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Bone defect regeneration and cortical bone parameters of type 2 diabetic rats are improved by insulin therapy☆

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ABSTRACT

Zucker Diabetic Fatty (ZDF) rats represent an established model of type 2 diabetes mellitus (T2DM) and display several features of human diabetic bone disease, including impaired osteoblast function, decreased bone strength, and delayed bone healing. Here, we determined whether glycemic control by insulin treatment prevents skeletal complications associated with diabetes. Subcritical femur defects were created in diabetic *(fa/fa)* and non-diabetic *(+/+)* ZDF rats. Diabetic rats were treated once daily with long-lasting insulin glargin for 12 weeks for glycemic control. Insulin treatment successfully maintained serum levels of glycated hemoglobin, while untreated diabetic rats showed a 2-fold increase. Trabecular and cortical bone mass measured by μCT were decreased in diabetic rats. Insulin treatment increased bone mass of the cortical, but not of the trabecular bone compartment. Dynamic histomorphometry revealed a lower bone formation rate at the trabecular and periosteal cortical bone in diabetic animals and decreased serum procollagen type 1 N-terminal propeptide (P1NP, −49%) levels. Insulin treatment partially improved these parameters. In T2DM, serum levels of tartrate-resistant acid phosphatase (TRAP, +32%) and C-terminal telopeptide (CTX, +49%) were increased. Insulin treatment further elevated TRAP levels, but did not affect CTX levels. While diabetes impaired bone defect healing, glycemic control with insulin fully reversed these negative effects.
In conclusion, insulin treatment reversed the adverse effects of T2DM on bone defect regeneration in rats mainly by improving osteoblast function and bone formation.
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1. Introduction

Diabetes mellitus alters bone metabolism, leading to a decreased bone quality and increased fracture risk in humans [1,2]. Type 2 diabetes mellitus (T2DM) is the most common form of diabetes affecting almost 344 million patients worldwide [3]. Therefore, treatment of T2DM is of utmost importance not only due to its high prevalence but also because of the high treatment cost of T2DM patients [1]. Besides macro- and micro-vascular complications such as stroke, retinopathy, neuropathy, and nephropathy, T2DM patients have an increased risk for fractures in spite of a higher bone mineral density [1,4,5]. While the mechanisms of diabetes-induced bone fragility remain largely unknown, increased cortical porosity has recently been found to contribute to low bone strength in humans with T2DM [1,5,6]. Additionally, fracture healing is delayed, which is associated with pain and prolonged immobility for the patient [1]. Thus, it is crucial to understand how T2DM alters bone homeostasis in order to maintain bone health in patients with T2DM and/or develop efficient therapies to improve bone mass and microarchitecture [1,7].

The Zucker Diabetic Fatty (ZDF) rat is an established rodent model for T2DM. Male ZDF *(fa/fa)* rats develop a metabolic syndrome with obesity and insulin resistance as found in humans [8–10]. By the age of nine to eleven weeks, the animals develop a full-blown T2DM. Diabetic ZDF *(fa/fa)* rats further display decreased bone mass and bone regeneration caused by an impaired osteoblastic function [10]. Recently, we have shown that bone-anabolic treatment of diabetic ZDF rats with the anti-sclerostin antibody improved both trabecular and cortical bone mass and bone defect healing [11,12]. By contrast, treatment with parathyroid hormone (PTH 1–84), was not able to fully reverse the effects of diabetes on bone homeostasis and bone defect healing.

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Although PTH treatment had bone anabolic effects on the trabecular compartment, cortical parameters were unaffected. Because the metabolic disturbance in these animals was left untreated, serum glucose levels were extremely high. Thus, it is unclear whether adequate glycemic control might have allowed for a complete reversal of the effects of diabetes on bone.

Despite lifestyle interventions and oral antidiabetic drugs, patients with T2DM often also require treatment with insulin for proper glycemic control. ZDF rats also develop pronounced insulin resistance with subsequent loss of insulin production, and are therefore suitable for an insulin therapy [8–10]. The differential effects of improved glycemic control on cortical vs. trabecular bone mass and bone defect regeneration, however, have not been studied in animal models of T2DM.

Here, we evaluated the effects of daily insulin treatment on bone metabolism, bone mass, and defect regeneration in type 2 diabetic ZDF *(fa/fa)* rats.

2. Materials and methods

2.1. Animals

At nine weeks of age male ZDF rats (ZDF *fa/fa*) and Zucker lean rats (ZDF +/+) were purchased from Charles River Laboratories and fed with high-fat, high-carbohydrate chow (Purina 5008) and water *ad libitum*. They lived in a light/dark cycle of 12:12 h at room temperature in makrolon type IV cages. The ZDF *(fa/fa)* rats develop spontaneously T2DM at the age of nine to eleven weeks by reason of a homozygous mutation of the leptin receptor [8,9]. ZDF *(+/+)* rats served as non-diabetic controls. All invasive procedures were approved by the local Institutional Animal Care Committee.

2.2. Subcritical size defect and insulin therapy

On the left femur of eleven-week-old rats a four-hole plate was fixed with screws (Stryker, USA) as previously described [10–12]. Between screw two and three, a 3-mm cross-sectional subcritical defect was created at the femur mid-shaft. After surgery, long-lasting insulin (insulin glargin, Sanofi, France) was s.c. administered daily over a time period of 12 weeks in diabetic ZDF *(fa/fa)* rats, resulting in three randomized groups of 8–10 rats, respectively. For the same ZDF rats, insulin concentrations were adapted individually for each animal ranging from 0.5 international units (IU) at week one to 13 IU at week 12 to titrate blood glucose concentrations to levels comparable to non-diabetic *(+/+)* ZDF rats (between 25 and 30 mmol/l after 24 h of administration). After 12 weeks, ZDF rats were sacrificed under general anesthesia, blood and urine samples and bones were collected, and tissues were removed for further analysis.

2.3. Serum and urine analysis

Serum levels of glucose, calcium, phosphate, urea, and fat parameters like triglycerides (TG), cholesterol, and high and low density lipoprotein (HDL, LDL) were measured using a Roche ModularPPEanalyzer. Glycated hemoglobin (HbA1c) levels in serum were detected using VARIANT TURBO instrumentation (Bio-Rad). Urinary levels of cyclic adenosine monophosphate (cAMP) and serum concentrations of bioactive intact parathyroid hormone (iPTH), tartrate-resistant acid phosphatase 5b (TRAP), C-terminal telopeptide (CTX), and type 1 procollagen amino-terminal-propeptide (P1NP) were detected using an immunoassay kit (cAMP: Biotrend, GER; iPTH: Immunotopics, USA; TRAP, CTX, and P1NP: Immundiagnostik Systems, GER) according to the manufacturer's protocol.

2.4. Assessment of bone mass and bone microarchitecture and bone fat content

Intact femur and vertebral body were analyzed blinded by micro-computed tomography (μCT) using a vivaCT40 (ScancoMedical, Switzerland) with an isotropic voxel size of 10.5 μm (70 kVp, 114 μA, 200 ms integration time). The metaphyseal scan region of the intact femur consisted of 150 slices beginning 1 mm proximal to and extending away from the growth plate. The diaphyseal region was located halfway between the femoral head and distal condyles and consisted of 400 slices. The fourth vertebral body (L4) was scanned and 60 slices around the center were analyzed. Trabecular and cortical compartments of the L4 vertebrae, femoral metaphysis, and mid-shaft were isolated by separate contouring and analyzed using established analysis protocols from ScancoMedical. The trabecular bone volume ratio (BV/TV) and bone mineral density (BMD) were assessed in the femoral metaphysis and the cortical BV/TV and BMD were calculated at the femoral mid-diaphysis. Micro-CT parameters were reported according to international guidelines [13].

To assess the bone marrow fat content, the second vertebral body (L2) was fixed in 4% paraformaldehyde (PFA, Carl Roth, GER), dehydrated in 80% ethanol and decalcified (Osteosoft ®) over a time period of four weeks. After scanning L2 with the μCT to ensure completed decalcification, the vertebral body was washed in PBS once for 5 min, stained for 1 h with 2% osmium tetroxide (Electron Microscopy Science, UK) diluted in 0.1 M sodium cacodylate buffer (pH 7.4) like reported elsewhere [14] and were transferred into PBS. L2 was scanned (70 kVp, 114 μA, 200 ms integration time) at a resolution of 10.5 μm isotropic voxel size and 150 slices around the center were analyzed using the established analysis protocols from ScancoMedical.

2.5. Biomechanical testing

The right intact femur was removed from 80% ethanol and rehydrated in PBS prior to three-point bending test (Zwick Roell, GER), which was performed blinded as previously described [11,12].

2.6. Bone histology and histomorphometry

Ten and three days before sacrifice, all rats received two i.p. injections of calcein (20 mg/kg). The right and left proximal tibia and the third lumbar vertebrae (L3) were fixed in 4% PFA and dehydrated with 80% ethanol. Left tibia and L3 were embedded in methylmethacrylate and either cut into 7-μm sections to analyze calcein labels (fluorescence) or 4-μm for the different staining. BV/TV, bone formation rate per bone surface (BFR/BS), mineralized surface per BS (MS/BS), and mineral apposition rate (MAR), were blinded determined for trabecular and cortical parts of the bones separately. The Osteomeasure software (OsteoMetrics) was used following the international standards [15]. To visualize mineralized bone areas von Kossa staining was applied. In brief, the 4-μm sections were rehydrated using alcohol gradient and then sequentially exposed to silver nitrate (Roth), sodium carbonate (Merck), and sodium thiosulfate (Roth). Afterwards slides were stained with toluidine blue (Waldeck) and then dehydrated. Adipogenesis was assessed in the medullary cavity. Adipocyte area (Adipo.Ar.) and number (N.Adipo) were used to quantify adipocytes. The right proximal tibia was decalcified (Osteosoft ®) and embedded in paraffin (Leica Biosystems, USA).

2.7. Assessment of bone defect healing

Bone inside the defect zone of the left femur was measured *ex vivo* using a μCT (vivaCT40, ScancoMedical) to detect BV/TV of newly formed bone by using an isotropic voxel size of 10.5 μm and X-ray energy of 70 kVp for 400 slices.

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