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Differences of bone healing in metaphyseal defect fractures between osteoporotic and physiological bone in rats

Ulrich Thormann [a,b,1], Thaqif El Khawassna [b,1], Seemun Ray [b], Lutz Duerselen [c], Marian Kampschulte [d], Katrin Lips [b], Helena von Dewitz [d], Sascha Heinemann [e], Christian Heiss [a,b], Gabor Szalay [a], Alexander C. Langheinrich [f], Anita Ignatius [c], Reinhard Schnettler [a,b], Volker Alt [a,b,*]

[a] Department of Trauma Surgery, University Hospital of Giessen-Marburg, Giessen, Germany
[b] Laboratory of Experimental Trauma Surgery, Justus-Liebig University, Giessen, Germany
[c] Institute of Orthopedic Research and Biomechanics, Centre of Musculoskeletal Research, University of Ulm, Ulm, Germany
[d] Department of Radiology, University Hospital of Giessen-Marburg, Giessen, Germany
[e] Institute of Materials Science, Technische Universität Dresden, Dresden, Germany
[f] Department of Diagnostic and Interventional Radiology, BG Trauma Hospital Frankfurt/Main, Germany

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ABSTRACT

Discrepancies in bone healing between osteoporotic and non-osteoporotic bone remain uncertain. The focus of the current work is to evaluate potential healing discrepancies in a metaphyseal defect model in rat femora. Female Sprague-Dawley rats were either ovariectomized (OVX, $n = 14$) and combined with a calcium-, phosphorus- and vitamin D3-, soy- and phytoestrogen-free diet or received SHAM operation with standard diet rat (SHAM, $n = 14$). Three months post-ovariectomy, DEXA measurement showed a reduction of bone mineral density reflecting an osteoporotic bone status in OVX rats. Rats then underwent a 3 mm wedge-shaped osteotomy at the distal metaphyseal area of the left femur stabilized with a T-shaped mini-plate and allowed to heal for 6 weeks. Biomechanical competence by means of a non-destructive three-point bending test showed significant lower flexural rigidity in the OVX rats at 3 mm lever span compared to SHAM animals ($p = 0.048$) but no differences at 10 mm lever span. Microcomputer tomography (μCT) showed bridging cortices and consolidation of the defect in both groups, however, no measurable differences were found in either total ossified tissue or vascular volume fraction. Furthermore, histology showed healing discrepancies that were characterized by cartilaginous remnant and more unmineralized tissue presence in the OVX rats compared to more mature consolidation appearance in the SHAM group. In summary, bone defect healing in metaphyseal bone slightly differs between osteoporotic and non-osteoporotic bone in the current 3 mm defect model in both 3 mm lever span biomechanical testing and histology.

Introduction

Osteoporosis is characterized by low bone mass with skeletal micro-architectural changes, resulting in fragility and thus an increased risk of fracture. Common osteoporotic fractures are of the hip and spine, which are associated in increasing mortality rate of 10–20% [1,2]. The spine and the ends of long bones consist mainly of cancellous bone, which is also affected in the case of a metaphyseal fracture. Several reports indicated that bone deteriorations resulting due to osteoporosis affect metaphyseal trabecular rather than cortical bone [3,4]. Moreover, studies reported higher osteoporotic fracture risk in metaphyseal regions of long bone such as the distal radius, proximal humerus, and proximal femur [5–7]. Despite their relevance to osteoporotic fracture research, and their overall importance in bone healing, metaphyseal fractures are less studied than diaphyseal fractures [8]. Nonetheless, reports of healing patterns in diaphyseal fracture model and drill-hole defects [9–13] suggested deviations between osteoporotic and normal bone. Metaphyseal fracture healing was tackled with several models, which studied either a small sized gap [14–16], a drilling-hole defect [17,18] or partial osteotomy [5]. Probably, the need of a clinically relevant fracture model and adequate fixation lay behind the lack of detailed studies of metaphyseal fracture. Recently we published a new metaphyseal fracture defect model [19], with a 3 mm defect under osteoporotic bone conditions to study fracture healing. Furthermore, our team has also recently reported a successful model

* Corresponding author at: Department of Orthopaedic Trauma Surgery, Justus-Liebig-University Giessen, Rudolf-Buchheim-Str. 7, 35385 Giessen, Germany. Tel.: +49 0641 985 44 601; fax: +49 0641 985 44 609.
E-mail address: volker.alt@chiru.med.uni-giessen.de (V. Alt).
[1] Both authors contributed equally to first authorship.

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of multi-deficiencies-diet-induced osteoporosis in rats [20]. By combining these two models we recently published a rat study showing that osteoporotic bone status influenced the osteogenic but not the angiogenic response in metaphyseal bone healing in rats compared to physiological bone status [21]. However, this study lacked of biomechanical, micro-CT and detailed histological analysis. Therefore, the purpose of the present study was to explore in-depth the influence of osteoporotic bone status on healing patterns in a 3 mm metaphyseal fracture model after 6 weeks in a detailed observation by means of biomechanical testing, histology, and μCT analysis. We hypothesized that bilateral ovariectomy combined with multi deficiencies-diet treatment lead to an osteoporotic bone status, which will negatively influence metaphyseal bone healing when compared to untreated control rats.

Materials and methods

Twenty-eight female Sprague-Dawley rats (Charles River, Sulzfeld, Germany) with an age of 4–5 months were randomly assigned to two groups, (14 animals each). In the experimental group, an osteoporotic bone status was induced by means of bilateral ovariectomy with multi-deficiencies diet (OVX) as previously described [20] [further details in the supplementary file], whereas animals of the control group only underwent SHAM operation (SHAM).

Animal experiments followed the animal welfare act of the national institute of health and the guide for care and use of laboratory animals. Experiments also accorded with the national animal welfare guidelines approved by the local regional government and conformed to German animal protection laws of the district government of Giessen (Ref. number: V 54-19 c 20-15 (1) GI 20/28 Nr. 92/2009).

Three months after ovariectomy or SHAM operation, surgery of the left distal femur was performed by one surgeon under aseptic conditions and general anaesthesia as described earlier [19]. Briefly, distal femur was approached anterolaterally, and then fixed laterally with 7-hole T-shaped mini-plate (Leibinger® XS-miniplate, Stryker®, Schoenkirchen, Germany). The metaphyseal region of the distal femur was then osteotomized to create a wedge shaped defect with a lateral gap of 3 mm and a medial gap of 1 mm using an oscillating saw (Piezosurgery® 3, Saw blade OT7S-3, Mectron, Germany)

Post-operatively, animals were individually housed with free access to feed and water each according to the assigned diet; OVX deficient-diet (OVX group) or standard rat diet (SHAM). 6 weeks post-osteotomy, the animals were euthanized under inhalation of CO_2 after general anaesthesia. Both operated and contralateral femurs of each group were harvested and samples were processed as later mentioned in the methods section.

Biomechanical testing

A non-destructive three-point-bending-test was used to evaluate the flexural rigidity of the femur, as described earlier [19]. Briefly, the femurs were placed in the testing device (Z10, Zwick, Ulm, Germany). Prior to testing the fixation plates were removed from the bones. Femoral condyles were positioned over a notch on a metal support block (3 mm in diameter) and the proximal femur rested on the trochanters thereby creating a three-point contact with the base. Load was applied at a distance of 3 mm from the condyles resulting in a shear test like testing of the healing zone and, alternatively, at 10 mm away from the femoral condyles, which resulted in a 3-point type of bending reflecting the flexural rigidity of the whole femur. The deflection rate was 1 mm/min. In order to evaluate the linear part of the load-deformation curves the osteotomized femurs were loaded up to 5 N and the not operated controls up to 25 N. Each sample was tested thrice: the first two test cycles conditioned the sample to avoid artefacts due to contact settlements. The third loading cycle of each sample was evaluated to define the flexural rigidity (EI) from the slope (k) of the linear region of the load–deflection curve. The flexural rigidity EI was calculated using the formula:

$$EI = k\frac{a^2b^2}{3l} \quad [\mathrm{Nmm}^2]$$

where (a) is the distance between the load vector and the proximal support and (b) is the distance between the load vector and the distal support, and is the support length ($l = a + b$).

Micro-CT analysis

To obtain the vascular volume formation (VVF) by means of μCT ($n = 6$/group), the abdominal aorta was cannulated and infused with heparinized saline (10 ml of 0.9% sodium chloride with 1000 IU Heparin) immediately after euthanasia until the venous effluent was free of blood. A lead-containing radiopaque polymer (Microfil® MV-122, Flow Tech, Carver, MA, USA) was used to carry out the perfusion. It was administered into the aorta and following polymerization of the compound, the femora with the indwelling mini-plate were removed and immediately fixed in 4% phosphate buffered paraformaldehyde.

New ossified tissue formation was assessed within the fracture gap and on the lateral periosteal site. Longitudinal cross-section parallel to the specimen's long axis was reconstructed using en-bloc and measurement of total ossified tissue was performed. Axial cross-sections within the fracture zone were identified and the cross-sectional area (mm^2) of total ossified tissue was measured using the ANALYZE software (ANALYZE 10.0, Mayo Clinic, Rochester, USA).

μCT analyses of the femur we performed using a micro-CT system (Sky Scan 1073, Kontich, Belgium). For all micro-CT analyses, the X-ray source was operated at 130 kV and between 0 and 100 μA reaching a minimum spot size of 5 μm at 8 W generating projection images irradiating X-rays in cone-beam geometry. Images were captured using a 12-bit digital, water-cooled CCD high-resolution (2240 × 2240 pixel) camera with fibre optic 3.7:1 coupled to an X-ray scintillator and digital frame-grabber. Samples were scanned on rotation stage with a rotation of 180° around the vertical axis in rotation steps of 0.675 degrees at 130 kV. Acquisition time for each view was 2.4 s. Measured regions of interest of both TOT and VVF are shown in Fig. 1. Briefly, scans of Microfil perfused thighs were performed with femora and soft tissue en bloc. Due to the overlapping absorption of calcified tissue and contrast vasculature, regions of interest (ROI) was manually identified and segmented to obtain: (i) cross sectional area of calcified tissue and (ii) vasculature percentage of soft tissue.since these areas contained both, calcified tissue and vasculature over a standardized volume of interest no bone-volume-fraction was calculated. Nevertheless, to estimate the dimension of calcified tissue we measured the surface of the contoured cross sectional plans. Further, soft tissue was identified by manual segmentation of the soft tissue within the fracture gap excluding calcified tissue. The resulting soft-tissue volume was used to obtain the vascular volume fraction by threshold-based segmentation.

Skeletal preparation and histological analysis

Movat pentachrome staining was used to describe dimensions and distribution of tissues coinciding in the fracture gap at the time of euthanasia. Directly after μCT scanning in, the samples were kept in 4% formalin at 4° until processed for histological examination. Samples were embedded in Technovit 9100 NEU