Microstructural changes in the bone tissue and in the bone callus of diabetic rats with and without insulin treatment

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Aims
The objective of this study was to investigate the microstructural changes in the bone tissue and in the bone callus in diabetic rats. Furthermore we aimed to examine the effects of insulin treatment on the bone structure and on the bone healing process in diabetic rats.

Method
All the experimental procedures used in this study were approved by the Animal Care and Use Committee of our University. Forty-eight female Wistar rats weighing approximately 200g were given single intravenous injection with streptozotocin (60 μg/g body weight in 0.1 M citrate buffer; Sigma, St. Louis, MO) to induce diabetes (DM). Control rats (CON, n=12) were injected with buffer alone. Blood glucose levels were examined 7 days after the streptozotocin injection by obtaining blood from the tail vein and measuring glucose concentration with a glucometer (Abbott, IL, USA). Diabetes in rats was diagnosed on the basis of blood glucose concentrations of ≥250 mg/dL on two consecutive days. Animals were monitored three times per week for body weight and blood-glucose concentration. Rats from groups DM+INS received daily insulin treatment when blood-glucose values exceeded 250 mg/dL. Thirty days after diabetes induction (or citrate buffer injection), half of the animals were anesthetized with an intramuscular injection of xylazin (0.2 mL/kg) and ketamine (0.4 mL/kg) mixture in order to perform bone fracture (CON+FRA, DM+FRA and DM+INS+FRA, n=12 per group). The right distal femur was shaved and then disinfected with 70% alcohol. Subsequently, bone was placed in a device especially manufactured to perform a closed fracture in the mid-femur (Figure 1). Immediately after fracturing bone, an incision was made parallel in the proximal extremity of the femur. A 1-mm-diameter Kirschner wire was introduced into the medullary canal in order to stabilize the bone fragments. The wound was closed with resorbable sutures. The status of the fracture was radiographically confirmed immediately after surgery and then followed-up weekly (Figure 2). Any fractures not consistent with standardized placement criteria (mid-diaphyseal) or grossly comminuted were excluded. On days 14 and 28 following surgery, six rats from each group were killed and the femurs harvested in preparation for tridimensional microstructure analysis of both trabecular and cortical bone, as well as the bone callus. Prior to microCT scanning the k-wire nail was removed while taking great care to not disrupt the fracture site and callus. A high-resolution, desktop micro-CT system (SkyScan 1174v2; Bruker-microCT, Kontich, Belgium) was used to quantify the BMD and the three-dimensional microarchitecture parameters in the femur. The specimens were scanned using 50 kV and 800 mA, with the aid of a 0.5-mm-thick aluminum filter to optimize the contrast, a rotation step of 1°, three-frame averaging and an isotropic resolution of 26.7 μm. Images of each specimen were reconstructed with dedicated software (NRecon version 1.6.3; Bruker-microCT), providing axial cross-sections of the inner structures of the samples.

In the non-fractured bone, two regions of interest were made, one at the femoral distal metaphysis, which mainly contains trabecular bone and, another at the mid-diaphysis, which
mainly contains cortical bone. The reconstruction of the metaphysis was selected manually starting just proximally from the growth plate for an extension of 3 mm. The reconstruction of the diaphysis was defined by a 2-mm region starting 8 mm proximally from the growth plate. Cortical and trabecular bone were isolated using manually drawn contouring. CTAn software (Bruker-microCT), version 2.2.1, was used for the determination of the optimal threshold from the image histograms and was set to exclude soft tissue but to include poorly mineralized bone. The same threshold was used in all of the samples, but differed between trabecular and cortical bone. The thresholded image was used as a mask to measure the BMD of the bone structures (trabecular). For the accurate calculation of BMD, appropriate calibration of the Skyscan CT analyzer was performed with known density calcium hydroxyapatite phantoms (0.25 and 0.75 g/cm³). Once the phantoms’ BMDs were calibrated in the CTAn software, a VOI of 3 mm was selected in the bone. Trabecular architecture of the distal metaphysis was characterized by determining trabecular bone volume (BV), trabecular bone volume fraction (BV/TV), specific bone surface (BS/BV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and connectivity density (Conn.D) (Figure 5). Cortical architecture was assessed in the diaphysis and was characterized by cortical volume (Ct.V and Ct.BV/TV), cortical thickness (Ct.Th) and cortical porosity (Ct.Po). All bone morphometric measurements and nomenclature are in accordance with recommendations of the ASBMR(1).

The volume of callus was analyzed by using the CTAn software, where the entire callus was selected as the ROI.

Results

The diabetic rats exhibited a substantial loss of bone tissue and uncoupled bone turnover, where resorption was up-regulated and formation was down-regulated, when compared to the control animals. These changes occurred on both the trabecular (Figures 3 and 4) and the cortical bone (Figures 5 and 6) and were mitigated by insulin administration. Additionally, bone healing was negatively affected by diabetes and ameliorated when insulin was given to the animals (Figure 7).

Figures 3 and 4 show that within 44 days of uncontrolled diabetes we observed a significant (p<0.05) decrease in the BMD (-22%) and a marked deterioration in the trabecular architecture in the proximal metaphysis (reduction of -49% in BV, -82% in BV/TV, -17% in BS/BV, -65% in Tb.N, -83% in Tb.Th and -71% Conn.D and, +79% in Tb.Sp). Within 58 days of uncontrolled diabetes, we observed a significant (p<0.05) decrease in the BMD (-24%), reduction of -83% in BV, -82% in BV/TV, -78% in Tb.N, -33% in Tb.Th and -74% in Conn.D and, +121% in Tb.Sp). Conversely, insulin treatment mitigated bone quality loss induced by diabetes, when compared to the uncontrolled diabetic rats (DM+INS vs DM). Within 44 days, insulin significantly increased in +10% the BMD, in +29% BV, in +347% BV/TV, in +44% Tb.Th, in +59% Tb.N, in +167% Conn.D and, in -26% Tb.Sp. Within 58 days, insulin significantly increased in +31% the BMD, in +437% BV, in +385% BV/TV, in +50% Tb.Th, in +267% Tb.N, in +261% Conn.D and, in -38% Tb.Sp.

Figures 5 and 6 show that within 44 days of uncontrolled diabetes, we observed a significant (p<0.05) decrease in the cortical volume and increased porosity (-27% in Ct.V and Ct.BV/TV, -19% in Ct.Th and, +23% in Ct.Po). Within 58 days of uncontrolled diabetes, we observed a significant (p<0.05) decrease of -41% in Ct.V, -10% in Ct.BV/TV, -25% in Ct.Th and, +7% in Ct.Po. Conversely, insulin treatment mitigated cortical bone loss induced by diabetes, when compared to the uncontrolled diabetic rats (DM+INS vs DM). Within 44 days, insulin significantly increased in +24% Ct.V, in +17% Ct.BV/TV, in +30% Ct.Th and, in -10% Ct.Po. Within 58 days, insulin significantly increased in +49% Ct.V, in +17% Ct.BV/TV, in +28% Ct.Th and, in -9% Ct.Po.

With regards to the bone callus, we observed a smaller callus in the diabetic rats, both within 14 and 28 days post-fracture (Figure 7). Within 44 days, the callus volume was 80% smaller in diabetic rats when compared to the control, however the insulin treatment increased in 39% the volume (DM+INS vs DM).
Figure 1: Device used to cause closed fracture in mid-femur.

Figure 2: X-ray images to confirm the fracture and alignment immediately following surgery (PO 0) and post-surgery; on the 7th (PO7) and on the 14th (PO14) days post-fracture.

Figure 3: Trabecular bone microstructures of femoral metaphysis showing a dramatic reduction of trabecular bone in diabetic rats (DM), which was ameliorated by insulin administration (DM+INS).
Figure 4: Quantitative analysis of trabecular bone microstructure. Asterisks indicate significant difference (p<0.05).

Figure 5: Cortical bone microstructures of femoral diaphysis showing decreased cortical bone in diabetic rats (DM), which was ameliorated by insulin administration (DM+INS).
Figure 6: Quantitative analysis of cortical bone microstructure. Asterisks indicate significant difference (p<0.05).

Figure 7: MicroCT images of bone callus, both in sagittal and axial planes. Uncontrolled diabetes (DM) induces decreased bone healing, where bone callus is smaller than in both the control and in the treated diabetic rats (DM+INS).

**Conclusion**

We concluded that uncontrolled diabetes leads to dramatic changes in both trabecular and cortical bone mass and microstructure. Additionally, bone healing is negatively affected by diabetes, where the volume of bone callus is smaller in the uncontrolled diabetic rats than in the controls. On the other hand, the administration of insulin mitigates the deleterious effects of diabetes not only on the bone tissue (trabecular and cortical), but also on the bone healing process.
References: