

**Microcirculatory aspects of bisphosphonate-related osteonecrosis
of the jaw**

Ágnes Janovszky, M.D.

Ph.D. Thesis

**Department of Oral and Maxillofacial Surgery,
Institute of Surgical Research,
University of Szeged, Hungary**

Szeged, 2014

INTRODUCTION

The oral cavity is particularly prone to inflammatory complications (e.g. periodontitis or abscesses), as it can be sensitively exposed to the external environment in the immediate vicinity of the teeth. The propagation of various infection-related inflammatory reactions within the soft tissues is at least partially due to the rich blood supply of the oral and maxillofacial region. A special nutritive aspect of the jaw (i.e. it is predominantly supplied by the periosteal circulation) also predisposes to various pathological conditions of the bones. The periosteal microcirculatory aspects of systemic and intraoral diseases, however, have not been well characterized. This is partially due to fact that observation of the periosteal compartment necessitates surgical exposure. The present thesis is based on the assumption that morphological and functional changes in the microcirculation in the periosteum play a decisive role in the pathogenesis of various mandibular pathologies. For this reason we developed a novel microsurgical method which makes examination of the mandibular periosteum possible using different *in vivo* microscopic methods.

The role of the periosteal integrity in bone physiology is well recognized, not only as it relates to the maintenance of the vascular supply, but also from the aspect of the active regulation of the bone metabolism and regeneration. Periosteal damage leads to perturbed bone healing with consequent delayed union or pseudoarthrosis formation. With regard to the mandible, clinical observations show that defective angiogenesis of the mandibular mucoperiosteal tissues is evoked by long-term treatment with bisphosphonate (BIS), resulting in severe conditions such as osteonecrosis of the jaw. It occurs mainly after invasive dental procedures, e.g. tooth extraction, with an increased incidence particularly after the use of third-generation BISs (e.g. zoledronate, ZOL). BISs together with other antiresorptive (e.g. denosumab) and antiangiogenic (e.g. bevacizumab and sunitinib) drugs induce necrosis of the oral bones, usually referred to as medication-related osteonecrosis of the jaw (MRONJ), but bone destruction is also seen less frequently in other bones of the skeleton. As for pathophysiology of BIS-induced MRONJ, the role of many potential contributing factors are presumed such as local contamination and infection, direct toxic effects and inhibition of bony and gingival healing processes. The role of BIS-induced mandibular periosteal microcirculatory changes in the pathogenesis of MRONJ has, however, not yet been examined. Herein, we hypothesized that a disturbed mandibular microcirculation may play a role in the pathogenesis of MRONJ. In this context, we designed a rat model where chronic

BIS treatment was combined with an invasive dental procedure and where the processes of mucosal healing and bone destruction resembled the clinical manifestations of MRONJ.

THE MAIN GOALS OF THE PRESENT STUDIES

1. To develop a novel microsurgical procedure for the *in vivo* visualization of the mandibular periosteal microcirculation through the use of different microscopy methods (fluorescence intravital microscopy, IVM; orthogonal polarization spectral imaging, OPS; and confocal laser scanning microscopy, CLSM) in rats.
2. To examine the systemic and local mandibular periosteal inflammatory microcirculatory reactions in comparison with those in the tibia in a clinically relevant model of BIS-induced MRONJ in rats.

MATERIALS AND METHODS

In Study 1, 10 male Sprague-Dawley rats were used. The periosteal microcirculation of the mandible was exposed by cutting the fascia between the anterior part of the deep masseter and the anterior superficial masseter muscles with microscissors. By this means, the periosteal membrane covering the corpus of the mandible laterally to the incisor tooth was reached. The tibial periosteum was prepared by using a previously described method of our research group. After exposure of the mandibular and tibial periosteum on both sides, recordings were performed on the right side with OPS (Cytoscan™, Cytometrics, Philadelphia, PA, USA), which does not require any fluorescence labeling. After this, the animals received intravenous (i.v.) injections of fluorescein isothiocyanate (FITC)-labeled erythrocytes and rhodamine-6G for the staining of leukocytes, and IVM (Zeiss Axiotech Vario 100HD microscope) recording was performed at the previous locations. Subsequently, 50 µL of the nuclear dye acriflavin (1 mM) was applied topically to the tibial periosteal surface on the left side, and rinsed off with warm physiological saline solution after an exposure time of 1 min, and CLSM (Five1 Optiscan device (Optiscan Pty. Ltd., Melbourne, Victoria, Australia) recording was then performed. The same staining procedure was carried out for the mandible on the left side. This was followed by an i.v. injection of the plasma dye FITC-dextran 150 kDa, and CLSM and IVM recordings were made on the tibia and the mandible on the right side 5 min after the injection of the tracer.

In Study 3, 20 male Sprague-Dawley rats were randomly allocated to saline vehicle-treated control (n=10), or i.v. ZOL-treated (n=10, ZOL) groups. ZOL was administered through a tail vein in a dose of 80 µg/kg once a week for 8 weeks. At the end of the 3rd week

of the protocol, the first two right mandibular molar teeth were extracted under ketamine and xylazine anesthesia. Mucosal healing processes were monitored continuously throughout the experimental period. Through IVM, the microcirculatory variables were compared in the mandibular and tibial periosteum in the 9th week of the protocol. FITC-labeled erythrocytes were used to stain red blood cells, and rhodamine-6G to stain leukocytes. Leukocyte function/activation and inflammation were examined by assessing the NADPH-oxidase activity of neutrophil leukocytes, whole blood free radical production (by chemiluminometric procedures), the expression of CD11b adhesion molecule on neutrophil leukocytes (by flow cytometric analysis) and the plasma TNF-alpha content (by enzyme-linked immunosorbent assay kit, R&D Systems, Minneapolis, MN, USA). The incidence and severity of mucosal lesions were determined on the basis of a new scoring system (on the basis of the osteonecrosis staging system provided by the American Association of Oral and Maxillofacial Surgeons), while jaw osteonecrosis was diagnosed by means of computed micro tomography and histological examinations.

Quantitative evaluation of the microcirculatory parameters (leukocyte–endothelial cell interactions: rolling and adherence, red blood cell velocity) was performed off-line by frame-to-frame analysis of the videotaped images taken for IVM and OPS (IVM Software; Pictron Ltd, Budapest, Hungary).

RESULTS

The novel microsurgical approach provided a simple and reproducible approach to the mandibular periosteum of the rat, where morphological and functional features of the microvasculature can be assessed by different *in vivo* visualization techniques (IVM, OPS and CLSM methods). This access to the mandibular periosteum offers an excellent opportunity for investigations of microcirculatory manifestations of dentoalveolar and maxillofacial diseases.

In vivo microscopy revealed homogenous microvascular perfusion in all of the periosteal tissues examined and were not influenced by chronic ZOL treatment. IVM revealed significantly increased leukocyte–endothelial interactions (leukocyte rolling and adhesion on the endothelial surface) in the mandibular periosteum, but not in the tibia. ZOL evoked similar rolling and adhesion values irrespectively of the presence of MRONJ. Only the leukocyte count and NADPH-oxidase activity of the leukocytes displays significant reductions, the other systemic inflammatory parameters not being affected by ZOL. In control rats, intact mucosa could be observed, but different degrees of mucosal healing disorders were

detected in all (10/10) of the ZOL-treated animals. Normal bony regeneration could be detected at the site of the earlier tooth extraction in all (10/10) of the saline-treated animals. In contrast, a certain degree of discontinuity of the cortical and spongy bone regions was found in 7/10 of the ZOL-treated animals. This higher incidence of impaired bony regeneration was accompanied by a significantly lower average bone density in this group as compared with that in the saline-treated group ($P < 0.01$). The radiological diagnosis of mandibular osteonecrosis was confirmed by standard histological examinations.

CONCLUSIONS

The periosteum of the jaw bones has a high impact in the pathogenesis of various orofacial diseases, mostly because of its nutritive functions and osteoprogenitor cells content. Although oral mucosal microcirculation can relatively easily be examined by different methods, specific, real-time examination of the mandibular microcirculation can be performed only after surgical exposure. To date, microcirculatory consequences of various diseases (gingivitis/periodontitis) and interventions (consequences of flap surgery, dental extraction, bony regeneration) are still completely unknown. Our novel method (presented in Study 1) provides an excellent opportunity for examination of the above processes.

After tooth extraction, normal healing of the jaw bones and the gingiva necessitates the contribution of many potential factors that can be compromised by chronic ZOL treatment. Apart from the direct effect of BISs on the balance of osteogenesis/osteolysis (through the RANKL/osteoprotegerin and mevalonate pathway), high degree of their accumulation in the mandible, their direct toxicity of many cell types, as well as inhibited cell migration may contribute to the pathogenesis of MRONJ induced by BISs. In Study 2, clinical characteristics, limited possibilities for early detection, surgical and adjuvant therapeutic possibilities of MRONJ were summarized. In Study 3, we also demonstrated that ZOL treatment causes a distinct microcirculatory inflammatory reaction in the mandibular periosteum, but not in the tibia. Since various indices of systemic inflammation (e.g. expression of the neutrophil-derived adhesion molecule CD11b) were not found to be influenced by chronic ZOL treatment, regional differences might therefore be explained by different degrees of endothelium-derived adhesion molecule expression at the different anatomical locations.