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Effects of insulin therapy on porosity, non-enzymatic glycation and mechanical competence in the bone of rats with type 2 diabetes mellitus

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ABSTRACT

Type 2 diabetes mellitus increases skeletal fragility; however, the contributing mechanisms and optimal treatment strategies remain unclear. We studied the effects of diabetes and insulin therapy on non-enzymatic glycation (NEG), cortical porosity (Ct.Po) and biomechanics of the bone tissue in Zucker Diabetic Fatty (ZDF) rats. Eleven-week old ZDF diabetic and non-diabetic rats were given insulin to achieve glycaemic control or vehicle seven days per week over twelve weeks (insulin dose adapted individually 0.5 international units (IU) at week 1 to 13.0 IU at week 12). The right femora were excised, micro-CT scanned, and tested in 3-point bending to measure biomechanics. NEG of the midshaft was determined from bulk fluorescence.

Diabetes led to increased NEG (+50.1%, $p = 0.001$) and Ct.Po (+22.9%, $p = 0.004$), as well as to reduced mechanical competence (max. stress: −14.2%, $p = 0.041$, toughness: −29.7%, $p = 0.016$) in the bone tissue. NEG and Ct.Po both correlated positively to serum glucose (NEG: $R^2 = 0.41$, $p < 0.001$, Ct.Po: $R^2 = 0.34$, $p = 0.003$) and HbA1c (NEG: $R^2 = 0.42$, $p < 0.001$, Ct.Po: $R^2 = 0.28$, $p = 0.008$) levels, while NEG correlated negatively with bone biomechanics (elastic modulus: $R^2 = 0.21$, $p = 0.023$, yield stress: $R^2 = 0.17$, $p = 0.047$). Twelve weeks of insulin therapy had no significant effect on NEG or Ct.Po, and was unable to improve the mechanical competence of the bone tissue.

A reduction of mechanical competence was observed in the bone tissue of the diabetic rats, which was explained in part by increased collagen NEG. Twelve weeks of insulin therapy did not alter NEG, Ct.Po or bone biomechanics. However, significant correlations between NEG and serum glucose and HbA1c were observed, both of which were reduced with insulin therapy. This suggests that a longer duration of insulin therapy may be required to reduce the NEG of the bone collagen and restore the mechanical competence of diabetic bone.

1. Introduction

Diabetes mellitus (DM) has become a global health problem with continually more cases reported every year worldwide, in both genders and all age demographics. Patients with type 2 diabetes mellitus (T2DM), which results from a combination of insulin resistance and insufficient insulin production, have an elevated fracture risk despite having normal or even high bone mineral density (BMD) [1]. This implies that the bone strength is compromised at the tissue level. Indeed, recent research shows that collagen cross-linking plays an important role in bone micro-damage formation, and may be altered by T2DM [2]. Increased advanced glycation end products (AGEs), which are collagen cross-links formed from non-enzymatic glycation (NEG), have been linked to increased micro-damage accumulation and inferior tissue mechanical properties in aging and osteoporotic bone [3–6]. Pre-clinical work with the WBN/Kob diabetic rat model has shown that an accumulation of AGEs leads to reduced bone strength in diabetic bone as well, independent of BMD [7]. In humans, recent studies suggest that T2DM also results in increased cortical porosity (Ct.Po) [8–10]. This may also contribute to inferior bone tissue strength in these patients; however no direct link between porosity and mechanics has been established to date in diabetic bone.

The male Zucker diabetic fatty (ZDF) rat has been established as a rodent model for T2DM, which replicates the obesity, insulin resistance and progressive loss of insulin production observed in humans [11–13]. ZDF rats have impaired bone mechanics resulting from reductions in bone mass, structure and tissue-level material properties [11,14,15]. We have recently shown that the anabolic agent parathyroid hormone

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(PTH) improves bone mass and micro-structure at the femur and vertebra in this model, which leads to increased extrinsic (whole-bone) mechanics [16]. However the intrinsic (tissue-level) bone mechanics, which are also impaired with diabetes, could not be improved. Since PTH was unable to reduce serum glucose and NEG levels in the bone tissue, the new bone formed with anabolic therapy likely contained elevated levels of AGE cross-links and porosity. In order to maximize the therapeutic effect on bone mechanics, agents such as insulin, which improve glycaemic control and could therefore reduce the formation of AGE cross-links, may be necessary.

As in humans with T2DM, insulin resistance and loss of insulin production are observed in the ZDF rat, making it a suitable model for studies of insulin therapy [11,13]. We previously showed that insulin treatment partially restored blood glucose and HbA1c levels as well as increased bone formation and bone defect regeneration in ZDF rats [17]. This improvement in glycaemic control may also lower collagen glycation and porosity in the cortical bone, which could preserve the tissue-level mechanics. In order to confirm this, we studied the effect of insulin therapy on bone tissue NEG, Ct.Po and intrinsic biomechanics in the ZDF rat.

2. Materials and methods

2.1. Animals and insulin therapy

A total of 26 male rats (18 ZDF fa/fa and 8 ZDF +/+) were used for this study (Charles River Laboratories), and were given a high-fat, high-carbohydrate chow (Purina 5008). ZDF fa/fa rats spontaneously develop T2DM between the age of weeks 9 and 11, and the ZDF +/+ served as non-diabetic control. All invasive procedures were approved by the local Institutional Animal Care Committee of the Technische Universität Dresden.

At the age of eleven weeks, ten of the ZDF fa/fa rats were administered long-lasting insulin (insulin glargin, Sanofi, France) s.c. daily over a time period of twelve weeks. Insulin concentrations were adapted individually for each rat ranging from 0.5 international units (IU) at week 1 to 13.0 IU at week 12 to reduce blood glucose to levels between 25 and 30 mmol/l after 24 h of administration. The remaining rats received vehicle (water) s.c. daily over the same twelve-week period resulting in three groups: [1] twelve weeks of simultaneous diabetes and insulin treatment ($N = 10$), [2] twelve weeks of diabetes only ($N = 8$) and [3] twelve weeks of normal development. At the end of the study, the rats were sacrificed under general anaesthesia and the right femora excised, fixed in 4% paraformaldehyde (PFA) and stored in 70% ethanol.

2.2. Micro-computed tomography analysis

The excised samples were analysed by micro-computed tomography (micro-CT) using a vivaCT 40 (70 kVp, 114 μA, 300 msec integration time, 1000 projections on 180° 2048 CCD detector array, cone-beam reconstruction, ScancoMedical, Brüttisellen, Switzerland). All scans were done at an isotropic voxel size of 10 μm. The scans were performed at the mid-diaphysis, cantered halfway between the femoral head and distal condyles, and consisted of 10 slices. An automated contouring method was used to isolate the cortical bone of the femoral midshaft [18]. The images were Gaussian-filtered ($\sigma = 0.8$; supp $= 2$) and thresholded (24% of maximal grayscale value) for analysis. The cortical porosity was determined using the following equation:

$Ct.Po = (1 - BV/TV) * 100$where BV/TV is the bone volume density, and was calculated as the bone volume divided by the total volume in the thresholded image using the built-in micro-CT software (IPL V5.15, ScancoMedical, Brüttisellen, Switzerland). The inner (endosteal) and outer (periosteal) diameters of the femoral cross-sections were then estimated by applying an ellipse-fit using custom-built C++ software with libraries from the Insight Segmentation and Registration Toolkit (ITK, Kitware Inc., New York). This analysis provided the long and short diameters of the ellipse for each surface (Fig. 1).

The regional tissue mineral density (TMD) within the femoral midshaft was then analysed. First, one layer of voxels was removed from the endosteal and periosteal surfaces to eliminate partial volume effects. Subsequently, sub volumes were defined that identified endosteal and periosteal surface (superficial) volumes that may be affected by bone remodelling following disease onset and treatment, and deeper bone that would be less likely to be affected by remodelling (Fig. 1). The superficial and deep zones were defined as bone tissue within 5 voxels (50 μm) from the surface. The deep zones were defined as 10 voxel thick volumes along the centreline. The superficial regions were chosen to include tissue within the approximate depth of a typical resorption cavity (40 μm) [19]. The deep zones were assumed to have mineralization that was not affected by disease and therapy and thus served as a reference mineralization. The ratio of TMD at the surface to deep zones was then calculated.

2.3. Biomechanical testing

The femora were removed from the ethanol and rehydrated in PBS for 2 h prior to mechanical testing (Zwick Roell, Ulm, Germany). A 3-point bend test was set up with a span length of 15 mm, and application of the load applied to the centre of the span. Femora were placed anterior side up and the midpoint of the femoral shaft identified with a calliper and aligned to the span centre. A force was applied at a rate of 0.05 mm/s to a preload of 2 N and held for 5 s before being loaded at a rate of 0.5 mm/s to failure. Force and displacement at the upper support were recorded and converted to stress (σ) and strain (ε) using the following equations [20]:

$$\sigma = \frac{FLc}{4I} \tag{1}$$

$$\varepsilon = \frac{12cd}{L^2} \tag{2}$$

where F is the applied force, L is the span length, c is the distance from the neutral axis to the periosteal surface in the direction of bending, I is the moment of inertia (MOI) of the cross-section at the midpoint of the sample and d is the vertical displacement of the upper point. The parameters c and I were calculated using the built-in micro-CT software. A custom Matlab script (V. R2014a, Matlab, Mathworks Inc., Massachusetts, USA) was written to identify the linear portion of the stress-strain curve and determine the elastic modulus (slope), yield stress (stress at which the stress-strain relationship deviates from linearity) and maximum stress. The toughness was calculated as the area under the stress-strain curve up to the maximum stress.

2.4. Assessment of non-enzymatic glycation

Following the mechanical tests, a 3.5 mm thick section was cut from the proximal end of the break in the femoral midshaft for the assessment of NEG. The specimens were flushed of marrow, demineralized and digested with papain collagenase (0.4 mg/ml in 0.1 mM sodium acetate buffer, pH 6.0, 16 h, 65 °C) [21]. AGE content was determined using an Infinite200 fluorescence plate reader (Tecan) with an excitation wavelength of 370 nm and emission of 440 nm, and normalized to a quinine sulphate standard. The amount of collagen in each specimen was determined from the hydroxyproline content, which was estimated by the absorbance of the digested samples against a hydroxyproline standard at the wavelength of 570 nm using a SpectraFluor Plus microplate reader (Tecan) [22].