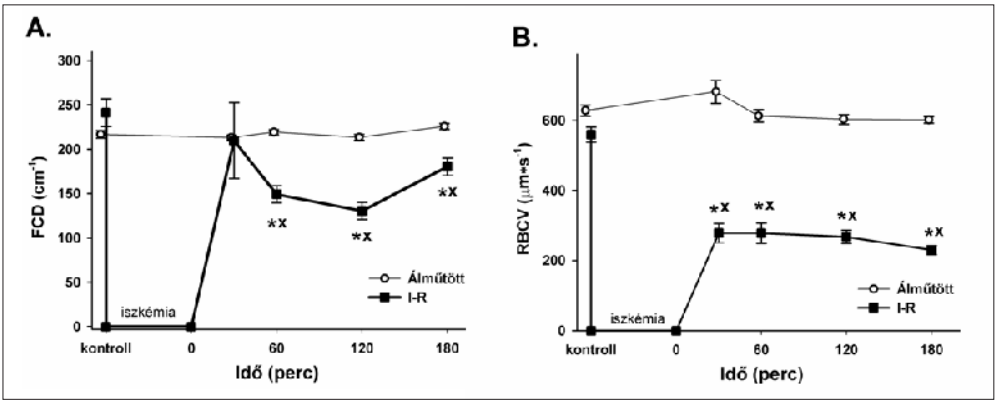
Az adatok analízise a SigmaStat for Windows (Jandel Scientific, Erkrath, Németország) szoftverrel történt. Csoporton belüli ismételt mérések analíziséhez one way ANOVA-t, csoportok közötti különbségek kimutatásához t–próbát használtunk. Statisztikailag szignifikánsnak $p<0.05$ esetén tekintettük a különbségeket.

EREDMÉNYEK

A makrohemodinamikai kontroll értékekben (szívfrekvencia és artériás középnyomás) nem volt szignifikáns eltérés az egyes csoportok között és az álműtött csoporton belül sem, a különböző mérési időpontokban. Az I–R csoportban a reperfusio kezdeti szakaszán átmeneti artériás középnyomás csökkenést észleltünk, de az értékek gyorsan (1–2 perc alatt) visszatértek a kiindulási szintre.

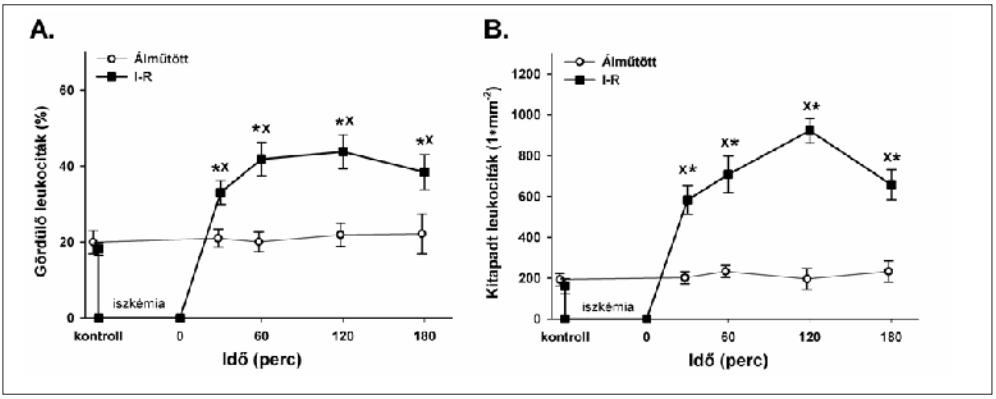
Intravitális mikroszkóppal a kiindulási állapotban homogén mikrokeringés volt látható mindkét csoportban. Az álműtött csoportban nem történt jelentős változás, míg az I–R csoportban a csonthártya FCD a reperfusio kezdetétől fokozatosan csökkent, a reperfusio 2. órájára érkezve a mélypontra ért (a kiindulási érték 60%-ra), majd lassan emelkedve a kísérlet végére is csak az eredeti érték 70%-át érte el (*2. A ábra*). A kiindulási vörösvértest áramlási sebesség (RBCV) hasonló volt az egyes csoportokban (560–620 µm s^{-1} között), és ezek az értékek az álműtött csoportban nem változtak a kísérlet alatt. A reperfusio ugyanakkor szignifikáns RBCV csökkenéshez vezetett az I–R csoportban (*2. B ábra*).

Kontroll állapotban a leukociták mintegy 30%-a mutatott gördülést a posztkapilláris venulák endotél felszínén az egyes csoportokban. Az álműtött kontroll csoportban nem volt szignifikáns változás a gördülő és a kitapadt leukociták számában egyik megfigyelési időpontban sem. Az I–R csoportban a reperfusio 60. percére szignifikánsan emelkedett a leukocita-endotélsejt interakciók száma. Mind a gördülő, mind a kitapadt leukociták száma jelentő-



2. ábra

Funkcionális kapilláris denzitás (A: FCD) és vörösvértest áramlási sebesség (B: RBVC) változás patkány csonthártyában 60 perces teljes végtag ischaemia és 180 perces reperfusiós fázis alatt az álműtött és I–R csoportokban. Az ábrán az átlagot ± S.E.M.-et ábrázoltuk. *$p < 0.05$ a kiindulási értékhez képest (ANOVA teszt); x $p < 0.05$ az álműtött csoporthoz képest (t–teszt).



3. ábra

Leukocita gördülés (A) és adhaesio (B) változások patkány tibia csonthártya posztkapilláris venuláiban 60 perces teljes végtag ischaemia és 180 perces reperfusiós fázis alatt az álműtött és az I–R csoportokban. A vizsgálatok a kiindulási időpontban és a reperfusio 30, 60, 120 és 180. percében történtek. Az ábrán az átlagot ± S.E.M.-et ábrázoltuk. *$p < 0.05$ a kiindulási értékhez képest (ANOVA teszt) ; x $p < 0.05$ az álműtött csoporthoz képest (t–teszt).

sen nőtt a preischaemiás értékhez képest, és az álműtött csoport egyes mérési eredményeihez viszonyítva is (*3. ábra*).

MEGBESZÉLÉS

A mikrokeringés vizsgálata IVM technikával

A mikrokeringési elégtelenség kimutatása a terápiás stratégia kialakítása és a kezelés hatásosságának megállapítása szempontjából is fontos lehet, ám ma még csak kevés eszközös lehetőség áll a diagnózis és az orvoslás rendelkezésére. A mikrokeringés anatómiai szerkezetének vizsgálatára szolgáló statikus módszerek (például korróziós öntvények, érfestési technikák, elektron mikroszkóp stb.) nem alkalmasak a keringés dinamikájának jellemzésére. Más, közvetett módszerekkel (például laser–Doppler véráramlás mérés, stb.) *in vivo* vizsgálhatjuk a keringés időbeni változásait, de ezekben az esetekben az egyes érképletek szummációiból, nagyobb, heterogén szövetmasszából származó jelek állnak csak rendelkezésre.

Intravitális mikroszkóp segítségével a felszíni szövetek felső rétegében elhelyezkedő különböző struktúrák keringése (arteriolák / venulák / kapillárisok) elkülöníthető, és a változások dinamikus megfigyelésére nyílik lehetőség. A hagyományos szövettani vizsgálatokkal szemben nem áthaladó fénnyel (transzilluminációval), hanem epi-illuminációs technikával vizsgáljuk a szöveteket. Ekkor a fény visszaverődik, és a mikroszkóp fókuszának változtatásával a szövetek különböző mélységben (általában 100–200 μm) vizsgálhatók. A képletek fluoreszcens marker alkalmazásával tökéletesen megjeleníthetők, a vörösvértesteket általában fluoreszcein izotiocianát (FITC) festéssel, a fehérvérsejteket pedig rhodamin 6G festéssel lehet láthatóvá tenni. A fluoreszcencia alkalmazása természetesen hátrányt jelent a klinikumban, de kísérletes körülmények között így jól vizsgálható a microvascularis perfusio és az érpermeabilitás, valamint számszerűsíthetők a leukociták és az endotélsejtek között kialakuló ideiglenes vagy tartós kapcsolatok (interakciók). Ugyancsak jól megítélhetők a vazodilatátor vagy vazokonstriktor stimulusokra adott érátmérő változások is.

Emberben a mikrokeringés közvetlen megfigyelése nem egyszerű, a problémák a fluoreszcens jelzésből és az eszközök méretéből adódnak. Ezeket a nehézségeket az intravitális videó-

mikroszkópia egyik új válfaját jelentő, úgynevezett „ortogonális polarizációs spektrális" (OPS) képalkotás kiküszöböli (6). Ekkor nem fluoreszcens markerekkel, hanem polarizált fénnyel történik a vizsgálat, és a készülék mérete lehetővé teszi az emberi műtétek alatti alkalmazást is. A módszer hátránya, hogy a hagyományos fluoreszcens IVM-mel szemben nem alkalmas a leukocita-endotélsejt interakciók megjelenítésére és vizsgálatára.

A csonthártya mikrokeringés zavarai

Az ischaemiát követő reperfusiós fázist a csonthártya mikrokeringés zavara jellemzi. Ha nem áll helyre a kiindulási állapot, a mikrokeringés romlása a szöveti oxigenizációt is csökkenti. A perfusiós paraméterek közül erre utal a vörösvértestek áramlási sebességének (RBCV) és az átjárt kapillárisok számának (FCD) csökkenése, ez utóbbi paraméterrel a véráramlási mintázat térbeli heterogenitását is nyomon lehet követni. Kísérletünkben az ischaemiát követő reperfusio alatt az FCD drámai csökkenését észleltük, az értékek csak a reperfusio 3. órájára kezdtek emelkedni. Az RBCV is jelentősen változott, a reperfusio kezdetén csak az eredeti érték 50%-át érte el, majd a későbbi mérési időpontokban további, fokozatos csökkenést észleltünk. Az FCD és RBCV értékek kapcsolatáról lényeges megjegyezni, hogy csakis az átjárható kapillárisokban lehet áramlási sebességet mérni, vagyis ahol van mikrokeringés. Az RBCV mérésekor e paraméter valódi jelentőségét könnyen túlbecsülhetjük, hiszen az egyes kapillárisokban mért sebességek átlagolásakor nem számítjuk azokat a kapillárisokat, ahol nincs keringés (vagyis ahol a sejtek sebessége zérus). Fontos tehát a fenti két paraméter együttes figyelembe vétele. Irodalmi adatok alapján az RBCV csökkenés hátterében az endotélium károsodása és a helyi érszűkítő és értágító erők közötti egyensúly felbomlása állhat (19). Az FCD csökkenés oka hasonló lehet – a pre-kapilláris szfinkterek fokozott vazokonstrikciója vagy a kapillárisok lumenének elzáródása fontos kóroki tényezők – utóbbiban az endotélium ödémája vagy gyulladásos sejtek kitapadása játszik lényeges szerepet (14).

A reperfusio alatt tulajdonképpen egy steril gyulladásos válaszreakciót figyelhetünk meg. A mikroér hálózatban áramló neutrofil leukociták egyre szorosabb kapcsolódása (gördülése, majd kitapadása) az endotéliumhoz végül szöveti migrációhoz és akkumulációhoz, a reperfusiós károsodás egyik karakterisztikus jelenségéhez vezet (19). E folyamat hátterében a reoxigenizációt követően képződő reaktív szabadgyökök és a leukocitákat marginációra késztető adhéziós molekulák fokozott expressziója áll (8, 17). Az aktivált leukociták maguk is termelnek szabadgyököket, melyek valamennyi sejtorganellumban károsodást okozhatnak. Kísérletes modellünkben kimutattuk, hogy mind azt elsődleges, mind a másodlagos leukocita-endotélsejt reakciók, vagyis a gördülő és kitapadó leukociták száma jelentősen növekedett a reperfusio alatt. A kontrollszintet többszörösen meghaladó kitapadási értékek csak igen mérsékelten csökkentek a reperfusio 3. órája végére.

A mikrohemodinamikában és a leukocita adhézióban bekövetkező változások között szoros összefüggés lehet. Kimutatták, hogy az endotél-függő vazoaktív anyagok (mint például a vazodilatátor nitrogén monoxid és a vazokonstriktor endotelin–1) képződése mellett a perfusiós változások és a következményes nyíróerő változás is befolyásolja a leukociták aktivációját és kitapadását. E folyamatban megváltozik az adhéziós molekulák expressziója (például CD11b/CD18), a molekuláris kötések dinamikája és fél-életideje (11). Ismeretes, hogy az érfalhoz közeli leukociták sebességének csökkenése megnöveli a kitapadás esélyét (1). Amennyiben a nyíróerő az optimum érték alatt van, ez elősegíti a leukociták gördülését. Ezzel ellentétben, a megnőtt nyíróerő fokozza a gördülő leukociták sebességét és ennek következtében csökken a kitapadások száma (10). Kísérleti modellünkben a nyíróerő közvetlen mérésére technikai korlátok miatt nem nyílt lehetőség, ám a romló mikrokeringési paraméterek (RBCV és

FCD) közvetett módon utaltak a kapilláris hálózat csökkenő perfusiójára. Bizonyos mértékben ez érinti a posztkapilláris venulákat is, így ez a folyamat hozzájárulhatott a leukocita-endotélsejt interakciók számának növekedéséhez.

Más szervekkel ellentétben a csonthártyát érintő I–R károsodás egyes tényezői még alig ismertek. Munkacsoportunk korábban már kimutatta az endotélium által termelt endogén endotelin–1 jelentős szerepét a reperfusiós károsodás közvetítésében (19). A károsodásban jelentős szerepet játszhat még az I–R folyamatához társuló fizikai membrán defektusa, a foszfolipidek degradációja és az endogén foszfatidilkolin (PC) források kimerülése (3, 9). Korábbi kísérleteink során kimutattuk, hogy a PC-kezelés is hatékonyan csökkenti a végtag I–R által kiváltott mikrokeringési elégtelenség káros következményeit, egyrészt a környező szövetek hízósejtjeinek stabilizációja, másrészt a leukociták kitapadásának gátlása révén (5). Itt kell megemlíteni, hogy kooperációs partnereink e mikrokeringési változásokat összetett csont–izom–bőr lebenyekben, illetve hő-sokk által kiváltott prekondicionálást követően vizsgálják (12–14).

Eredményeinket összefoglalva elmondhatjuk, hogy az intravitális fluoreszcens videómikroszkópia alkalmas az I–R csonthártya károsodás számszerűsítésére, a perfusio zavarának leírására és a gyulladásos reakciók minőségi és időbeli változásainak jellemzésére. E technika alkalmazásával lehetőség nyílik az egyes terápiás beavatkozások hatékonyságának elemzésére és tárgyszerű összehasonlítására is.

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PAPER II.

CASE REPORT

MICROCIRCULATORY CONSEQUENCES OF OSTEOPOROSIS

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REZUMAT

Introducere. Etiologia sindromului de osteoporoza senila este plurifactorial, dar nivelul scazut de estrogeni la femeile aflate in perimenopauza este clar asociat cu o pierdere accelerata de material osos. In cadrul studiului nostru experimental, au fost investigate consecintele osteoporozei si a terapiei cronice cu estrogeni asupra reactiilor inflamatorii de la nivelul microcirculatiei periostale si a expresiei receptorilor de estrogeni. **Material si metoda.** Dupa sase luni de la initierea terapiei de substitutie cu estrogeni cu 17β-estradiol (E2, 20 $\mu g^{-1}kg^{-1}zi^{-1}$) la sobolani Sprague-Dawley ovarectomizati (OVX), au fost examinate consecintele inflamatorii la nivel de microcirculatie la 60-min de ischemie totala a membrului posterior si dupa reperfuzie la 180-min. Modificarea expresiei receptorului de estrogen de la nivel leucocitar a fost de asemenea evaluata la o luna de la OVX sub tratament cu E2. **Rezultate:** In afara de inversarea efectelor osteopenice ale OVX, tratamentul amelioreaza semnificativ cresterea aderentei leucocitelor neutrofile indusa de reperfuzarea zonei ischemice la nivelul venulelor postcapilare de la nivelul periostului (vizualizat prin microscopie cu fluorescenta). Acest efect nu a fost asociat cu reducerea expresiei CD11b de la nivelul leucocitelor neutrofile (analiza FACS). OVX a determinat o scadere moderata a expresiei proteinei ER-beta de la nivelul receptorului de estrogen leucocitar, care tinde sa fie inversata de E2. Nivelele mRNA ER-alpha si ER-beta (RT-PCR) a leucocitelor si expresia proteinei ER-alpha (Western blotting) au fost sub limita de detectie. **Concluzii:** Suplimentarea cronica de estrogeni amelioreaza eficient osteoporoza si consecintele microcirculatorii inflamatorii ale ischemiei tranzitorii de la nivelul membrului. Se pare ca acest efect nu este mediat de leucocite CD11b sau de modificari ale expresiei receptorilor de estrogeni.

Cuvinte cheie: ovarectomie, ischemie-reperfuzie, neutrofil, microscopie in vivo, recptor de estrogen

ABSTRACT

Introduction. The etiology of senile osteoporosis syndrome is multifactorial, but the reduced estrogen levels in peri-menopausal women are clearly associated with an accelerated bone loss. In our experimental studies, the consequences of osteoporosis and chronic estrogen supplementation were investigated on the periosteal microcirculatory inflammatory reactions and estrogen receptor expressions. **Material and method.** Six months after the initiation of estrogen replacement therapy with 17β-estradiol (E2, 20 $\mu g^{-1}kg^{-1}day^{-1}$) in ovariectomized (OVX) Sprague-Dawley rats, the microcirculatory inflammatory consequences of 60-min total hindlimb ischemia followed by 180-min reperfusion were examined. Leukocyte estrogen receptor expression changes were also assessed 1 month after OVX plus E2 treatment. **Results.** Apart from reversing the osteopenic effects of OVX, E2 treatment significantly ameliorated the ischemia-reperfusion-induced elevation of neutrophil leukocyte adherence to the postcapillary venules of the periosteum (visualized by means of intravital fluorescence microscopy). This effect was not associated with a reduction of CD11b expression of the neutrophil leukocytes (FACS analysis). OVX caused a moderate decrease in leukocyte estrogen receptor (ER)-beta protein expression which tended to be reversed by E2. Leukocyte ER-alpha and ER-beta mRNA levels (RT-PCR) and the ER-alpha protein expression (Western blotting) were below the detection limit. **Conclusions.** Chronic estrogen supplementation efficiently ameliorates osteoporosis and the inflammatory microcirculatory consequences of transient limb ischemia. It appears that this effect is not mediated by leukocyte CD11b, or estrogen receptor expression changes.

Key words: ovariectomy, ischemia-reperfusion, neutrophil, intravital microscopy, estrogen receptor

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INTRODUCTION

Osteoporosis is the most common disease in the elderly population which progressively weakens bones and often leads to painful accidental fractures. The primary causative therapy is chronic estrogen supplementation which prevents or reverses osteopenic bone loss, and also exerts protection against different inflammatory processes[1]. Apart from their pivotal role in sexual development and reproduction, estrogens (in particular 17β-estradiol, E2) play a remarkable role in the modulation of the cardiovascular system. Some of circulatory effects of E2 are mediated by the

influencing of endothelial production of nitric oxide and prostacyclin[2]. Besides, E2 also promotes endothelial healing and angiogenesis.[3] Both acetylcholine-induced and flow-dependent vasodilation are preserved or potentiated by estrogen treatment.[4]

The role of estrogen receptors in the mediation of microcirculatory reactions is yet largely unknown. Estrogen actions are essentially mediated by two molecular targets: estrogen receptor-alpha (ER-alpha) and ER-beta. The impact of ER-beta has been addressed in vitro only, but a prominent role has been attributed to these receptors in the vascular biology[5] (for review see at Arnal JF et al.[6]). E2 causes vasodilation via ER-alpha receptors which effect is lost in ovariectomized (OVX) rats.[7] Besides, it has been shown that OVX leads to the down-regulation of ER-alpha expression in the vasculature.[8]

As the prevalence of skeletal injuries increase after menopause, our studies were designed to determine the potential microcirculatory benefit of E2 in a limb ischemia-reperfusion model of osteoporosis. An ovariectomized rat model with clinically relevant time frame was used which shares many similarities with the human disease[9]. Our aims were (1) to examine the consequences of osteoporosis on the postischemic periosteal microcirculatory reactions, (2) to evaluate the effects and the consequences of chronic estrogen on these microcirculatory reactions, and (3) to clarify whether the mechanisms of microcirculatory reactions provided by E2 are mediated by expression changes of ER-alpha or ER-beta.

MATERIALS AND METHODS

Animals and experimental design

The experiments were carried out on female 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 experimental protocol was approved by the local animal rights protection authorities and followed the EC Directive 86/609/EEC and the National Institutes of Health guidelines for the care and use of laboratory animals.

The experiments were performed in two series. In the first experimental series, 3-months-old animals (weighting 200-210 g) were ovariectomized (Group 1, OVX, n=12), or sham-operation was performed (Group 2, SH-OVX, n=12; see methods at Szabó *et al.*[9]). Five months later (at the age of 8 months), chronic estrogen therapy[10] was initiated 5 days per week with 20 μg kg^{-1} sc 17ß-estradiol (E2, (Sigma, St. Louis, MO, USA) in 6 animals of the OVX group (Group 1a, OVX+E2). The E2 substitution was continued weekly until the end of the experiments (see time scheme in Fig. 1).

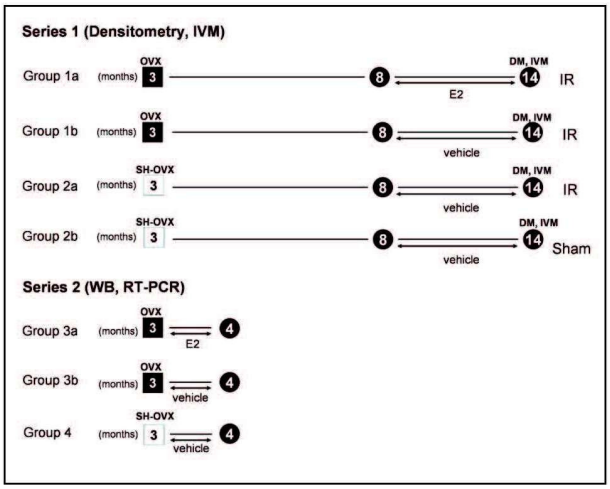

Figure 1. Experimental protocol. The time sequence of surgical interventions, treatments and measurements in Series 1 and Series 2. OVX=ovariectomy; SH-OVX= sham operation for ovariectomy; DM=densitometry; IR=ischemia-reperfusion; Sham=non-ischemic control; IVM=intravital microscopy, E2=17ß-estradiol treatment; WB=Western blotting; RT-PCR=real-time PCR.

The development of osteoporosis was detected by means of ultrasonic densitometry eleven months after OVX, and then microcirculatory consequences of a 60-min complete hindlimb ischemia followed by a 180-min reperfusion period (IR) were investigated using intravital video microscopy (IVM, in groups OVX+IR, OVX+E2+IR and SH-OVX+IR). Total limb ischemia was induced by applying a tourniquet around the thigh and placing a miniclip on the femoral artery. Six animals in the SH-OVX group served as non-ischemic controls SH-OVX+SH. Recordings of the tibial periosteal microcirculation were performed before ischemia and at the end of the 180-min reperfusion period.

A second series of experiments was performed to detect changes in expression of ER-alpha and beta in polymorphonuclear leukocytes (PMNs, by western blotting and real time-PCR). OVX was induced in 8 rats, 4 of which received 20 μg kg-1 E2 supplementation (Group 3a) (in the former dose), starting 2 days after the OVX, while the remaining 4 rats were injected with vehicle (Group 3b). Vehicle-treated sham-operated animals served as controls (Group 4; n=5). 28 days after the OVX, PMNs were isolated from these animals for the determination of ER-alpha and ER-beta protein contents with Western blot analysis and mRNA expressions.

Intravital video microscopy

At the age of 14 months of the animals, the periosteum of the medial surface of the right tibia was exposed under a Zeiss 6x magnification operating microscope, using an atraumatic surgical technique[11] using pentobarbital anesthesia (45 mg kg^{-1} ip). The right hindlimb with the exposed tibial periosteum was positioned horizontally on an adjustable stage, and the microcirculation of the proximal tibia was visualized by IVM (Zeiss Axiotech Vario 100HD microscope, 100 W HBO mercury lamp, Acroplan 20x water immersion objective, Carl Zeiss GmbH, Jena, Germany), using fluorescein isothiocyanate (Sigma, St. Louis, MO, USA)-labeled erythrocytes (0.2 ml iv) for red blood cell staining, and rhodamine 6G (Sigma, St. Louis, MO, USA, 0.2%, 0.1 ml iv) for PMN staining. The microscopic images were recorded with a charge-coupled device video camera (AVT HORN-BC 12, Aalen, Germany) attached to an S-VHS video recorder (Panasonic AG-MD 830, Matsushita Electric Industrial Co., Tokyo, Japan) and a personal computer.

Video analysis

Quantitative assessment of the microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images, using image analysis software (IVM, Pictron Ltd., Budapest, Hungary). Leukocyte-endothelial cell interactions were analyzed within 5 postcapillary venules (diameter between 11 and 20 μm) per animal. Adherent leukocytes (stickers) were defined in each vessel segment as cells that did not move or detach from the endothelial lining within an observation period of 30 s, and are reported 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 given as the number of cells/vessel circumference in mm.

Flow cytometric analysis of CD11b expression on leukocytes

The surface expression of CD11b on peripheral blood granulocytes was determined by whole blood flow-cytometric analysis (a modification of a literature method)[9]. CD11b is the M part of the CD11b-CD18 complex which binds to its endothelial counterpart ICAM-1 resulting in leukocyte adhesion to the venular endothelium (a process which can directly be observed with IVM.

Isolation of PMN leukocytes from the blood

PMN leukocytes were isolated by a method described by Russo-Racolante EMS et al..[12] The viability of the PMNs was found to be 80-95% (by the trypan blue exclusion test) and the purity of the suspension was >80% (with the May-Grünwald/Giemsa staining on cell smears). The cell number in 0.1 ml of suspension was in the range 3.5-5.8x10^6.

The suspension was divided into two portions. Fifty μl of suspension was frozen and later used for the detection of ER-alpha and ER-beta protein by Western blot analysis. The remaining suspension was dissolved in TRIzol buffer and frozen for the later evaluation of ER-alpha and ER-beta mRNA expression by real-time PCR.

Western blotting procedure

SDS-polyacrylamide gel electrophoresis was performed on 10% gels; samples were applied without prior heating. Membrane proteins were transferred onto nitrocellulose membrane (Amersham Hybond-C Extra). After quenching with 5% Blotto/Tween (5% milk powder in Tris-buffered saline containing 0.2% Tween 20) for 1.5 h, blots were incubated with the primary antibody overnight. The anti-estrogen receptor alpha rabbit polyclonal antibody (Thermo Fischer Scientific, Fremont, CA, USA) was used in a dilution of 1:150, while the dilution factor for the anti-estrogen receptor beta mouse monoclonal antibody (Leica Biosystems Newcastle Ltd, Newcastle, UK) was 1:50. As secondary antibodies, either HRP-conjugated goat anti-rabbit immunoglobulins (DAKO Glostrup, Denmark) or HRP-conjugated anti-mouse IgG (R&D Systems, Minneapolis, MN, USA) were employed at a dilution of 1:1000 for 1 h. All the antibodies were diluted in 1% Blotto/Tween (containing 1% milk powder), and the washing steps were performed with Tris-buffered saline containing 0.2% Tween 20. Labeled membrane proteins were visualized through a chemiluminescence reaction (Immobilon Western HRP substrate, Millipore, Billerica, MA, USA).

Real-time PCR

Total RNA was isolated from cell lysates with TRIzol™ reagent (Gibco, Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. RNA concentration was determined by measuring the A260 values. First-strand cDNA was synthetized from 1 mg of total RNA, using the iScriptTM cDNA Synthesis Kit from Bio-Rad (Hercules, CA, USA). Real-time RT-PCR experiments were performed to quantify the relative abundance of each mRNA by using the iCycler IQ Real-Time PCR machine of Bio-Rad. The Universal Probe Library (UPL) system (Roche, Basel, Switzerland) was used to design primers and probes (see Table 1). Real-time RT-PCR reactions were performed in the FastStart TaqMan Probe Master mix (Roche, Basel, Switzerland), following the

Table 1. Probes and primers used in the study

	Probe no.	Left primer	Right primer
18S	77, Cat. no. 04689003001	ctcaacacgggaaacctcac	cgctccaccaactaagaacg
ER-alpha	130, Cat. no. 04693663001	ttctttaagagaagcattcaaggac	tcttatcgatggtgcattgg
ER-beta	111, Cat. no. 04693442001	ggctgggccaagaaaatc	tctaagagccggacttggtc

manufacturer's instructions.

Statistical analysis

All data are expressed as means±standard error of the mean (SEM). Data analysis was performed with the SigmaStat statistical software (Jandel Corporation, San Rafael, CA, USA). Changes in variables within and between groups were analyzed by one-way ANOVA followed by the Bonferroni test. P values < 0.05 were considered statistically significant.

RESULTS

Bone mineral density changes

A decrease in bone density in the proximal portion of the tibias was observed 11 months after the ovariectomy (proving the development of osteopenia). This reaction could be reversed by the chronic E2 supplementation (Figure 2).

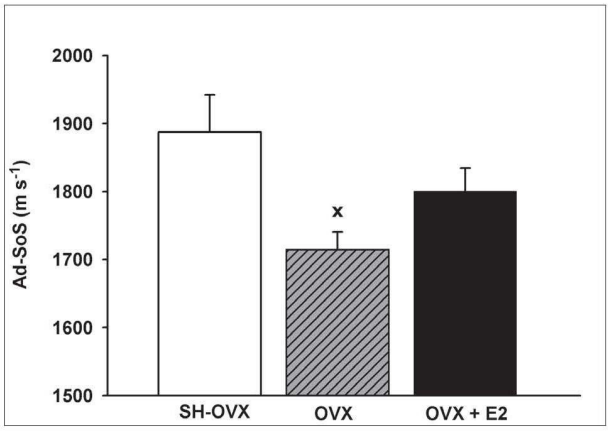

Figure 2. Bone density. Bone density changes in the proximal part of the tibia in sham-operated (SH-OVX), ovariectomized (OVX) or 17ß-estradiol-treated ovariectomized animals (OVX+E2) animals. Data are expressed as Ad-SoS and presented as means±SEM. ANOVA was followed by the Bonferroni test. X P<0.05 vs SH+OVX group.

Leukocyte-endothelial interactions

Using intravital microscopy, a significant increase in the number of firmly adherent leukocytes (as compared to the baseline values or data of the sham-operated SH-OVX animals) was observed in the OVX+IR group by the 180th min of reperfusion (Figure 3). No major differences, however, between the OVX+IR and OVX+SH+IR animals could be observed. These leukocyte-endothelial interactions were significantly ameliorated by E2 (see OVX+E2+IR group).

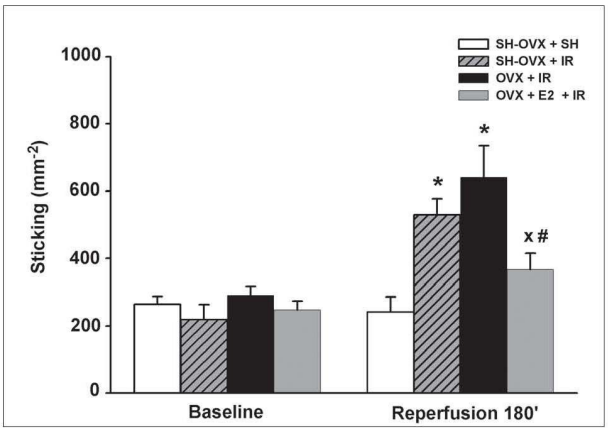

Figure 3. Leukocyte adherence. Firm adhesion (sticking) of neutrophil leukocytes in the postcapillary venules of the tibial periosteum in response to IR injury in sham-operated (SH-OVX+IR), ovariectomized (OVX+IR) or 17ß-estradiol-treated ovariectomized animals (OVX+E2+IR) animals in comparison with sham-operated animals without IR (SH-OVX+SH). Data are presented as means±SEM. ANOVA was followed by the Bonferroni test. * P<0.05 vs baseline, X P<0.05 vs OVX+IR group, # P<0.05 vs SH-OVX+IR group.

CD11b expression changes

The surface expression of adhesion molecule CD11b did not change significantly after sham operation, but the activation of the PMNs was increased approx. 1.8-fold in the OVX group subjected to IR (Figure 4). Similar increases were observed when SH-OVX animals or E2-treated OVX animals were subjected to IR.

Changes in ER-alpha and beta protein expression in the PMNs

On the basis of the available literature, a very low extent of ER expression was expected on the PMNs, hence relatively high amount of protein (75 µg/lane) was loaded during the Western blotting procedures.

Nevertheless, only low ER-alpha and ER-beta signals were detected, and we did not find statistically significant differences in ER-alpha expression within the different experimental groups (data no shown). ER-beta also afforded a very weak signal, which was weaker in the OVX animals in comparison with the Sham or OVX+E2 animals (Figure 5).

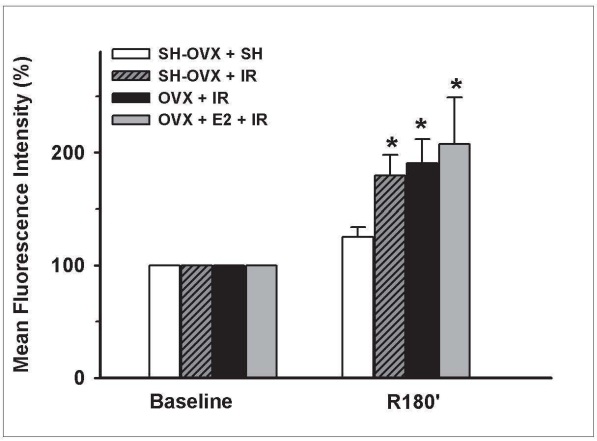

Figure 4. CD 11b expression. Changes in expression of the CD11b adhesion molecule on the surface appearance of PMN leukocytes in response to 60 min of ischemia followed by 180 min of reperfusion in sham-operated (SH-OVX+IR), ovariectomized (OVX+IR) or 17ß-estradiol-treated ovariectomized animals (OVX+E2+IR) animals in comparison with sham-operated animals without IR (SH-OVX+SH). Data are presented as means±SEM. * P<0.05 vs baseline.

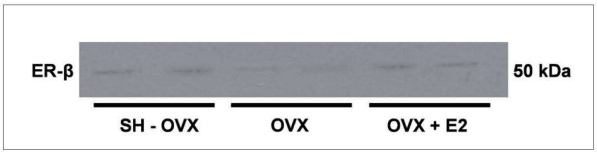

Figure 5. Estrogen receptor-beta protein expression. Representative Western blot demonstrating the expression of estrogen receptor beta (ERβ) in neutrophil leukocytes obtained from sham-operated (SH-OVX) or ovariectomized (OVX) or ovariectomized plus 17ß-estradiol treated (OVX+E2) animals.

Changes in ER-alpha and beta mRNA in the PMNs

The ER-alpha mRNA content of the samples did not reach the limit of detection (Figure 6A). The ER-beta mRNA was detected in most of samples of the Sham group, but never in the OVX groups, while in the OVX+E2 group the data were inconclusive (Figure 6B).

The possibility of technical failure could be excluded, since the expression of 18S RNA was very high and even in each cases, indicating that the amount and quality of the total RNA isolated from the cell lysates and the cDNA samples were satisfactorily high (Figure 6C).

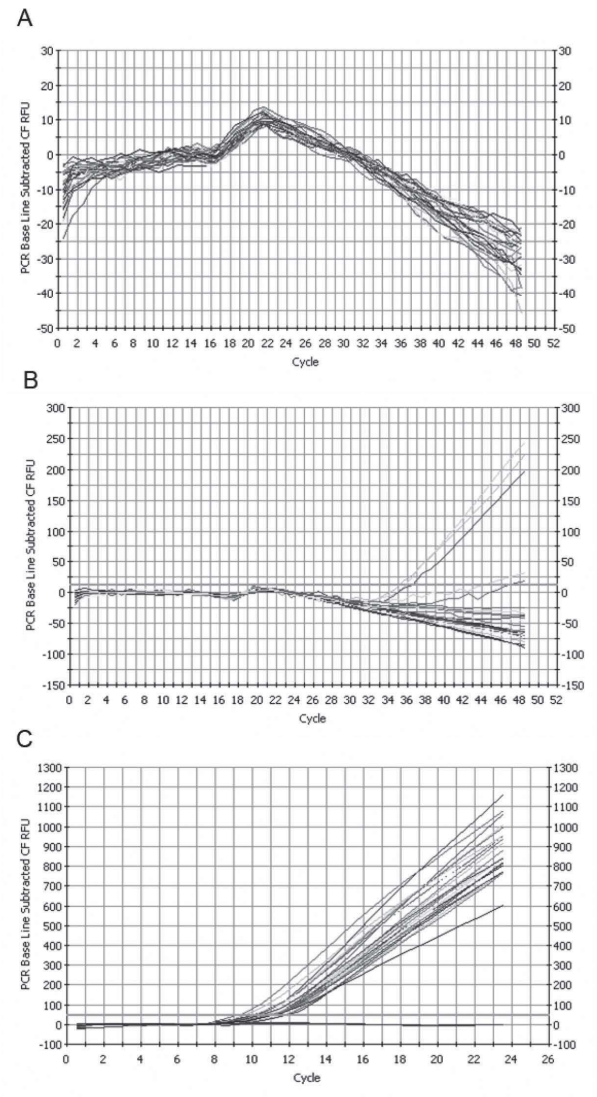

Figure 6. Estrogen receptor-alpha and beta mRNA expressions. Real-time PCR data for ER-alpha (A) and ER-beta (B) and 18S RNA signals of the samples (C).

DISCUSSION

The incidence of osteoporotic fractures remains to be high in the aging population.[13] So far it has not been clarified whether osteoporotic women are more prone to post-ischemic inflammatory bone complications than postmenopausal women receiving estrogen replacement therapy. In this study, we targeted this issue by applying an animal model of osteopenia combined with chronic estrogen replacement therapy. The periosteal microcirculatory consequences of tourniquet-caused ischemia were quantified by IVM after an adequately long-term follow-up of osteoporosis. The data show that osteoporosis or chronic estrogen deficit does not change the magnitude of the PMN-associated microcirculatory menace in

the event of transient ischemia of the osteoporotic limb. In this condition, however, chronic estrogen supplementation ameliorates bone demineralization and also decreases systemic and PMN-driven local periosteal inflammatory reactions. The protective, anti-inflammatory effects of E2 regarding the PMN-endothelial interactions were evident without influencing the expressions of the adhesion molecule CD11b which plays a role in the adhesion process of the PMNs to the venular endothelium. In our previous studies we have shown the alleviation of ischemia-reperfusion-induced activation of leukocyte-endothelial interactions after E2 treatment,[9] but not the effect on ICAM-1 expression, the endothelial adhesion molecule being responsible to a final stage of leukocyte-endothelial interactions. Hence we extended the examinations on the effects of E2 on estrogen receptor expression changes in the PMNs.

To date little is known about the role of ER receptors during the course of neutrophil-mediated microcirculatory inflammatory reactions. In vitro studies generally agree on the positive effect of E2 and the modulatory role of ER receptors regarding the outcome of leukocyte-driven inflammatory processes.[14] There are studies demonstrating that these reactions are dependent on ER-beta receptors.[5] Others emphasized the significance of a biological (transcription) modulator effect, because E2 can modify (via ER-alpha) the phosphorilation of genes being responsible for the transcription of adhesion molecules that play roles in the process of leukocyte-endothelial interactions.[15]

In our studies, the expression of both ER receptor mRNA in the PMNs was under the detection limit of RT-PCR. Only a few papers were published on this issue, and the data are controversial. Considerable species differences were detected and Yu *et al.* demonstrated tissue-specific expression of ER-alpha and beta.[16] Specifically, only the ER-beta protein was found in cows, but both subtypes could be traced at the mRNA level.[17] In a human study only ER-beta mRNA was found,[18] while in another study neither ER-alpha nor ER-beta mRNA were detected, though the proteins could be traced by Western blotting analysis.[19] The levels of ER proteins were similarly low in our studies. In humans, both ER-alpha and ER-beta are present on PMNs, and E2 enhances the expression of both proteins.[20]

CONCLUSION

In summary, chronic estrogen supplementation reduced the inflammatory complications caused by ischemia-reperfusion injury in the periosteal microcirculation, but the anti-inflammatory effects were mediated by mechanisms other than estrogen receptor expression changes on the circulating PMN leukocytes. As far as we are aware, this is the first report of leukocyte ER receptor expression in rats, and it is clear that further experiments are needed to improve the sensitivity of the methods to clearly define the potential role of estrogen receptors during the course of PMN-endothelial interactions.

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PAPER III.

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Periosteal microcirculatory action of chronic estrogen supplementation in osteoporotic rats challenged with tourniquet ischemia

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ABSTRACT

Aims: Transient ischemia of osteoporotic bones during elective orthopedic surgery or fracture repair carries risks for serious complications, and estrogen loss or replacement has a potential to influence ischemia–reperfusion-induced inflammatory activation. To clarify this, we investigated the periosteal inflammatory changes in a clinically relevant time frame in ovariectomized rats, an experimental model of postmenopausal bone loss. Furthermore, the effects of chronic estrogen supplementation on the postischemic local and systemic inflammatory reactions were assessed.

Main methods: Bilateral ovariectomy or sham operation was performed in 3-month-old female Sprague–Dawley rats. Five months later, estrogen replacement therapy with 17β-estradiol ($20\ \mu g^{-1}\ kg^{-1}\ day^{-1}$) or vehicle treatment was initiated. The microcirculatory inflammatory consequences of 60-min total hindlimb ischemia followed by 180-min reperfusion were examined 11 months after ovariectomy and were compared with those in 3-month-old animals.

Key findings: The osteoporosis that developed 5 months after ovariectomy was significantly ameliorated by estrogen replacement therapy. Both in ovariectomized and in non-ovariectomized animals, ischemia–reperfusion elevated the neutrophil adherence ~3-fold in the postcapillary venules of the periosteum (intravital microscopy), with an ~50–60% increase in intravascular neutrophil activation (CD11b; FACS analysis), an enhanced TNF-α release (ELISA) and periosteal expression of ICAM-1 (the endothelial ligand of CD11b; immunohistochemistry). Exogenous 17β-estradiol considerably reduced TNF-α release and the number of neutrophil–endothelial interactions in the periosteum, without affecting the CD11b and ICAM-1 expression changes.

Significance: Osteoporosis itself does not increase the magnitude of the limb ischemia–reperfusion-associated periosteal inflammatory reaction. Chronic estrogen supplementation, however, reverses osteoporosis and significantly ameliorates the microcirculatory consequences of transient ischemia.

Introduction

The etiology of senile osteoporosis syndrome is multifactorial, but the reduced estrogen levels in peri-menopausal women are clearly associated with an accelerated bone mineral density loss (Richelson et al., 1984). The structural bone deficit is thought to be attributable to enhanced resorption and simultaneously decreased bone formation, and several early studies provided convincing evidence that these changes can be suppressed or even prevented by restitution of the pre-menopausal estrogen levels (Christiansen et al., 1982).

The systemic and local consequences of endogenous estrogen loss and exogenous replacement in the circulatory system are more ambiguous. The incidence of septic, inflammatory complications is significantly lower in many trauma-hemorrhage conditions in females than in males (Sperry et al., 2008; Choudhry et al., 2005), and short-term estrogen pretreatment confers significant protection from ischemia–reperfusion (IR) injury and leukocyte activation in males (Burkhardt et al., 2008). However, other lines of evidence support the notion that estrogen supplementation may increase the risk of intravascular clotting complications in post-menopausal women (Cushman, 2002). Although the underlying mechanistic details are

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still unclear and the concept is debated (Seelig et al., 2004; Canonico et al., 2008), it is obvious that the skeletal and anti-inflammatory benefits of hormone replacement therapies should be carefully weighed against the possible circulatory side-effects. Given this background, it is important to note that the consequences of chronic estrogen depletion and repletion on the human bone circulation are still unclear. Under certain compromised flow conditions, the periosteal microcirculation may be a good indicator of the perfusion changes of the whole bone, but the microcirculatory effects of osteoporosis in this tissue layer are also largely unknown. The patency of the periosteal microcirculation is of particular importance in transient ischemic states such as traumas, fractures, soft tissue injuries or limb operations involving tourniquet application (Varga et al., 2008; Zhang et al., 2003). In these cases, both the bone and the periosteum undergo microvascular events reflected by perfusion insufficiencies and severe antigen-independent inflammatory reactions. These processes are mainly initiated by the increased adhesion of polymorphonuclear (PMN) granulocytes to the microvascular endothelium (rolling and then firm adhesion), followed by their migration to and accumulation in the perivascular tissues (Wolfárd et al., 2002).

As the prevalence of skeletal injuries increases after menopause, our studies were designed to determine whether hormonal replacement therapy might be of microcirculatory benefit in this subset of the osteoporotic population. The ovariectomized (OVX) rat is a well-established animal model for osteoporosis research, as it shares many similarities with the human disease. These include an increased rate of bone turnover (Wronski et al., 1986), and an initial rapid phase of bone loss and similar skeletal responses to treatment with estrogen, calcitonin, bisphosphonates and many other agents (Fleisch, 1993; Allen et al., 2004). In this context, our first objective was to observe the effects of osteoporosis and the consequences of estrogen therapy in a clinically relevant time frame. To this end, we first determined if chronic treatment with estrogen influences the OVX-triggered local periosteal microcirculatory reactions.

Secondly, we hypothesized that the periosteal microcirculation of osteoporotic rats would be more sensitive to the detrimental consequences of transient limb ischemia than that of estrogen-treated, age-matching controls. The results suggest that, even though osteoporosis itself does not amplify the IR-induced inflammatory responses, the periosteal granulocyte recruitment can be significantly reversed by chronic estrogen supplementation.

Materials and methods

Animals and experimental design

The experiments were carried out on female 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 experimental protocol was approved by the local animal rights protection authorities and followed the EC Directive 86/609/EEC and the National Institutes of Health guidelines for the care and use of laboratory animals.

In the first experimental series, 3-month-old animals (weighing 200–210 g) were ovariectomized (Group 1, OVX, n = 12), or sham-operation was performed (Group 2, Sham, n = 6). Five months later (at the age of 8 months), chronic estrogen therapy (Sims et al., 1996) was initiated 5 days/week with 20 μg kg^{-1} sc 17β-estradiol (E2, Sigma, St. Louis, MO, USA) in 6 animals of the OVX group (Group 1a). The E2 substitution was continued weekly until the end of the experiments (see time scheme in Fig. 1). The remaining OVX (Group 1b) and Sham animals received the vehicle for E2 (100% ethanol diluted in corn oil) in the same volume. Body weight changes and the development of osteoporosis were continuously followed in the proximal tibiae by means of ultrasonic densitometry (see later).

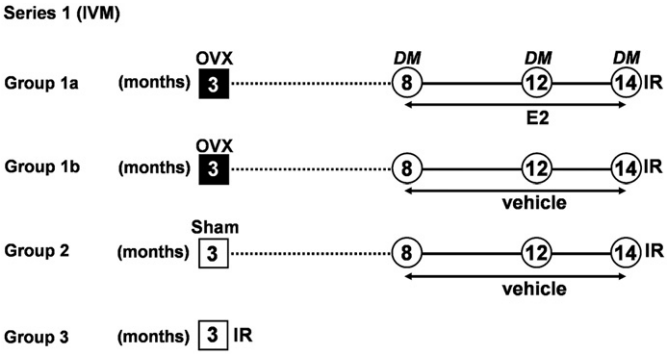

Fig. 1. The time sequence of surgical interventions, treatments and measurements in Series 1. OVX = ovariectomy; Sham = sham operation; DM = densitometry; IR = ischemia–reperfusion; IVM = intravital microscopy, E2 = 17β-estradiol treatment. In Series 2 identical protocols were applied to detect adhesion molecule changes.

Eleven months after OVX, the animals were subjected to a 60-min complete hindlimb ischemia followed by a 180-min reperfusion period (IR). Limb ischemia was induced by applying a tourniquet around the thigh and placing a miniclip on the femoral artery. Control hindlimb IR was conducted on another group of 3-month-old female rats (Group 3, n = 6). In this group the microcirculatory consequences of limb IR alone were investigated using intravital video microscopy (IVM).

A second series of experiments (Groups 4a, 4b, 5, and 6), with identical protocols (OVX or Sham combined with IR, n = 7–10), was performed to detect changes in the pro-inflammatory cytokine TNF-α concentrations in the plasma and in the expressions of adhesion molecules known to play a role in the process of PMN leukocyte adhesion to the endothelium. This was necessary because the fluorescent dyes used for IVM interfere with the flow-cytometry technique, and blood samples for the assessment of adhesion molecule expressions were therefore obtained separately. Tissue biopsies were also taken for detection of the tissue ICAM-1 changes by immunohistochemistry (see later).

Surgical procedure of ovariectomy

Animals were anesthetized with a combination of ketamine and xylazine (25 mg kg^{-1} and 75 mg kg^{-1} ip, 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. Thereafter, the abdomen was filled up with warm sterile physiological saline and the abdominal wall was closed in two layers. Sham-operated animals underwent identical procedures except that the Fallopian tubes and ovaries remained intact.

Quantitative ultrasound bone densitometry (QUS)

Under ketamine–xylazine anesthesia (as discussed earlier), bone density measurements were performed at the tibia and the tail with a DBM-Sonic 1200 (IGEA, Carpi, Italy) ultrasonic bone densitometry device; the changes in the average of the amplitude-dependent speed of sound (AD-SoS) were determined (Joly et al., 1999). After calibration, the AD-SoS values of the soft tissues (muscle and skin above the thigh) were determined, and the system deducted this value from the bone density. The AD-SoS values were calculated via a computer program and the average of 5 measurements was used at each time points. Twenty-one weeks after OVX, statistically significant density alterations were observed in the proximal tibia, and this location was therefore used for further QUS measurements at the ages of 8, 9, 12 and 14 months.

Surgical procedure for IVM

Intravital analysis of the periosteal microcirculation was performed at the end of the experimental protocol on 14-month-old animals. Anesthesia was induced with sodium pentobarbital (45 mg kg^{-1} ip) and sustained with small supplementary iv doses when necessary. The right carotid artery and the jugular vein were cannulated for the measurement of mean arterial pressure (MAP) and the administration of drugs and fluids, respectively. The animals were placed in a supine position on a heating pad to maintain the body temperature between 36 and 37 °C. 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 dissected free, 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 (Varga et al., 2008).

Experimental protocols

In the first series, after a 30-min stabilization period, the baseline cardiovascular and microhemodynamic parameters were determined (baseline; −60 min). The periosteal microcirculation was observed every 60 min during the 180-min reperfusion period.

In the second experimental series, blood samples from the carotid artery were taken at baseline and during the reperfusion period for the detection of changes in the plasma concentrations of TNF-α and in the expression of the adhesion molecule CD11b. TNF-α levels were determined from plasma samples taken at the 60th min of reperfusion. In a pilot study, the dynamics of the CD11b expression changes in response to limb IR were characterized; significant elevations were not found earlier than 120 or 180 min in the reperfusion period. Accordingly, this time frame was selected for flow cytometric evaluations of blood samples (see later).

Intravital video microscopy

The right hindlimb with the exposed tibial periosteum was positioned horizontally on an adjustable stage, and the microcirculation of the proximal tibia was visualized by IVM (Zeiss Axiotech Vario 100HD microscope, 100 W HBO mercury lamp, Acroplan 20× water immersion objective, Carl Zeiss GmbH, Jena, Germany), using fluorescein isothiocyanate (Sigma, St. Louis, MO, USA)-labeled erythrocytes (0.2 ml iv) for red blood cell staining, and rhodamine 6G (Sigma, St. Louis, MO, USA, 0.2%, 0.1 ml iv) for leukocyte staining. The microscopic images were recorded with a charge-coupled device video camera (AVT HORN-BC 12, Aalen, Germany) attached to an S-VHS video recorder (Panasonic AG-MD 830, Matsushita Electric Industrial Co., Tokyo, Japan) and a personal computer.

Video analysis

Quantitative assessment of the microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images, using image analysis software (IVM, Pictron Ltd., Budapest, Hungary). Leukocyte–endothelial cell interactions were analyzed within 5 postcapillary venules (diameter between 11 and 20 μm) per animal. Adherent leukocytes (stickers) were defined in each vessel segment as cells that did not move or detach from the endothelial lining within an observation period of 30 s, and are reported 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 given as the number of cells/vessel circumference in mm.

In the examined anatomical fields of the tibia, the majority of the vessels were not capillaries, but venules (see later); hence the total vascular density was calculated from the length of recognized vessels divided by the corresponding area (an average of 3 measurements per observation field was used).

Immune labeling and flow cytometric analysis of CD11b expression

The surface expression of CD11b on the peripheral blood granulocytes was determined through flow-cytometric analysis of whole blood in duplicate (Varga et al., 2008). One-hundred μl of whole blood was incubated with 20 μl of (50 μg ml^{-1}) mouse anti-rat monoclonal antibody (BD Pharmingen, San Jose, CA, USA) for 20 min. Negative controls were obtained by omitting the monoclonal antibody. The cells were then washed twice in Hanks buffer and centrifuged at 13,000 rpm for 5 min and the resuspended pellet was incubated with fluorescein isothiocyanate-conjugated polyclonal rabbit anti-mouse immunoglobulin (10 μg ml^{-1}; DAKO Cytomation, Glostrup, Denmark; 20-μl aliquots of reagents to 180-μl aliquots of blood cells). The cells were again washed twice, and the erythrocytes were lysed with a Lysing kit (Biodesign, Saco, ME, USA), after which the cells were washed twice again (6000 rpm, 5 min) and resuspended in 200 μl 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 labeled (activated) granulocytes (relative to the overall marker-bearing cells) and the mean fluorescence intensity (average marker density) were calculated.

TNF-α measurements

TNF-α levels were measured in duplicate, using a commercially available anti-rat TNF-α ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Detection of tissue ICAM-1 by immunohistochemistry

At the end of the experiments, both the limb subjected to IR and the contralateral one were removed and the tibias and the surrounding muscles were fixed in 4% phosphate-buffered formalin for 2–3 days. The tissues were then decalcified with an electrophoretic apparatus (for 2×4 h) using a special decalcifying solution (Sakura TDE30; Sakura Finetek Corp. Torrance, CA, USA). The samples were embedded in paraffin, and the 4-μm sections were placed on silanized slides. After conventional methods of dewaxing and rehydration (initiated in xylene, followed by decreasing concentrations of ethanol and methanol), tissue endogenous peroxidase was blocked with a mixture of methanol and 1% H_2O_2 for 5 min, and the nonspecific tissue antigens with conventional 2.8% cow milk. For ICAM-1 immunohistochemistry, mouse monoclonal anti-rat ICAM-1 antibody (BD Pharmingen, BD Biosciences, San Jose, CA, USA) was used as primary antibody (1:200; 30 min), this being followed by a biotinylated goat anti-mouse antibody conjugated to HRP polymer (Envision® System; Dako, Glostrup, Denmark) for 30 min which employs 3,3′-diaminobenzidine as chromogen. The sections were counterstained with hematoxylin (for 1 min) and examined by two independent histologists by means of light microscopy at 200× magnification. During the semiquantitative analysis, periosteal and intramuscular vessels were evaluated separately and the percentage of ICAM-1-positive vessels was calculated. The samples were allotted to one or another of the following semiquantitative categories (scores 1–8) (Table 1).

Statistical analysis

All data are expressed as means ± standard error of the mean (SEM). Data analysis was performed with the SigmaStat statistical software (Jandel Corporation, San Rafael, CA, USA). Changes in

Table 1
Scoring system for the analysis of ICAM-1 positivity in vessels from periosteal samples processed for immunohistochemistry.

Score	% of ICAM-1-positive vessels	Staining
1	<5%	Local
2		Diffuse
3	5–25%	Local
4		Diffuse
5	25–50%	Local
6		Diffuse
7	>50%	Local
8		Diffuse

variables within and between groups were analyzed by one-way ANOVA followed by the Bonferroni test. P values<0.05 were considered statistically significant.

Results

During the course of the experiments, the body weight of the animals increased in all groups (Fig. 2), but OVX was followed by a significantly higher weight gain as compared with that in the Sham group. After the initiation of the E2 therapy (at the age of 8 months), the weight gain decreased to the level for the non-OVX animals.

As shown by bone densitometry on the proximal tibia, osteoporosis developed 21 weeks after bilateral ovariectomy. The AD-SoS was significantly lower than that for the sham-operated animals ($1674 \pm 33\ m\ s^{-1}$ *vs* $1850 \pm 101\ m\ s^{-1}$, respectively). The OVX-induced osteopenia was completely restored by E2 therapy (Fig. 3).

At the beginning of IVM, the baseline values of the macrohemodynamic variables (including heart rate and MAP) in the various groups did not differ significantly and there were no significant hemodynamic changes relative to the baseline values during the experimental period. In all groups subjected to IR, a moderate decrease in MAP was observed in the first few minutes of reperfusion, but MAP thereafter stabilized at the control level (data not shown).

The number of firmly adherent (sticking) leukocytes was significantly increased in the postcapillary venules of the tibial periosteum in the 3-month-old controls, the Sham rats and the OVX animals at the end of the reperfusion phase (120–180 min). This phenomenon was nearly completely prevented by chronic E2 supplementation in the OVX animals (Fig. 4).

In the examined anatomical fields of the tibia, the majority of the vessels were not capillaries, but venules (Fig. 5). No significant differences were found in total vascular density between the 3-month-old controls ($268.1 \pm 10.1\ cm^{-1}$), the Sham ($262.6 \pm 7.1\ cm^{-1}$), the OVX ($261.9 \pm 7.6\ cm^{-1}$) or the OVX + E2 ($265.1 \pm 1.1\ cm^{-1}$) groups.

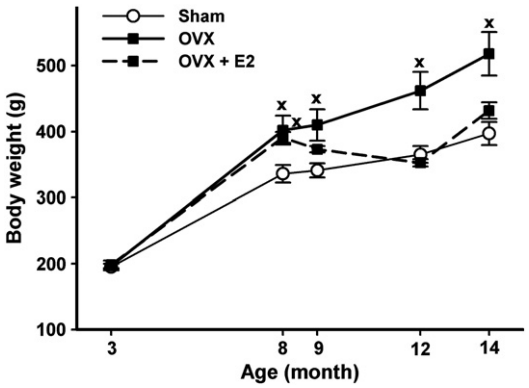

Fig. 2. Body weight changes in sham-operated (Sham) or ovariectomized animals treated with 17β-estradiol (OVX + E2) or vehicle (OVX). Data are presented as means ± SEM. ANOVA was followed by the Bonferroni test. $^{X}P<0.05$ *vs* Sham group; differences *vs* baseline (3 months of age) are not shown.

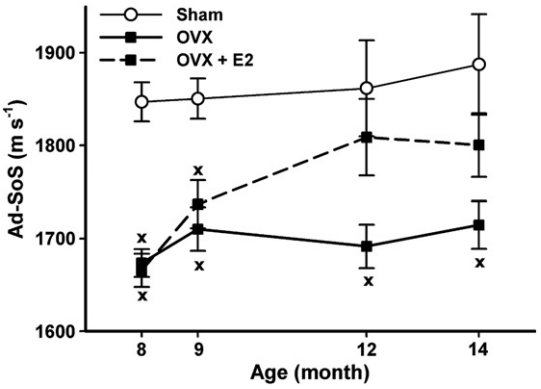

Fig. 3. Bone density changes in the proximal part of the tibia in sham-operated (Sham) or ovariectomized animals treated with 17β-estradiol (OVX + E2) or vehicle (OVX). Data are expressed as Ad-SoS and presented as means ± SEM. ANOVA was followed by the Bonferroni test. $^{X}P<0.05$ *vs* Sham group.

An increased surface expression of adhesion molecule CD11b was observed after 120 and 180 min of reperfusion; no major differences could be detected between the findings for the OVX and the Sham or the 3-month-old rats (Fig. 6). Chronic E2 administration did not influence this parameter.

The plasma TNF-α reached similar concentrations (young animals: $21.24 \pm 3.30\ pg\ ml^{-1}$; Sham: $22.16 \pm 5.79\ pg\ ml^{-1}$; OVX: $19.84 \pm 1.35\ pg\ ml^{-1}$) by the 60th min of reperfusion. E2 treatment, however, resulted in significantly lower TNF-α values (OVX + E2: $11.14 \pm 1.19\ pg\ ml^{-1}$) as compared with that for the OVX-challenged rats ($P<0.05$). No TNF-α was detected in control samples taken before the ischemic insult.

As assessed by immunohistochemical analysis (Fig. 7), the tissue ICAM-1 density in the vessels of the periosteum was significantly higher in the limbs subjected to IR (young animals: 1.67 ± 0.67; Sham: 1.83 ± 0.60; OVX: 2.17 ± 0.48; OVX + E2: 2.0 ± 0.52) than in the intact contralateral limbs in all experimental groups (young animals: 0.33 ± 0.21; Sham: 0.50 ± 0.22; OVX: 0.67 ± 0.21, OVX + E2: 0.50 ± 0.22). The intensity of the IR-induced ICAM-1-positive reaction was only moderate in the muscle tissue, and the data for the 3-month-old controls, the Sham, OVX and the OVX animals treated with E2 were also similar.

Discussion

Females are more resistant to circulatory shock and inflammation than males, but this gender-related anti-inflammatory protection is

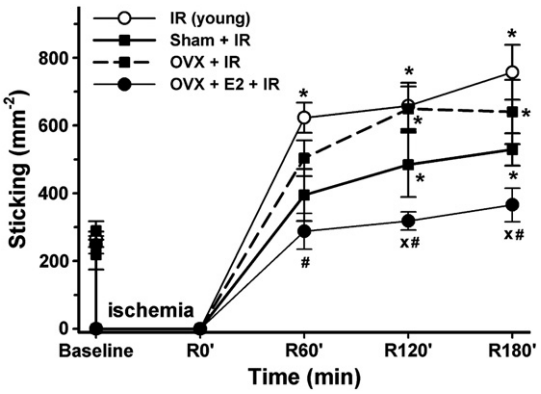

Fig. 4. Secondary leukocyte–endothelial cell interactions (sticking) in the postcapillary venules of the tibial periosteum in sham-operated (Sham + IR) or ovariectomized animals treated with 17β-estradiol (OVX + E2 + IR) or vehicle (OVX + IR) in comparison with 3-month-old (young) female rats (IR). Data are presented as means ± SEM. ANOVA was followed by the Bonferroni test. $^{*}P<0.05$ *vs* baseline, $^{X}P<0.05$ *vs* OVX + IR group, $^{\#}P<0.05$ *vs* IR group.

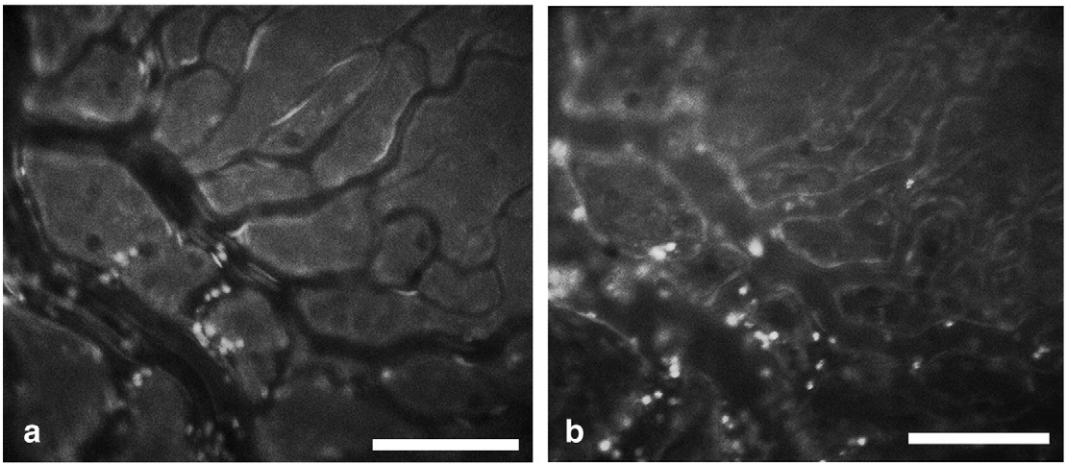

Fig. 5. Representative micrographs of the venular network of the tibial periosteum. Panel a: FITC-labeled erythrocytes, panel b: same field with rhodamine 6G-labeled leukocytes. Bar represents 100 μm.

lost during menopause or after OVX (Wichmann et al., 2000; Angele et al., 2000). As the incidence of traumas and osteoporotic fractures steadily rises in the elderly population (Gullberg et al., 1997), the question arises of whether osteoporotic women are more susceptible to postoperative or post-traumatic inflammatory bone complications than postmenopausal women receiving estrogen replacement therapy. We set out to answer this question by employing a chronic rat model of osteoporosis where the consequences of a long-term female hormone deficiency can be adequately estimated (Tan et al., 2003). The results showed that osteoporosis was reproducibly evolved after OVX, and the reversing efficacy of E2 therapy on osteopenia, together with the periosteal microcirculatory status of the tibia, could subsequently be evaluated in the long term. The data also showed, however, that the postischemic periosteal microcirculatory PMN recruitment and the expressions of the adhesion molecules CD11b and ICAM-1 (which play a role in the process of adhesion of the PMNs to the venular endothelium) undergo similar changes in this condition, irrespective of the age or the endogenous estrogen status of the animals. Thus, the primary message of our study is that a chronic estrogen deficit does not change the magnitude of the PMN-associated microcirculatory menace in the event of transient ischemia of the osteoporotic limb.

IVM allows direct observation and quantitative analysis of the microcirculation of the exposed tissues. With this technique, the consequences of an estrogen deficit and estrogen replacement have already been demonstrated in certain organs (Santizo et al., 2000; Watanabe et al., 2001), but never in the periosteum, though the protecting and feeding functions of the periosteum are hallmarked by the fact that restoration of periosteal microcirculation guarantees bone survival even in an environment with limited blood supply (Berggren et al., 1982). Additionally, osteoporosis has been shown to develop on the basis of an imbalance between bone resorption and formation, the effector cells being derived from mesenchymal osteoprogenitor cells found in the periosteum and endosteum, the former displaying predominance in this respect (Brighton et al., 1992). Furthermore, the experimental and clinical evidence suggests an important role of the periosteum not only in the pathogenesis of osteoporosis itself, but also in the effects of different anti-osteoporotic approaches (Allen et al., 2004). The periosteal region of the cortical bones exhibits a better anti-fracture efficacy than that of the endosteal region and it is proposed that the major target of different bone-reinforcing therapies is the periosteal site (Ferretti et al., 1995). Together with the nutritive functions of the periosteum, these observations underline the importance of the periosteum in the pathogenesis of different disorders also affecting the bone metabolism.

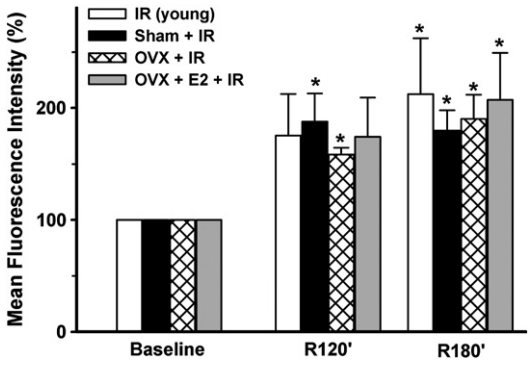

Fig. 6. Changes in expression of the CD11b adhesion molecule on the surface appearance of PMN leukocytes in response to 60 min of ischemia followed by 180 min of reperfusion in sham-operated (Sham + IR) or ovariectomized animals treated with 17β-estradiol (OVX + E2 + IR) or vehicle (OVX + IR) in comparison with 3-month-old (young) female rats (IR). Data are presented as means ± SEM. *$P<0.05$ *vs* baseline.

In our study, IR induced considerable increases in the periosteal leukocyte–endothelial interactions during the reperfusion phase, but these changes were similar in the aged OVX, the sham-OVX groups and the young animals. This observation adds a new aspect to the previous finding of enhanced PMN adhesion in the venules of the femur in the short run, i.e. 2 weeks after OVX in unstressed animals (Kasiyaphat et al., 2008). Similarly, an early enhancement of PMN infiltration has been demonstrated in the muscle of OVX rats after IR (Stupka and Tiidus, 2001). In fact, there is currently no consensus on the magnitude or pathologic role of leukocyte adhesion in non-skeletal tissues after OVX. OVX has been reported to enhance PMN accumulation in the brain of resting animals (Santizo et al., 2000) but other data indicated that OVX does not influence the PMN adherence in the brain tissue after transient ischemia (Xu et al., 2004). We suggest that these differences in baseline leukocyte adhesion might be explained by the different timelines. Use of a standardized experimental setup with matching sex, age and bone density conditions would solve this question.

The microcirculatory patency is influenced by functional and morphological changes in the microvasculature. As concerns the structural aspects, it has been shown that OVX leads to reduced blood flow in the bone (Kapitola et al., 1995) and a lower total capillary density in the heart (Jesmin et al., 2002) and brain (Jesmin et al., 2003), a phenomenon probably related to the well-known effect of estrogen on angiogenesis. In our chronic OXV rat model, the periosteal vessel density was not affected by OVX or E2 replacement therapy. It

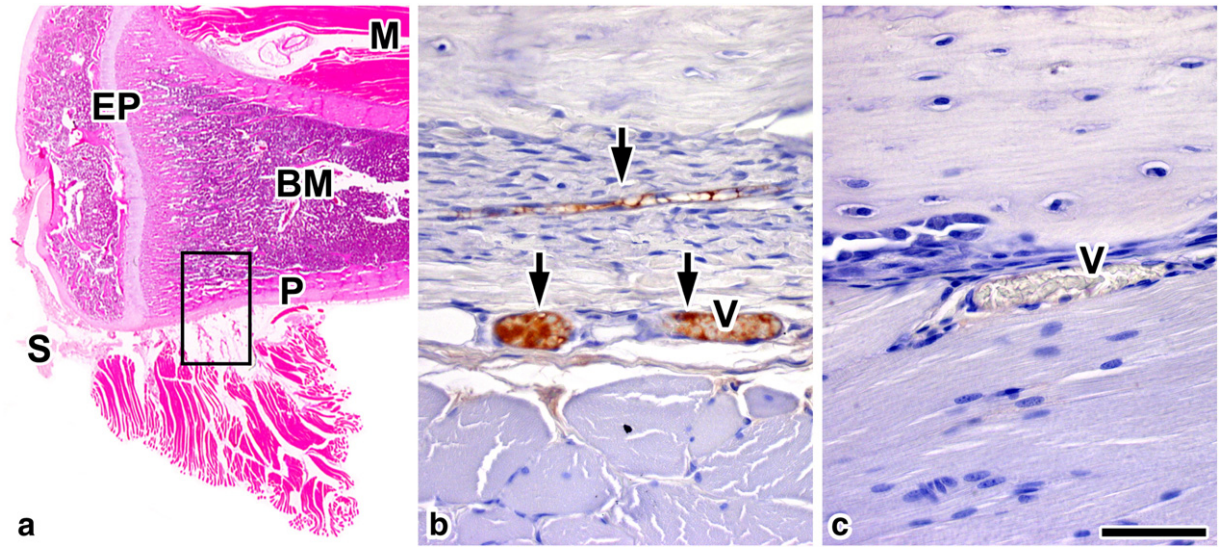

Fig. 7. Representative longitudinal section of the rat tibia surrounded by soft tissues (stained with ICAM-1 antibody plus hematoxylin). Left panel (a): tibial epiphysis (EP), bone marrow (BM), knee-joint synovia (S), muscle (M) and tibial periosteum (P) are indicated. Middle panel: positive staining for ICAM-1 (arrows) was found in the periosteal venules (V) after IR (b), but not in the contralateral (non-IR) limbs (c). Bar represents 50 μm.

is important to note that these changes were followed in the proximal tibial periosteum, where venules are the predominant vessel type. The postcapillary venules are predilectory sites for PMN–endothelial interactions, and thus a higher density may predispose to local inflammatory complications. The particularly high venular density of the periosteal microarchitecture may account, at least in part, for a tissue-specific response.

Although relatively little information is available on the effects of chronic OVX itself, there are numerous experimental studies on the microcirculatory changes of hormone substitution (e.g. E2). Since estradiol is protective in many forms of injury, it is possible that E2 is protective independently of the presence of OVX. Nonetheless, E2 has been shown to reduce leukocyte accumulation, infarct size (Jeanes et al., 2006) and oxygen free radical production (Florian et al., 2004) in OVX rats; PMN adherence and infiltration for instance can be effectively prevented by estrogen supplementation after OVX (Stupka and Tiidus, 2001).

The mechanism of action by which E2 influences PMN–endothelial interactions is not known in detail. It has been suggested that E2 reduces the expression of endothelium-derived adhesion factors such as P-selectin, vascular cell adhesion molecule-1, and ICAM-1 (Miller et al., 2004). Interestingly, some of these molecules also have a role in bone development, since LFA and ICAM-1 have been shown to influence the osteoclast function (Kurachi et al., 1993). It is hypothesized that an estrogen deficiency results in increased ICAM-1 expression on osteoclast precursors, which may contribute to the bone loss following menopause or OVX (Gao et al., 2000). The smaller diameter of the medullary resistance arterioles in OVX rats also suggests that endogenous estrogen exerts a significant dilator influence within the bone circulation (Soukhova-O'Hare et al., 2005). We investigated the changes in expression of CD11b, a key determinant of PMN-mediated injuries (Hentzen et al., 2000). Upon activation, CD11b (the αM part of the CD11b–CD18 complex) is transferred to the cell surface from preformed intracellular pools (Jones et al., 1988) and, as opposed to the constitutive CD11a, its expression is increased several-folds in response to IR stimuli. The considerably increased CD11b expression implies that complete limb ischemia leads to severe systemic inflammatory consequences in this model. The local expression of ICAM-1, the endothelial counterpart of CD11b, was also enhanced in the postischemic periosteum, where the PMN–endothelial interactions were simultaneously and visibly increased. These reactions were not influenced by E2 repletion, which suggests that E2 exerts its anti-adhesive effect independently of CD11b or ICAM-1. The mechanism of how estrogen can modulate *in vivo* PMN reactions demands further attention, but our results are in agreement with those of *in vitro* studies, where OVX did not influence the CD11b expression of the PMNs (Deitch et al., 2006). There is evidence of a cellular sexual dimorphism in the activation of PMNs, implying that PMNs from females respond to trauma and humoral stimuli to a lower extent than do those from males (Deitch et al., 2006). Further, estrogen directly modulates the expression of the neuronal isoform of nitric oxide synthase in PMNs (García-Durán et al., 1999). In our study, E2 treatment clearly reduced the IR-induced secondary PMN–endothelial interactions (i.e. sticking), but this potentially protective effect was not mediated by a mechanism involving tissue ICAM-1 expression changes. Although an increased PMN-derived NO production might reduce PMN adhesion, this reaction is ICAM-1 dependent (Dal Secco et al., 2006). Overall, the earlier findings may implicate that chronic estrogen supplementation modulates PMN activation directly, through a novel, specific, estrogen-sensitive pathway.

In our rat model, OVX was followed by definite diagnostic signs of osteoporosis 21 weeks later. In this condition, the postischemic periosteal microcirculatory complications were not aggravated further relative to the non-OVX age-matched controls. Similar changes were observed in TNF-α release, providing further evidence that OVX itself does not modify the inflammatory complications, but E2 supplementation greatly reduces this reaction. It has also been demonstrated by others 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 present data indicating the positive effect of prolonged E2 substitution on the PMN reactions and TNF-α release, however, points to another clinical implication of our findings; ovarial hormone deprivation supplemented with estrogen therapy (apart from the well-known positive effect in reducing the risk of osteoporotic fractures) also affords marked protection against inflammatory mediator release.

Conclusion

The periosteal microcirculatory consequences of tourniquet-caused ischemia were first quantified by IVM in an adequately long-term follow-up of osteoporosis. The efficacy of E2 therapy was

objectively judged in this condition. The overall message is that chronic estrogen supplementation not only ameliorates bone demineralization, but also decreases systemic and PMN-driven local periosteal inflammatory reactions.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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PAPER IV.

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ORIGINAL ARTICLE

Periosteal microvascular reorganization after tibial reaming and intramedullary nailing in rats

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Abstract
Background Intramedullary reaming and nailing of long bones impairs the endosteal circulation, often causing necrosis of the inner region of the bone cortex. We hypothesized that compensatory hypertrophy of the periosteal microcirculation may develop in response to mechanical destruction of the endosteum, and that this may affect bone survival in these circumstances. In these studies, nailing was performed with materials that affect regeneration of the endosteum differently, and the effects on the tibial periosteal microvasculatory organization were examined.
Methods In male Wistar rats, the right tibia was reamed and implanted with an inert titanium nail or a less osseointegrative polyethylene nail; the contralateral tibial endosteum was destroyed by reaming. Reaming without nailing or sham operation was performed on both extremities in two other groups of rats. Twelve weeks later, the anteromedial and anterolateral surfaces of the tibias were exposed by a microsurgical technique. The structural characteristics of the periosteal microcirculation (vessel density and distribution of vessel diameters) were determined by intravital videomicroscopy and computer-assisted analysis. The stability of the implants was assessed on the basis of grades 0–2 on a qualitative scale.
Results Tibial reaming alone caused significant increases in overall blood vessel and capillary densities in the periosteum compared with those of the intact tibias. Implantation with a titanium nail resulted in firm embedding of the nail and caused changes in the periosteal vasculature similar to those after reaming alone. In contrast, implantation of a polyethylene nail was followed by the development of marked instability of the endomedullary implant and significant increases in the percentage of capillaries and the vessel density in the periosteum.
Conclusions Destruction of the endosteal microcirculation per se brings about an increase in periosteal vascular density, which is further augmented if implantation is performed with a material which delays regeneration of the endosteal circulation.

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Introduction

The arterial blood supply of long bones is provided by three major sources: the nutrient artery sends branches to the endosteum, and the epiphyseal and metaphyseal arteries supply both ends and form anastomoses with branches of the nutritive artery. The blood vessels of the periosteum, however, run in a crosswise manner, supplying the superficial cortical bone and usually do not enter the deeper layers [1–3]. As a result, the connection between the periosteal and endosteal circulations under physiological conditions is relatively poor.

Restoration of the blood supply is a prerequisite of proper bone healing. Dislocated fractures in particular cause the destruction of periosteal or epiphyseal and metaphyseal blood vessels, and the normal regeneration of these systems is achieved only if healing is ensured by appropriate fracture management [2–5]. However, fracture-reduction techniques involving plate osteosynthesis or intramedullary nailing may also induce microcirculatory

disturbances by damaging the periosteum or the endosteum. Similar iatrogenic endosteal circulatory insufficiency may be a consequence of total hip arthroplasty. Intramedullary reaming and nailing, and the implantation of the femoral component of a total hip prosthesis also lead to cessation of the endosteal blood supply. This is important, because major nutritional defects to the cortical bone may give rise to partial bone necrosis [2–4, 6].

Microangiography has revealed that early hyperemia and the proliferation of blood vessels may develop in the periosteum in response to endosteal circulatory disturbances [2, 4, 7–9]. This phenomenon is usually detectable only during the first 2 weeks, whereas endosteal regeneration and remodeling of the inner cortical bone occur 8–16 weeks later, and it has been suggested that this is of major importance in bone healing after the implantation of prostheses [4–6, 9].

The objective of this experimental study was to clarify whether destruction of the endosteal microcirculation changes the vascular characteristics of the periosteum after standardized intramedullary damage. To this end, we designed a chronic rat model of tibial reaming and nailing with study of the periosteal microvascular consequences by intravital videomicroscopy (IVM). We hypothesized that intramedullary healing conditions would significantly affect the outcome of reactive periosteal microcirculatory changes. For this reason, we used inert materials which have different osseointegration properties and which are generally and safely used in clinical practice. Specifically, titanium was chosen because it induces nearly complete osseointegration and relatively fast remodeling of the surrounding bone [10]. Polyethylene was the other test material, because we hypothesized that in-growth of vascular elements and regeneration of the endosteal vessels would be different after use of this material. Thus, we compared and characterized the periosteal microvascular alterations caused by titanium or polyethylene nails, prosthesis materials expected to result in higher or lower intramedullary stability, respectively, 12 weeks after implantation into the tibia.

Materials and methods

All in-vivo animal experimental procedures were approved by the Animal Welfare Committee of the University of Szeged and were performed in accordance with NIH Guidelines (Guide for the Care and Use of Laboratory Animals).

Surgical technique

Male Wistar rats (380 ± 40 g) were randomly allotted to one or another of 4 groups. After intraperitoneal (ip) injection of Na-pentobarbital (45 mg/kg), skin incisions were made above the proximal metaphysis of both tibias under sterile conditions. In the sham-operated group ($n = 5$), the bones were left intact (control group). In the reamed group ($n = 5$), both tibias were reamed with a specially-designed flexible microdrill series of increasing diameter (from 0.2 to 0.8 mm) after a small drillhole had been made over the proximal metaphyses. The endosteum and the inner cortical bone were destroyed by reaching the required drill diameter and the skin incision was then closed. Destruction of the endosteal circulation was confirmed histologically in supplementary experiments ($n = 3$; Fig. 1b).

In the titanium nail-implanted group ($n = 6$), both tibias were reamed and a 13-mm-long, 0.88-mm-wide titanium nail (Protetim, Hódmezővásárhely, Hungary) was implanted into the right tibia with a special small impactor so that the nail fitted tightly into the medullary cavity. The left tibia was only reamed; nail implantation was not performed. The optimum dimensions of the implants were chosen in pilot studies on the basis of X-ray radiographs of the tibias of rats of similar weight and age. In the polyethylene nail-implanted

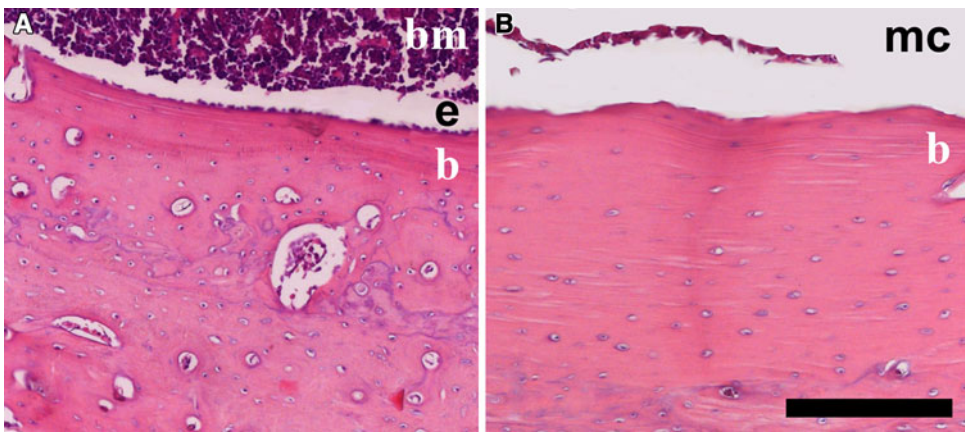

Fig. 1 Representative micrograph showing the tibial endosteum in sham-operated rats (**a**) (longitudinal section, H&E staining). The effect of reaming on the structure of the tibial endosteum (after 1 week) is shown in part **b**. *bm* bone marrow, *e* endosteum (with osteoblasts on the surface of the cortical bone), *b* bone, *mc* medullary cavity (with fragments of bone marrow). The *bar* denotes 200 μm

group ($n = 6$), specially designed polyethylene implants (Protetim) were inserted intramedullarily into the right tibia, using an identical surgical approach, and again the left tibia was reamed, but not implanted. The animals were not immobilized after the procedures; the reaming of both tibias imposed similar loads on the legs.

Examination of the periosteal vasculature

Twelve weeks after surgery, the animals were anesthetized with Na-pentobarbital (45 mg/kg ip), the trachea was intubated and the left jugular vein was cannulated for drug and fluid administration. Under an operating microscope at 4× magnification, the anteromedial and anterolateral surfaces of the tibial periosteum were exposed by use of an atraumatic microsurgical technique. The limbs were positioned horizontally on a special stage to expose periosteal vessels suitable for IVM. After IVM, each implant was subjected to a manual examination of stability by the same investigator (FG).

Intravital videomicroscopy

The periosteal vessel structure and microcirculation were examined by use of a Cytoscan A/R (Cytometrics, USA) intravital videomicroscope via the orthogonal polarization spectral (OPS) imaging technique (Fig. 2). The images were recorded with an S-VHS videorecorder (Panasonic AG-MD 830) on a minimum of 3 non-overlapping areas on the upper, middle, and lower thirds of the diaphysis surfaces [11, 12].

Video analysis

The captured intravital microscopy images were analyzed with the aid of a computer-assisted analysis system (IVM software; Pictron, Budapest, Hungary). The contours of capillaries, arterioles, and venules were marked, and the vessel density (defined as the length of vessels per observation area) was recorded separately. Capillaries were defined as vessels in the diameter range 5–7 μm. The proportion of capillaries was calculated by the software and expressed as small vessel length relative to total vessel length, as a percentage.

Stability tests

At the end of the experiments, each implant was subjected to manual traction 5 times for 2 s under microscopic visual control by the same investigator (FG). The reaction was categorized on a qualitative scale according to the following criteria: firmly anchored, stable: 2; loose movements, cannot be removed: 1; unstable, removable: 0.

Histology

Reamed or control tibias were harvested 1 week after surgery, fixed in 4 % phosphate-buffered formalin for 3 days, and decalcified with an electrophoretic apparatus (for 2 × 4 h) using a special decalcifying solution (Sakura TDE30; Sakura Finetek, Torrance, CA, USA). The samples were embedded in paraffin, sectioned (4 μm), and stained with hematoxylin–eosin.

Statistical analysis

Data analysis was performed with SigmaStat statistical software (Jandel, San Rafael, CA, USA). Differences between groups were analyzed by two-way ANOVA, followed by the Holm–Sidak test or the ANOVA test (stability testing). All data are expressed as means ± standard

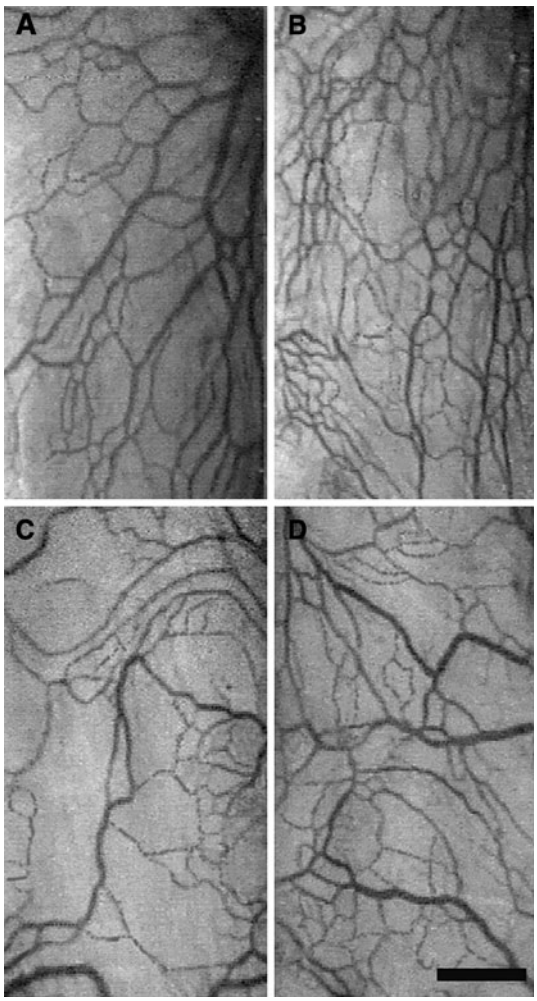

Fig. 2 Representative micrograph of the microvascular architecture of the anteromedial (**a**) and anterolateral (**c**) surfaces of the tibial periosteum of animals after reaming and after nailing with polyethylene (**b**, **d**). The *bar* denotes 250 μm

error of the mean (SEM). p values <0.05 were considered statistically significant.

Results

The effects of reaming on the vascular and capillary densities in the periosteum

In the sham-operated control animals, the vessel density was significantly higher on the anteromedial side of the tibial periosteum than on the anterolateral side (Fig. 3a). As was confirmed histologically, the reaming method used in this study caused destruction of the endosteum and the cortical bone (Fig. 1). In response to reaming, a significant increase in anterolateral periosteal vessel density was observed (Fig. 3a). The capillary density (the proportion of capillaries) proved to be significantly lower on the anterolateral side than on the anteromedial side. This density became markedly higher in response to reaming on both the anteromedial and anterolateral sides (Fig. 3b).

The effects of implantation with a titanium or a polyethylene nail on the vascular and capillary densities in the periosteum

In comparison with the contralateral side, nailing with polyethylene resulted in significantly higher vascular densities on both the anteromedial and the anterolateral sides of the tibial periosteum (Fig. 4a, c). In response to polyethylene nailing, the capillary density in the tibia was found to be higher on the anterolateral side than after reaming alone (Fig. 4d). Although vascular density was higher in the anteromedial periosteum for animals implanted with a titanium nail (Fig. 4a), this material did not affect vessel density i.e. the overall vascular or capillary density, at any of the other locations (Fig. 4b–d).

The stability of the titanium or polyethylene implant

Macroscopically visible loosening of the implanted nails was not observed. Micro-movements could not be evoked in the titanium nail-implanted group, demonstrating that anchoring of the implant in the tibial medullary cavity was sufficient (grade 2.0 ± 0). In the rats implanted with a polyethylene nail, however, the stability was significantly lower (0.33 ± 0.21), and high instability was observed for 4 implants.

Discussion

A variety of methods have been used to study the microcirculation of the bones, e.g. microangiography [2, 3] and electron microscopy to examine structural characteristics, and laser–Doppler flowmetry [13, 14] and radioactive microsphere techniques [7] to characterize functional details and dynamic aspects of the blood supply. Most of these techniques, however, have inherent limitations. The loading of capillaries during conventional microangiographic examinations can be incomplete, which limits assessment of the structure of the periosteal vessels [2, 9]. Electron microscopy or histology with light microscopy is mostly used for qualitative assessments, because quantitative statistical analysis with serial sectioning of the tissues is particularly difficult. Laser–Doppler flowmetry provides bulk information only on dynamic changes, in a large tissue mass [13, 14]. In contrast, IVM and intravital microscopic techniques enable computer-based quantitative analysis, and well-defined vessel structures can be studied. IVM after microsurgical exposure of the periosteum provides an opportunity for simultaneous visualization of microvascular structure and function [11, 12, 15].

Microangiography has revealed that after reaming the inner half or two-thirds of the cortical part of long bones

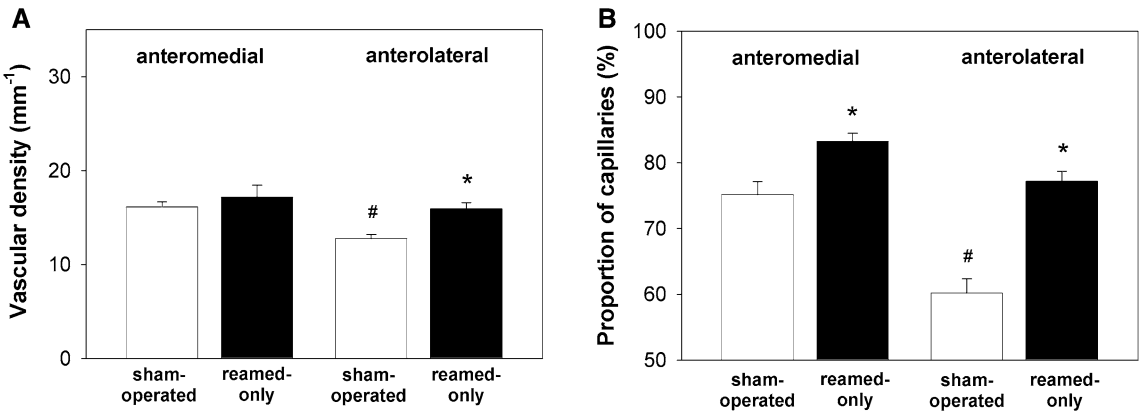

Fig. 3 The effects of reaming on vessel density (**a**) and on the proportion of capillaries (**b**) in the anteromedial and anterolateral periosteum of the rat tibia. $^{*}p < 0.05$ between corresponding sham-operated and reamed-only groups; $^{\#}p < 0.05$ between anteromedial and anterolateral sides

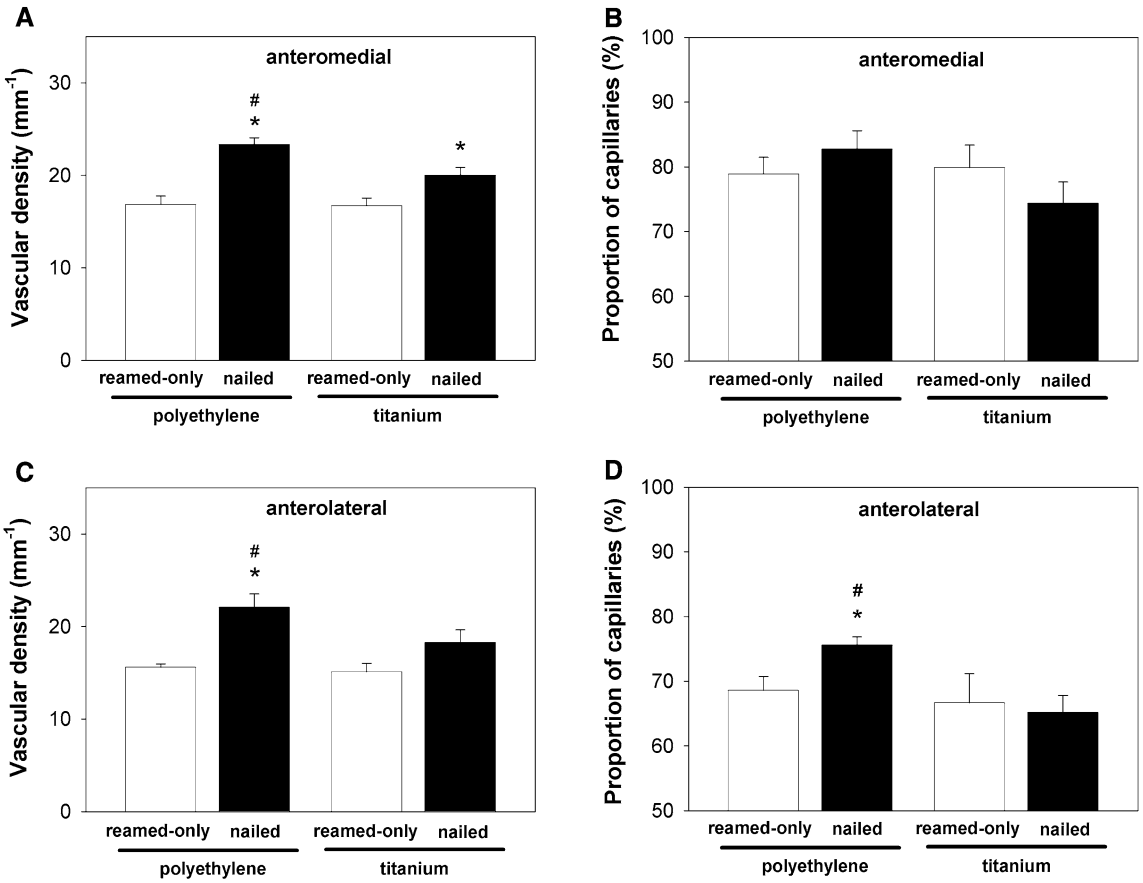

Fig. 4 The effects of nailing with polyethylene and titanium on the vascular (**a**, **c**) and capillary densities (**b**, **d**) on the anteromedial (**a**, **b**) and anterolateral sides (**c**, **d**) of the tibial periosteum in rats. $^{*}p < 0.05$ between corresponding reamed-only and nail-implanted groups, $^{\#}p < 0.05$ between corresponding groups implanted with a polyethylene or a titanium nail

becomes avascular and partially necrotic. This is thought to be caused by destruction of the endosteal blood supply and obstruction of the perforating arteries by fat embolism [3–6, 16–18]. Such observations also suggest that a significant proportion of the cortical blood flow is provided by the endomedullary vessels. The contribution of the periosteal microcirculation in the time frame of healing is controversial. Microangiographic studies by Rhinelander et al. [2, 4, 18] revealed the predominance of the periosteal blood supply during the early phase of non-dislocated fracture healing; this is later taken over by the endosteal blood supply. Reicher et al. [16] reported enhanced periosteal blood flow in the first 2 weeks after reaming without subsequent implantation, and this was supported by the observations of Pazzaglia et al. [9] that neovascularization and an increased periosteal vascular density lasted for only 10–14 days after surgery. In contrast, Gustilo et al. [5] reported that the periosteal blood vessel density remained elevated for 2–3 months after reaming of long bones in dogs.

After reaming, the marrow space is filled with hematoma, which is gradually followed for 30–40 days by the formation of reactive new bone and loose connective tissue [9]. This area has been found to be free of vessels for 4–8 weeks [5, 6]. Thus, revascularization from the metaphysis is initiated relatively late by the proliferation of metaphyseal and epiphyseal arteries and this process lasts for 8–12 weeks. The periosteal vessels make little or no contribution to this process [3, 5]. In our study, however, signs of neovascularization were clearly observed in the tibial periosteum: the proportion of capillaries was significantly increased on the anterolateral side 12 weeks after intramedullary reaming.

A similar process occurs in long bones after reaming and implantation with primary stable implants [9]. During the first 2 postoperative weeks, intense periosteal neovascularization and microcirculatory improvement occur, this process later progressively decreasing. Revascularization has been shown to be virtually complete by approximately 12–16 weeks after implantation; this seems to be because of the proliferation of metaphyseal and epiphyseal vessels [6].

To the best to our knowledge, this is the first study to reveal the periosteal microcirculatory reactions evoked by

intramedullary polyethylene nail implantation. The results furnish evidence of marked instability of the nail and the non-occurrence of osseointegration in the host bones. Implant loosening is well known to induce connective tissue formation [9, 19] and micromovements of the implant inhibit adequate revascularization around the endoprosthesis. However, we detected a significant increase in blood vessel density and, in particular, capillary density in the tibial periosteum in rats implanted with a polyethylene nail. An explanation of this finding is that a polyethylene implant is not integrated into the medullary canal. The chemical composition and the smooth surface, may contribute to the lack of stable fixation and to micromovements, thereby leading to inadequate restoration of the endosteal circulation [9]. The endosteal blood supply is probably insufficient to supply the cortical bone, and may be compensated by increased periosteal sources.

Wear of polyethylene implants is an important clinical problem [20, 21]; products of polyethylene wear after total hip arthroplasty cause formation of granulation tissue and loosening of an endomedullary implant. These observations indicate the need for development of new or more enduring polyethylene implants to prevent the micromovements leading to the wear process and the formation of granulation tissue. Complete implant stability was achieved in our study by use of a titanium nail, after which micromovements were not detected, and the proportion of small vessels and the blood vessel density did not differ significantly from those of the reamed-only tibias. Stable implantation with a titanium nail does not affect periosteal blood vessel formation, and the lack of microcirculatory reorganization on the surface of bones may therefore be regarded as a sign of a preserved or restored endovascular microcirculation.

Conclusions

This study has demonstrated the possibility of qualitative and quantitative analysis of the periosteal vasculature and microcirculation in rats by use of OPS videomicroscopy. The periosteum of the long bones does not have significant vascular connections with the endosteal circulation under physiological conditions, but reaming leads to neovascularization, with increased vessel density in the periosteum 12 weeks after surgery. This process is basically unaltered after endomedullary implantation of a titanium nail, whereas a polyethylene nail significantly increases the periosteal vessel density. This phenomenon is affected by the features of bone healing: insufficient osseointegration is associated with enhanced periosteal vessel formation. Hence, it can be concluded that destruction of the endosteal microcirculation in long bones gives rise to moderate compensatory reactions, but an endomedullary implant with poor osseointegration properties induces a marked compensatory increase in periosteal vessel density.

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Conflict of interest None.

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KÖSZÖNTŐ

A csont mikrokeringési változásainak kísérletes vizsgálati lehetőségei

Experimental studies for the examination of microcirculatory changes in the bone

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Bevezetés/Célkitűzés: Kísérleteink célja a periosteum mikrokeringési változásainak feltérképezése, valamint a velőűrfelfúrás által okozott periostealis mikroér-reakciók modellezése, időbeli változásuk megismerése volt. További vizsgálatainkban jellemeztük és összehasonlítottuk a humán csípőízületi endoprotetikában használatos anyagok mikrokeringési hatásait a csonthártyában. *Anyag és módszer:* Hím Wistar-patkányok jobb oldali tibiájába endomedullaris titán-, acélötvözet, illetve polietilén implantátumot helyeztünk mikrosebészeti módszerrel. Intravitalis videomikroszkóp segítségével vizsgáltuk a tibia anteromedialis és anterolateralis felszínén a periosteum érstruktúráját (össz-érdenzitás, kapillárisok aránya), valamint mikroszkópos mechanikai teszt segítségével az implantátumok stabilitását. Kontrollcsoportban vizsgáltuk a velőűrfelfúrás hatását a periostealis érstruktúra változásaira a műtét után 6 és 12 héttel. *Eredmények:* A velőűr felfúrása önmagában szignifikánsan növelte az anteromedialis periosteum érdenzitását, valamint a kapillárisok sűrűségét a beavatkozás után 12 héttel. Mindhárom implantátum fokozta az érdenzitást az anteromedialis oldalon, az anterolateralis oldalon azonban csak a polietilén implantátum esetében észleltünk jelentős periostealis kapilláris- és érdenzitásfokozódást. A titán- és acélimplantátumokkal szemben a polietilénből készült implantátum nem váltott ki érdemi osseointegrációt. *Következtetés:* A patkány tibiavelőűr felfúrása kompenzációs érújdonképződést eredményez, amely megnöveli a periostealis érdenzitást 12 héttel a beavatkozás után. A hosszú csöves csontok endostealis mikrokeringésének roncsolása által okozott periostealis érreakciót a teljes osseointegrációval járó implantátumok nem befolyásolják lényegesen, míg az osseointegrációra képtelen implantátum nagyon jelentős érújdonképződést okozhat.

Kulcsszavak: periosteum, mikrokeringés, implantátum, osseointegritás

Introduction/aims: Our aim was to characterize the periosteal microvascular reaction induced by the destruction of endosteal vasculature by reaming, and to monitor the time sequence of the events. We have also compared the microcirculatory effects of different implant materials that are most frequently employed in human endoprosthetics. *Materials and methods:* The right tibia of male Wistar rats was reamed by microsurgical means and implanted with titanium, steel-alloy or polyethylene nails. Intravital videomicroscopic examinations of the anteromedial and anterolateral surfaces of the tibial periosteum were performed to evaluate the changes in the overall vascular and capillary densities. Microscopic mechanical tests were used to assess the stability of the implants. In control groups, reaming without nailing was performed and the microvascular changes were examined 6 and 12 weeks after surgery. *Results:* Reaming alone caused a significant increase in the vascular density of the anteromedial periosteum and a bilateral increase in capillary density. Vascular density at the anteromedial side was increased after all of the implant materials applied, while only polyethylene induced remarkable increases in the capillary and vascular densities at the anterolateral side. Furthermore, polyethylene did not bring about osseointegration. *Conclusions:* Enhanced periosteal angiogenesis could be demonstrated after 12 weeks following tibial reaming. The compensatory microvascular reactions evoked by destruction of endosteal microcirculation of long bones are not influenced by osseo-integrative implant materials, but materials of poor osseointegration properties induce considerable compensatory increases in the microvascular density of the periosteum.

Keywords: periosteum, microcirculation, implantation, osseointegration

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Bevezetés

A hosszú csöves csontok vérellátását a periostealis és endostealis mikroér-rendszerek biztosítják. Utóbbiak az arteria nutritiva velőűri ágainak és a csont két végpontját ellátó epiphysealis és metaphysealis artériák anastomosisából integrálódnak egységes endostealis érhálózattá.[1–3] A nagyobb dislocatióval járó törések durván károsítják mindkét érstruktúrát, melyek megfelelő töréskezelés esetén, a csontgyógyulás során regenerálódhatnak.[2,4–6] Ortopédiai, traumatológiai beavatkozások során, pl. a velőűr felfúrásakor, az endostealis érrendszer önmagában is károsodhat, ekkor a periosteum vérellátása biztosíthatja a csont túlélését jelentő revascularisatiót. A periostealis mikrokeringés jelentőségét támasztják alá azok a klinikai megfigyelések is, melyek szerint a jelentős lágyrész-sérüléssel és periosteumkárosodással járó traumák után elhúzódó gyógyulással vagy pseudoarthrosis kialakulásával kell számolni.[7–9]

Az osteosynthesis során alkalmazott velőűrszegzés az endosteum szükségszerű, szelektív roncsolásával jár. Az endostealis keringés megszűnése következtében a corticalis belső fele-kétharmada érmentessé válik, élő alakos elemei elhalnak, részben necrotizál, de a mészváz megőrzi struktúráját.[2,4,5,10] Az endostealis keringés károsodását markáns változások követik a periosteumban, hyperaemia és érproliferatio alakul ki, gyakori a periostealis csontképződés.[2,4,11–13] Mintegy 8–16 héttel később az endostealis keringés a periostealis és az epi- és metaphysealis erek felől regenerálódik, és a necroticus corticalis csontállomány szerkezete is remodellálódik. Az endostealis revascularisatio elengedhetetlen a törések végső rögzüléséhez, és az implantátum és a törés stabilitásának alapvető feltétele az endostealis keringés regenerációja.[4,6,10,13]

A humán ortopédiai-traumatológiai gyakorlatban alkalmazott implantációk mikrokeringési hatásai pontosan nem ismertek, de korábbi vizsgálatainkban felvetettük, hogy az implantátumok anyaguktól függően eltérően befolyásolhatják a csontok mikroér-struktúrájában bekövetkező reaktív változásokat.[14] Jelen kísérleteink elsődleges célja a velőűrszegzés által kiváltott periostealis reakciók modellezése, jellemzése és időfüggésük vizsgálata volt. További célkitűzésünk szerint meghatároztuk és összehasonlítottuk a csípőízületi endoprotetikában használatos főbb alapanyagok (titán, polietilén, valamint acélötvözet) mikrokeringési hatásait kísérletes körülmények között.

Anyag és módszerek

Kísérleteinket a NIH irányelvei alapján (Guide for the Care and Use of Laboratory Animals) végeztük a Szegedi Tudományegyetem Munkahelyi Állatkísérleti Bizottsága jóváhagyásával.

Műtéti technika

Felnőtt hím Wistar-patkányokat (380 ± 40 g) öt csoportba osztottunk. Az *A* jelzésű (n = 10) csoportban nem végeztünk műtétet (álműtött kontroll), a *B* (n = 10) csoport esetében 45 mg/kg Na-pentobarbital intraperitonealis adását követően az állatok mindkét tibiáját a proximalis metaphysis közelében a corticalison készített nyíláson át mikrofúró sorozattal felfúrtuk. A flexibilis fúrósorozat átmérője 0,2 mm-től 0,8 mm-ig terjedt. A felfúrás során roncsoltuk az endosteumot és a corticalis belső felszínét, és a velőűrt a kívánt átmérőig tágítottuk (az endostealis mikrokeringés destructióját szövettani vizsgálattal igazoltuk[14] korábbi vizsgálatainkban). A bőrön ejtett metszést öltéssel zártuk, immobilisatiót nem alkalmaztunk a műtétet követően. Az állatok további alcsoportjaiban (n = 5-5) a periostealis érstruktúrát a 6. posztoperatív héten, illetve 12 héttel a felfúrás után vizsgáltuk (n = 5-5) mind az álműtött, mind a felfúrt tibiák esetében. A *C* jelű (n = 5) csoportban a felfúrást követően 0,8 mm átmérőjű 13 mm hosszú, speciálisan a kísérletes célra készített titánimplantátumot (Protetim Kft., Hódmezővásárhely) ültettünk a jobb oldali tibia velőűrébe. A bal oldali tibiába nem került implantátum, csupán felfúrtuk. Előzetes kísérleteinkben hasonló testtömegű és fejlettségű állatok tibiájáról készített radiológiai felvételek alapján választottuk ki az implantátum optimális méretét. A beültetéshez speciális impaktort használtunk, ügyelve arra, hogy az implantátum feszesen illeszkedjen a felfúrt velőűrbe, és teljes egészében intraosseális helyzetű legyen. A negyedik *D* (n = 5) csoportban a velőűrt a felfúrást követően hasonló módon, acélötvözetből készült implantátummal láttuk el (Protetim Kft., Hódmezővásárhely). Az ötödik *E* (n = 6) csoport esetében a velőűrt hasonló módon készítettük elő, és polietilén implantátumot ültettünk be, azonos technikával. A *C–E* csoportokba tartozó állatok bal oldali tibiáját minden esetben felfúrtuk, de nem implantáltuk (így az állat mindkét operált alsó végtagját azonos módon terhelte). A periostealis mikrokeringést a *C–E* csoportokban a műtétek után 12 héttel vizsgáltuk.

A periostealis érstruktúra vizsgálata

A primer műtét után 12 héttel Na-pentobarbital (45 mg/kg i.p.) anaesthesiában a légúti biztosítást követően a bal vena jugularisba kanült helyeztünk, majd operációs mikroszkóp segítségével (4×) a tibia anteromedialis és anterolateralis felszínén atraumatikus mikrosebészeti technikával feltártuk a periosteumot. A végtagokat vízszintes helyzetben pozicionáltuk, hogy alkalmasak legyenek az intravitális mikroszkópos vizsgálatra.

Inravitális videomikroszkópia

A periostealis microcirculatio vizsgálatára Cytoscan A/R (Cytometrics, USA) intravitális videomikroszkópot alkalmaztunk, orthogonalis polarizációs spektrális (OPS) technikával. A felvételeket videorekorderrel (Panasonic AG-MD 830) rögzítettük, minden tibia diaphysis felszínén legalább 3 (felső, középső, alsó harmad) régióban.

Videoanalízis

Az intravitális mikroszkópos képeket komputerasszisztált képanalizáló rendszerrel elemeztük (IVM szoftver, Pictron Kft., Budapest). A kapillárisok, arteriolák, venulák kontúrját kijelöltük, és látóterenként meghatároztuk az érdenzitást (érhossz/terület). Kapillárisnak minősítettük az átlagosan 5–7 mikrométer átmérőjű ereket; ezek hányadát a szoftver segítségével határoztuk meg, összevetve arányukat a teljes mért érhosszal (kapillárisok hossza / teljes érhossz).

Stabilitási vizsgálatok

Az érstruktúra elemzését követően valamennyi implantátum stabilitását ugyanaz a vizsgáló (G. F.) mikroszkóp alatt, kézi trakcióval (ötszöri húzás, egyenként 2 másodpercig ismételve) elemezte a következő semikvantitatív kategóriák alapján: stabil, rögzült, nem mozgatható implantátum (2 pont); mozgatható implantátum, de nem távolítható el (1 pont); instabil, eltávolítható implantátum (0 pont).

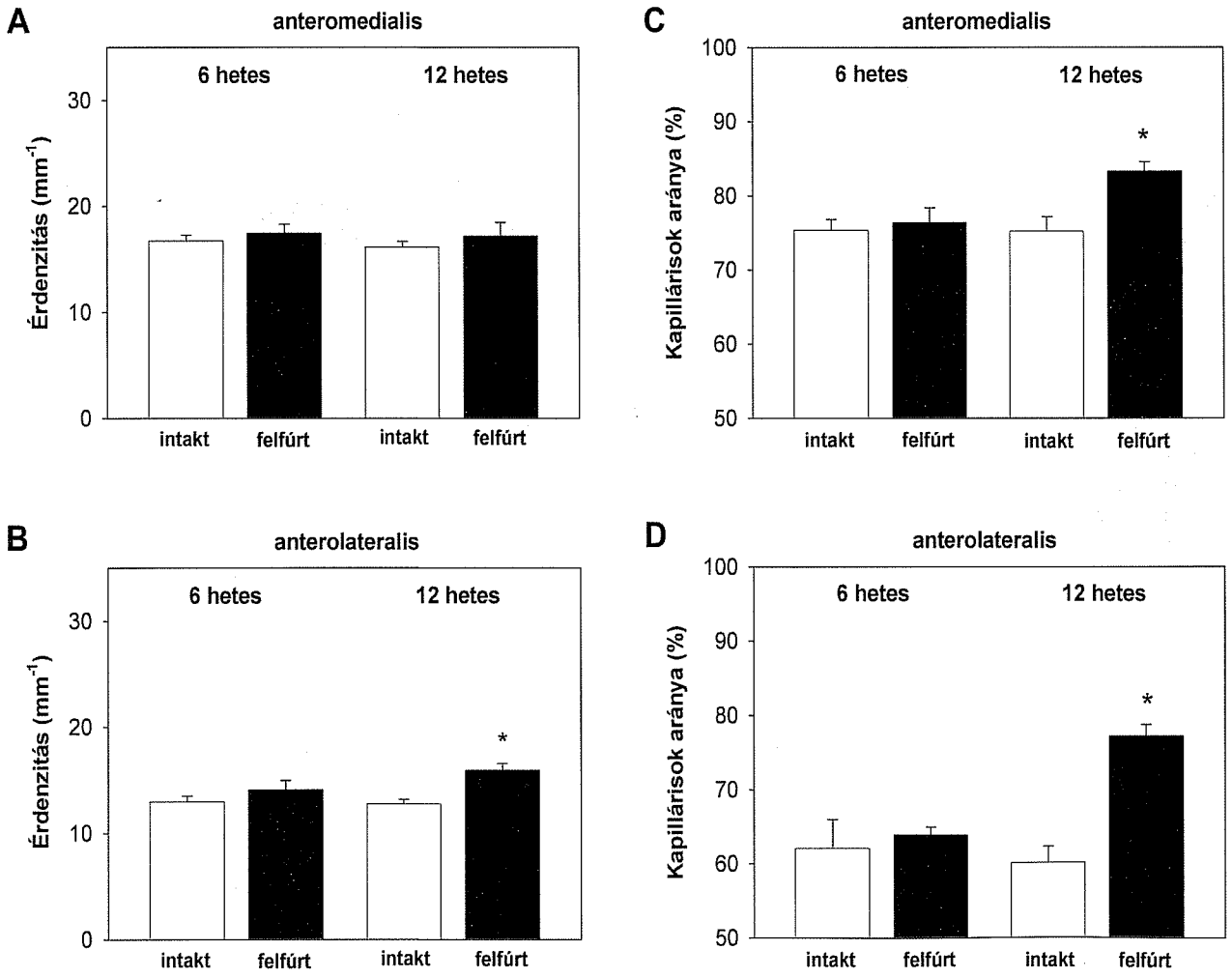
Statisztikai analízis

Az adatok analíziséhez SigmaStat statisztikai szoftvert (Jandel Corporation, San Rafael, CA, USA) alkalmaztunk. Az érdenzitás vizsgálatoknál két szempontos ANOVA tesztet használtunk, melyet a Holm–Sidak-teszt *post hoc* követett, a stabilitási vizsgálatoknál ANOVA tesztet használtunk. Statisztikailag szignifikánsnak tekintettük a különbségeket $p < 0,05$ esetén.

Eredmények

A velőűrfelfúrás hatása a periosteum mikrokeringésére

Hat héttel a velőűr felfúrását követően nem észleltünk szignifikáns változást sem az érdenzitásban, sem a kapillárisok százalékos arányában a tibiák anteromedialis és anterolateralis oldalán *(1.A–D ábra)* az álműtött csoporthoz képest.



1. ábra. A felfúrás hatása a periosteum érdenzitására (A, B) és kapilláris sűrűségére (C, D) a tibia anteromedialis (A, C) és anterolateralis (B, D) oldalán 6 és 12 héttel a beavatkozást követően, patkányon. Átlag ± SEM. $*p < 0,05$ vs intakt álműtött állatok

Tizenkét héttel később a periostealis össz-érdenzitás magasabb volt az anterolateralis oldalon *(1.B ábra)*, míg a kapillárisok denzitása mindkét vizsgált oldalon szignifikánsan magasabb volt *(1.C–D ábra)*. Itt meg kell jegyezni, hogy az intakt tibiák anterolateralis oldalán lényegesen alacsonyabb volt az össz-érdenzitás és a kapillárissűrűség az anteromedialis oldalhoz képest *(1.A–D ábra)*, ami a periostealis érellátás anatómiai sajátossága lehet patkányon.

Az implantációs anyagok hatása a periosteum mikrokeringésére

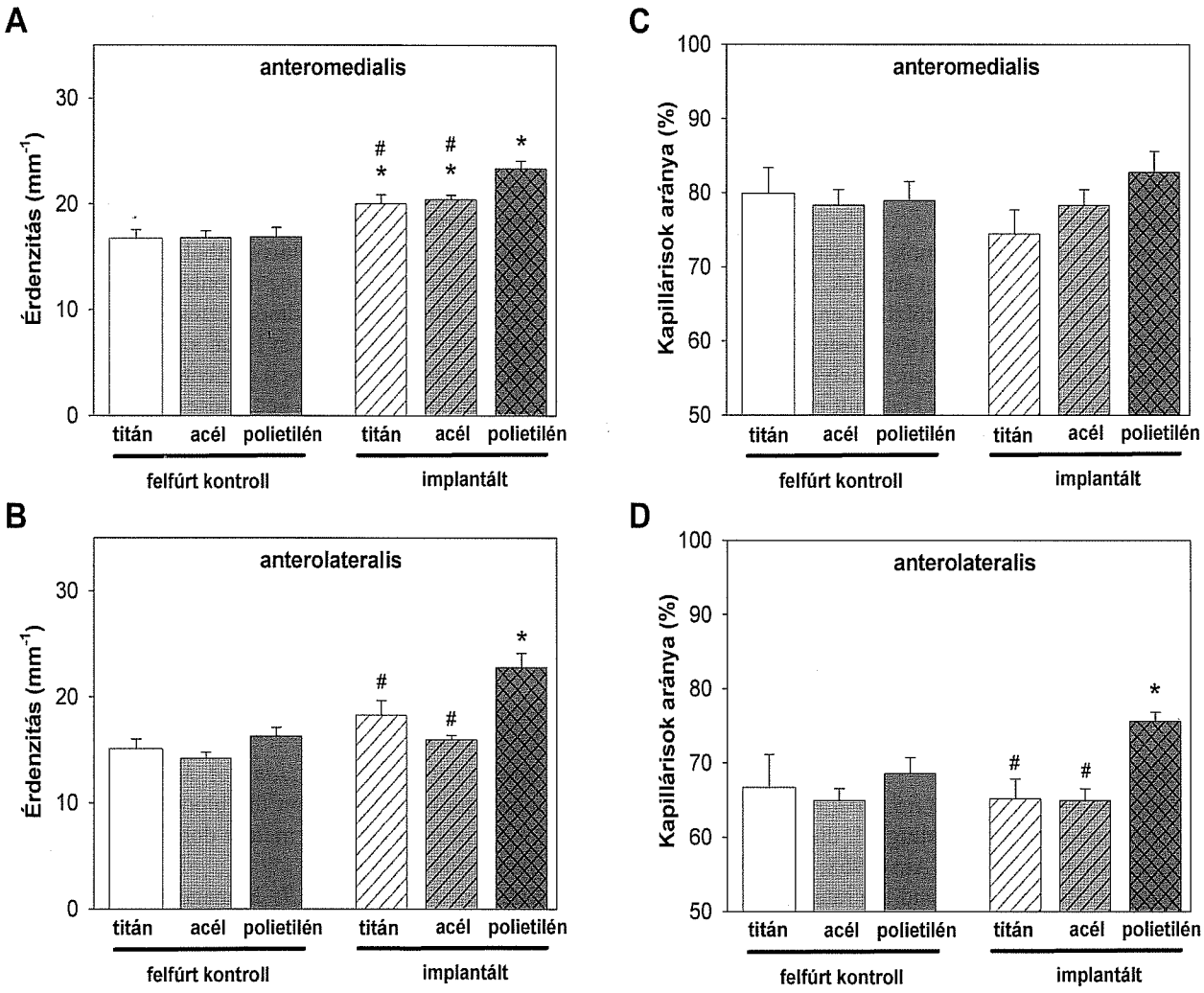
Az ellenoldali, csupán felfúrt tibiájú végtaghoz viszonyítva mindhárom implantátum szignifikáns érdenzitás-fokozódást okozott az anteromedialis oldalon *(2.A ábra)*. Az érdenzitás fokozódásán túl más hatást nem észleltünk sem a titán-, sem az acélötvözetből készült implantátumok hatására, a polietilén azonban nemcsak itt, hanem az anterolateralis oldalon is fokozta az érdenzitást, valamint a kapillárisok relatív arányának növekedését *(2.B, D ábra)*.

Az implantátumok stabilitása

Makroszkópos vizsgálattal instabilitást egyik esetben sem észleltünk. Mikroszkópos vizsgálattal a titánimplantátumok esetében mikromozgást sem lehetett kiváltani (2 ± 0). Az acélötvözetből készült implantátumok esetében hasonló eredményt kaptunk (2 ± 0), mely az implantátum stabil rögzülését, osseointegrációját jelzi. A polietilén implantátumok stabilitása ettől szignifikánsan eltért, kisebb volt (0,33 ± 0,21), 4 esetben nagyfokú instabilitást észleltünk.

Megbeszélés

Állatkísérletes modellünk további diagnosztikus és terápiás vizsgálatokhoz szolgáltat alapot. Kimutattuk, hogy a tibiavelőűr felfúrása és az endosteum roncsolása kompenzációs érdenzitás-növekedést okoz a periosteumban, és az újonnan képződött erek zöme kapilláris méretű. Adataink sze-



2. ábra. A velőűrbe helyezett titán-, acélötvözet és polietilén implantátumok hatása a periosteum érdenzitására (A, B) és kapilláris denzitására (C, D) a tibia anteromedialis (A, C) és anterolateralis (B, D) oldalán, patkányon. Átlag ± SEM. $^{*}p < 0{,}05$ vs csak felfúrt contralateralis oldal, $^{\#}p < 0{,}05$ vs polietilén az implantált oldalon

rint ez a folyamat a műtétet követő 6. hét után válik kimutathatóvá, és a 12. héten még észlelhető patkányokon.

Ismert, hogy a velőűr felfúrását követően a haematoma és csonttörmelék felszívódik,[13] majd a beavatkozást követő 4–8 héttel később a metaphysis felől elkezdődik a revascularisatio.[6,10] Ez a folyamat a meta- és epiphysealis erek proliferatiója révén feltételezhetően 8–12 héten át tart, és a korábbi irodalmi adatok szerint a periostealis erek csak minimális mértékben vesznek részt az endostealis regenerációs folyamatban.[5,6]

A csöves csont felfúrását követően behelyezett titán- és acélötvözet implantátumok stabilan rögzültek, mikromozgást nem észleltünk, és a periostealis ér- és kapillárisdenzitás sem különbözött lényegesen az ellenoldali kontrollértékektől. Ezek alapján feltételezzük, hogy a biológiailag inert anyagok (így a titánimplantátum) esetében csak a felfúráshoz hasonló mértékű endostealis érregeneráció jön létre, mely nem vált ki fokozott periostealis érdenzitás-növekedést. A titánimplantátummal azonos eredményeket kaptunk az acélötvözetek esetében is. Ugyanakkor a polietilénnel történt implantációk esetében szignifikáns érdenzitás-fokozódást észleltünk a periosteumban az anteromedialis és az anterolateralis oldalon is, és az anterolateralis oldalon a kapillárisok denzitása is jelentősen megnőtt. Feltételezzük, hogy az osteointegrációt nem eredményező polietilén implantátumok körül szabályos endostealis érhálózat helyett szabálytalan érstruktúrájú kötőszövet alakulhat ki,[15,16] mely megmagyarázhatja a periostealis érstruktúrák kompenzációs proliferatióját.

A polietilén implantátumok kopástermékei által kiváltott lazulást ma széles körben kutatják, hiszen a jelenség jelentős problémát okoz a csípőízületi endoprotetikában.[17,18] A képződött granulatiós sarjszövet osteolysist okozhat, ami a protézis kilazulásához vezethet, melynek során megváltozik és károsodik az újonnan kialakult endostealis keringés. A létrejövő microvascularis viszonyok a vizsgálatunk során alkalmazott polietilén implantátumok esetében ehhez hasonlóak lehetnek.

Cement nélküli protézisek esetében gyakori tünet a terhelési combfájdalom, melynek hátterében a fém-csont eltérő elaszticitása, a protézis mikromozgása áll. A tünetnek nincs lényeges terápiás jelentősége, átmeneti tehermentesítés megszünteti a panaszokat.[19] Radiológiai felvételen ilyen estekben nem észlelhető lazulás, de a 3 fázisú scintigraphia enyhe denzitásfokozódást mutathat a szárvég körül, valamint diffúzan, periproteticusan a cortexben és a periosteumban. A jelenség hátterében álló microvascularis változások összhangban állhatnak a titánimplantátum esetében észlelt enyhe periostealis érdenzitás- és kapillárissűrűségfokozódással *(2.A ábra)*. Radiológiailag is meglazultnak tűnő protézis esetében egyértelmű dúsulás észlelhető periproteticusan, a cortexben és a periosteumban.[20] A periproteticus endomedullaris dúsítást a kialakult granulatiós sarjszövet érbősége, a cortex és a periosteum intenzív dúsítását a fokozott periostealis ér- és kapillárisdenzitás okozhatja, melyet modellkísérletünkben a polietilén implantátummal ellátott tibiák esetén észleltünk *(2.A, B, D ábra)*.

A meglazult csípőízületi szárkomponens cseréjére számos műtéti módszer áll rendelkezésre. Wagner és munkatársai[21] technikája során a femur longitudinalis osteotomiáját követően a csontot hosszában felezik, megnyitják és eltávolítják a femoralis komponenst. A szerzők a periosteum szigorú kíméletét és húzóhurkos osteosynthesist ajánlanak, mivel a periosteum leválasztása és az azt károsító rögzítőlemez-felhelyezés esetén az osteotomia gyógyulása elhúzódik. Megfigyelésük hátterében az állhat, hogy ilyen esetben a csontgyógyulás csak a periosteum felől várható, mivel a lazulás miatt károsodott endostealis mikrokeringés nem játszik érdemi szerepet a csontgyógyulásban.[22]

Bemutatott új adataink szerint a cementes protézisek alapanyagául szolgáló acélötvözetből készült implantátumok meglepően jó, a titánhoz hasonló stabilitást és microvascularis változásokat eredményeztek kísérletes körülmények között. A közelmúltban hazai szerzők radiológiailag igazolták az osseointegrációt a cementes protézisek cement nélküli technikával történő beültetése során,[23] és középtávú vizsgálatukban nem észleltek jelentős lazulási arányt. Habár a szakmai állásfoglalás elvetette a cementes alapanyagú protézisek cement nélküli implantációjának alkalmazását, jelen kísérletes adataink is alátámasztják az acélötvözet alkalmazásakor kapott korábbi, kedvező eredményeket.[23]

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PAPER VI.

Microvascular disturbances in the periosteal and endosteal membranes following excessive osteosynthesis. Perfusion characteristics in the proximity of osteosynthesis materials in a pseudoarthrotic patient.

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Case Report

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This study was conducted to examine the tibial microcirculatory disturbances induced in the periosteum and the endosteum by long-term fixation modalities simultaneously involving the periosteum and endosteum in an orthopaedic patient. Two years previously, two long plates had been applied on both sides of the tibia and extension shaft of a cemented total knee endoprosthesis, and pseudoarthrosis had developed as a consequence of the long-term fixation. The roles of microcirculatory disturbances were assumed behind this phenomenon, and microcirculatory measurements were therefore performed to detect the concomitant microcirculatory perfusion deficit in the nearest proximity of the osteosynthesis materials during their surgical removal upon re-operation. Intraoperative perfusion measurements revealed significantly lower perfusion values in both the periosteal and the endosteal membrane compartments in the pseudoarthrotic tibia as compared with those observed on the contralateral side. This study provides important quantitative details regarding the long-term microcirculatory compromise elicited by excessive orthopaedic prostheses.

Previous medical history: During the period 1999-2007, the patient underwent a sequence of prosthesis implantations in both knees to treat osteoarthrosis: unicondylar knee arthroplasties followed by total endoprostheses, and finally two revision prostheses with extension shaft and homologous spongiosa grafts were implanted. In 2009, a left tibia diaphysis fracture occurred below the end of the prosthesis shaft; treatment: non-weight-bearing plaster for 8 weeks, plus physiotherapy. In February 2011, the patient displayed left limb pain and tibial hypertrophic pseudoarthrosis (by X-radiography); treatment involved DC plate osteosynthesis plus a circular plaster. After 6 months, the plate was broken and pseudoarthrosis of the tibia occurred; treatment: the fracture ends were refreshed, the fracture gap was filled with homologous spongiosa, and two long, angular stable plates were placed on the medial and lateral sides of the tibia. After a further 6 months (at time when the patient came into our field of view), pseudoarthrosis was diagnosed again.

Status at the time of the current operation: Pain, fracture of both plates, and hypertrophic pseudoarthrosis of the tibia (by X-radiography).

Description of the current surgical intervention: A special extension shaft with distal holes serving for the placement of locking screws was designed and manufactured. The plates that had previously been placed on the pseudoarthrosis of the left tibia were removed, together with their partially broken screws and the tibial component of the knee prosthesis. Subsequently, a new extension shaft was inserted into the tibial medullary cavity by bridging the fracture, and this was compressed with two locking screws distally. The polyethylene plate in the metallic backing of the new tibial component was replaced with a new one 15 mm

in width. The bone ends of the pseudoarthsosis were refreshed and the fracture gap was tightly filled with autologous spongiosa from the left iliac crest and platelet-rich plasma concentrate (Symphony^{TM}, DePuy) (Sheth U et al 2012) in order to induce osteogenesis. Microcirculatory measurements were conducted during removal of the old implants.

Present status (6 weeks after the last operation): The X-ray shows callus formation in the dorsal part of the fracture; good angulation of the fracture ends; the patient uses a knee-ankle-toe orthosis and avoids loading of the osteosynthesis.

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Methods

Intraoperative microcirculatory measurements were performed with the permission and signed consent of the patient and with the approval of the Ethical Committee of the Medical Faculty of the University of Szeged

Measurement of the tibial periosteal and endosteal microcirculatory variables by using laser-Doppler flowmetry

The blood flow in the periosteum and endosteum was recorded with a laser-Doppler flowmetric device (supplied by a 780-nm laser diode; PeriFlux System 500, Perimed, Järfälla, Sweden) with a sterilized fibre-optic probe (# 416, “dental probe”; fibre separation: 0.25 mm, penetration depth ~ 1 mm). The tibial periosteum was explored via a conventional anterior incision on the re-operated limb and via a small (~ 2 cm long) skin incision on the contralateral limb. The flow probe was held perpendicularly to the surface of the periosteum by means of a plastic holder which reduced the contact pressure on the observed area and restricted the angular movements of the probe. The endoprosthesis was then removed and the endosteal membrane compartment was approached through the bone cavity. A small hole was drilled in the anterior cortex, providing access to the endosteum at the opposite (inner surface of the posterior) medullary wall (Figure 1). The size of the drilled hole allowed perfect fitting of the flow probe. Since an endoprosthesis was present on the contralateral (non-operated) side, the endosteal circulation could be approached distally to the local shaft, somewhat below the level of the measurement on the re-operated side; the difference was ~ 1 cm. Given the examination depth of the laser-Doppler device and the thickness of the periosteal and endosteal membranes, mostly the periosteum and the endosteum plus a portion of the underlying cortex could be examined.

Characteristic flow curves paralleling the heart circles were reproducibly detected in the $\tau = 0.2$ s mode, showing that pressure artifacts were avoided. After the required signal quality had been reached, recordings were made in 30-s periods and were repeated 3 times. Tissue perfusion was expressed in arbitrary Perfusion Units (PU); before the measurements, the probe was calibrated with the special Motility Standard supplied by the manufacturer. Data were collected and stored on a computer and subsequently analysed with the computer software supplied together with the device.

Throughout the entire observation period, the room temperature (20±2 °C) and the core temperature of the patient were maintained constant; the stable macrohaemodynamic parameters were recorded continuously.

Statistical considerations

Since this study relates to only one patient and the measurements were conducted repeatedly at basically the same locations, no statistical comparisons were performed. Raw data (expressed in arbitrary units) are presented in Figure 2E to illustrate the potential differences.

Results

Considerably lower periosteal blood flow values were measured in the re-operated (pseudoarthrotic) tibial periosteum (Figure 2B) than in the contralateral non-operated limb (Figure 2A) (average levels of 76 and 106 PU, respectively) (Figure 2E). Much lower perfusion values were observed in the proximity of the endosteum, however. Specifically, an average endosteal perfusion of 30 PU was found in the non-operated tibia (Figure 2C). An even lower perfusion level was measured in the re-operated tibial endosteal region (average 9 PU; Figure 2D) even in the presence of a characteristically good signal quality (Figure 2E).

Radiographs taken 6 weeks after the operation revealed callus formation in the dorsal part of the fracture and good angulation of the fracture ends (Figure 3). The patient now walks with the aid of a knee-ankle-toe orthosis, without loading the osteosynthesis.

Discussion

Fracture healing is a complex, multifactorial process requiring the anatomical and functional integrity of the surrounding biological membranes of the bone (Macnab and Dehoas 1974).

The fracture reduction and osteosynthesis techniques applied for the fixation of fractures (plate osteosynthesis and intramedullary nailing) therefore cause circulatory disturbances by impairing the periosteum or the endosteum (Barron et al. 1977; Kowalski et al. 1996; Pazzaglia 1996; Mueller et al. 2009, Koo et al. 2010). Both experimental and clinical observations suggest that disruption of the continuity of the periosteum or the endosteum delays bone healing, but impairment of only one or other of these structures does not hinder the overall fracture healing (Kregor et al. 1995; Seibold et al. 1995). The anatomical connection between the two systems is most extensive in young animals, where the arterial supply and venous drainage traverse both the endosteal and periosteal surfaces, and either system is sufficient to sustain adequate bone circulation (Whiteside et al. 1978). We earlier demonstrated that destruction of the endosteum and nailing with different materials cause distinct changes in the periosteal vascular organization (Greksa et al. 2012). The dependence of the healing capacity of the cortex after osteosynthesis on the relative contributions of the endosteal and periosteal systems in supplying the cortex has been demonstrated in different species (Lippuner et al. 1992), but little is known as regards the human characteristics. In our study, laser-Doppler flowmetry was used for the estimation of local blood perfusion rates and the characterization of the alterations taking place in the circulation in response to intramedullary implants and plates in a clinical setting.

In the present case history, the patient underwent a sequence of distinct osteosynthesis techniques that resulted in excellent mechanical properties at the time of the operations. From a physiological/biological aspect, however, the endosteal and periosteal flow values were found to be diminished in the disabled limb (as compared with the contralateral limb) after excessive osteosynthesis applications. In the preoperative phase, the radiographic findings were consistent with the modest circulatory conditions seen at the non-union of the fracture, but the postoperative X-ray revealed a satisfactory healing process. We believe that prudent preoperative planning, with preservation of the local biology (i.e. circulation) carefully borne in mind, may have contributed to the results. Relying on the regeneration capacity (including recovery of the blood supply of the cortex) of the periosteum (Colnot 2009), we decided to remove the long plates and to apply a new prosthesis shaft designed with distal holes for the placement of locking screws (serving as a long intramedullar nail bridging the fracture ends). Additionally, the fracture gap was filled with a platelet concentrate originating from the autologous blood (containing growth factors) in order to support new bone formation (Symphony^{TM}, DePuy) (Sheth et al 2012). The postoperative radiographs taken 6 weeks after this operation clearly indicated new bone formation, proving evidence of the efficacy of this

biological approach.

As concerns long bones, endochondral bone repair is the primary form of bone healing when micromotions exist between the fractured parts of bones covered with periosteum (Shapiro et al. 2008). This process is based on the ability of periosteum-derived cells to proliferate and to form periosteal callus in the initial phase of fracture healing (Knothe Tate et al. 2007). In line with this, in an ovine "periosteal sleeve" model, the periosteum alone has been shown to be capable of regenerating the long bone defect when the endosteum has been completely destroyed (the medullary cavity was completely occupied by a nail) (Knothe Tate et al. 2007). With regard to the importance of periosteal integrity, careful periosteal stripping resulting in a relatively low alteration in blood supply is the widely accepted method for plate insertion (Whiteside and Lesker 1978). Probably because of the decisive role of the periosteum, the importance of the endosteum in bone healing is generally underestimated (Kojimoto et al. 1988). Similarly to the periosteum, endosteum covering all interior surfaces of bones (including all of the inner surfaces of the cortex and the spongiosa) also provides a richly vascular environment and harbours osteoprogenitor cells for fracture healing, but the cellular mechanisms differ greatly (Colnot et al. 2012). While periosteal injuries heal by endochondral ossification, bone marrow injuries heal by intramembranous ossification (Colnot 2009). It is observed clinically that fracture healing is faster in metaphyseal or epiphyseal regions (which are rich in spongiosa) than when the fracture occurs in a compact bone. Furthermore, considerable endosteal callus formation has been detected between bone ends in studies relating to bone lengthening (Kawamura et al. 1968). Such observations underline the importance of the endosteum in the bone healing processes. Our present study demonstrates the simultaneous impairment of both systems. This may explain the negative clinical outcome, i.e. pseudoarthrosis formation, after excessive prosthesis application in long bones.

In consequence of the ethical and technical limitations, the relationship and compensatory reactions between the periosteal and endosteal circulations can not be investigated in adequate numbers under clinical circumstances. A further potential flaw of this study is that no follow-up of the examinations was feasible. However, the present study provides a model for estimation of the circulatory consequences of long-term simultaneous iatrogenic cessation of both the interior and the exterior blood supply of a long bone.

Contribution of authors

FG, DG, HG: data collection and analysis, first draft of the manuscript. KT: manuscript revisions. AS, PH: data interpretation on graphs and manuscript revisions

Figure legends

Figure 1. Scheme of the laser-Doppler flowmetry approach used to observe the perfusion of the periosteum (A) and the endosteum (B) of the human tibia in the present study. The periosteum (*p*) was observed through a small incision made in the skin and the underlying tissues, while the endosteal region (*e*) was reached through a small hole drilled in the anterior cortex, providing access to the endosteal region at the opposite (inner surface of the posterior) medullary wall.

Figure 2. Original laser-Doppler flowmetric recordings of the periosteal (A and B) and endosteal regions (C and D) in the non-operated (A and C) and operated (pseudoarthrotic; B and D) tibia. Data relating to the perfusion of the above compartments are presented in part E. Data are the means of three measurements.

Figure 3. Anteroposterior and lateral views of the left tibia before the re-operation (A) and 6 weeks after the surgery (B). Callus formation was detected in the dorsal part of the fracture, with good angulation of the fracture ends.

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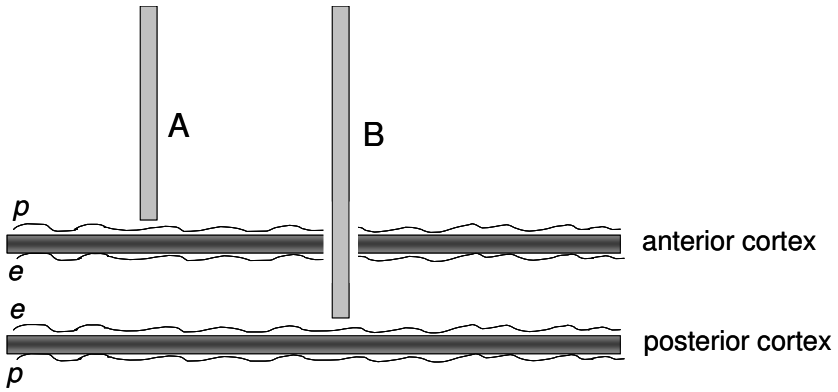

Figure 1.

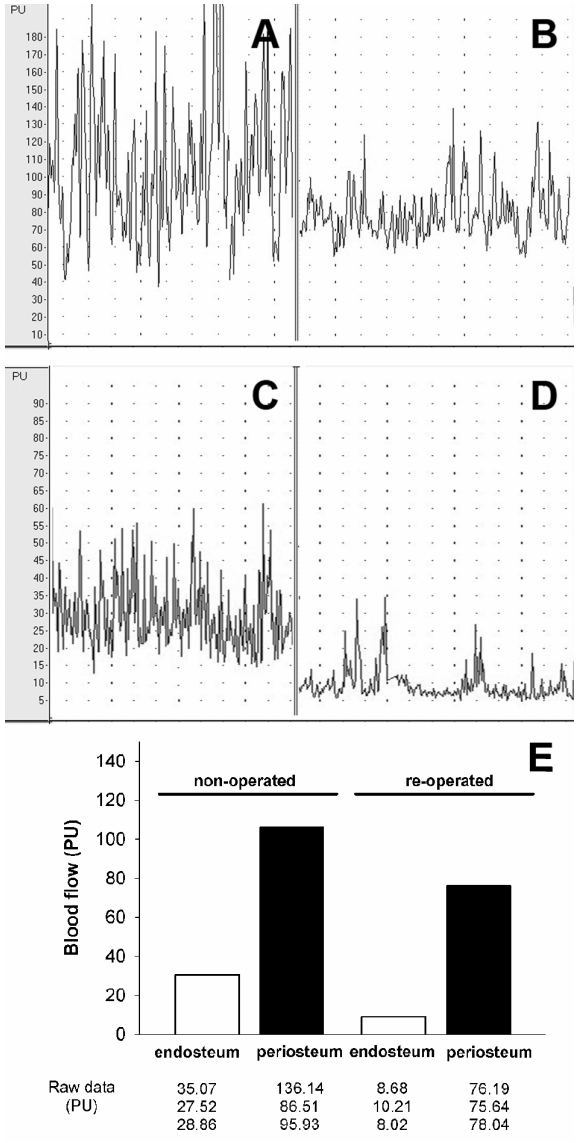
PU
A
B
C
D
E
non-operated
re-operated
Blood flow (PU)
endosteum
periosteum
endosteum
periosteum
Raw data (PU)
35.07
27.52
28.86
136.14
86.51
95.93
8.68
10.21
8.02
76.19
75.64
78.04

Figure 2.

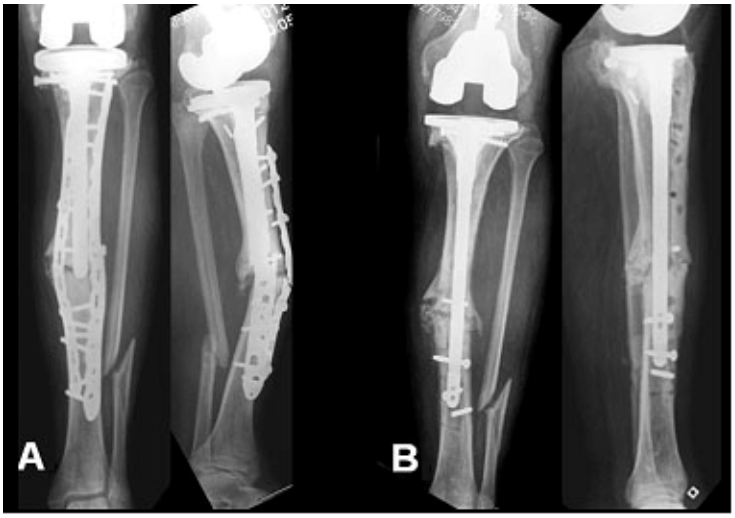

Figure 3.