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Structural study using micro-CT of the femur of Goto-Kakizaki rats, experimental model for non-overweight type 2 diabetes

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Summary

Background: The effects of type 2 diabetes on the microstructure and mass of bone are not clearly defined. The objective of this study has been to assess the microstructural properties and volumetric bone mineral density of Goto-Kakizaki rats, the rat model for non-overweight type 2 diabetes which tries to circumvent the influence of obesity on bone mass.

Material and methods: An experimental study was designed using Goto-Kakizaki rats compared with a control group of non-diabetic Wistar rats of similar weight and with normal glycemia, with densitometric and microstructural studies being carried out on the distal region of the femur using computerised X-ray microtomography (micro-CT).

Results: In the volumetric densitometry no significant differences were found between the two groups. The microstructural study showed that the BV/TV and trabecular connectivity were reduced in the diabetic rats, while the tube-like trabeculae increased to the detriment of plaque-like trabeculae.

Conclusion: The deterioration trabecular bone quality could explain the decrease in biomechanical bone resistance in type 2 diabetes.

Key words: *type 2 diabetes, X-ray microtomography, bone mineral density, bone microstructure.*

Introduction

Skeletal disorders in diabetes are different according to whether one is dealing with type 1 or type 2 diabetes. Those patients with type 1 diabetes have a decrease in bone mass which implies an increased risk of fracture^{1,2}, whilst type 2 diabetes may present bone mass which is increased, reduced or within normal limits^{3,4}. However, the risk of fracture in type 2 diabetes is increased^{5,6}. This fact could be due to causes both inside, and outside, the bone (retinopathy, neuropathy, drugs, etc) which determine a higher incidence of falls. One fact common in type 2 diabetics is overweight, which constitutes a confusion factor due to the fact that weight is a determining factor of bone mass. Obese people normally have raised bone mineral density. To avoid the influence of this confusion factor an experimental study was designed with Goto-Kakizaki (GK) rats, a substrain of non-obese Wistar rats which develop type 2 diabetes. GK rats have a light to moderate type 2 diabetes which occurs after birth, and develop chronic complications of the disease such as neuropathy and nephropathy⁷.

The main objective of this study was the assessment using computerised micro-tomography of the densitometry and the trabecular and cortical microstructural properties of GK rats, and to compare them with a group of Wistar rats used as a control, with the intention of evaluating, and in their case, defining, the changes which in these variables induce obesity.

Material and methods

Animal model

An experimental study was carried out with 4 male GK rats as against a control group of 4 male non-diabetic Wistar rats of a similar weight and with normal glycemia (Taconic Farms Inc. Lille Skensved, Denmark), due to the fact that the Goto-Kakizaki substrain was developed from the Wistar rats. The treatment of the animals and all the experiments were carried out in accordance with Law 14/2007 and with Royal Decree 1201/2005, and following the guidelines of the UNE-EN 30993-3:1994 rules and ISO 10993-2:2006. The rats were fed with a standard diet and had free access to water, did not received any drug treatment, and were sacrificed at 12 weeks in a chamber with CO₂.

Microstructural analysis of the bone using micro-CT

Once the animals were sacrificed, the right femurs were extracted in order to carry out the structural analysis. After the extraction, the samples were wrapped in gauze soaked in saline solution and conserved at -20°C until the last moment before analysis. The microstructural analysis of the samples was carried out using computerised X-ray microtomography (micro-CT), using the commercial equipment SkyScan 1172 (SkyScan NV, Aarstelaar, Belgium) in the Trabeculae® research laboratory, Empresa de Vase Tecnologia, S.L (Ourense, Spain).

The distal region of each femur was scanned with an X-ray source of 50 KV and an intensity of 200 µA, using a voxel size of 8.95 µm. An aluminium filter of 0.5 mm in thickness was positioned to reduce beam hardening artefacts. The rotation step used was 0.4° up to a total of 180° and the exposure time was 1250 ms.

The images obtained were reconstructed using the modified Feldkamp algorithm⁸, using the NRecon 1.5 software application (SkyScan NV, Aarstelaar, Belgium). The transverse sections resulting from the reconstruction stage were used for the quantitative analysis of the trabecular and cortical bone microstructure, using the application CTAn 1.10.0.1 (SkyScan NV, Aarstelaar, Belgium), after their segmentation into binary images using locally adapted thresholding⁹.

For the analysis of the trabecular bone a metaphyseal-diaphyseal region of interest (cortical bone excluded) of 2.5 mm was selected, starting at a distance of 1.0 mm from the growth plate in a proximal direction. For the analysis of the microstructural properties of the cortical bone a region of interest of 1.0 mm was taken, starting at 4.0 mm from the growth plate (Figure 1).

The quantitative variables which were determined for the trabecular region were: volumetric bone fraction (BV/TV), specific bone surface (BS/BV), bone surface density (BS/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N), trabecular pattern factor (Tb.Pf), structural model index (SMI), and degree of anisotropy (DA). The different variables were measured directly using methods described in the literature^{10,11}. The non-metric variables, SMI and Tb.Pf, were calculated directly from the three dimensional model. The SMI indicates the relative prevalence of plate-like or rod-like trabeculae, with a higher presence of plates being indicated the nearer its value is to zero¹². For its part, Tb.Pf is an inverse index of connectivity, measured from the calculation of the relative convexity or concavity of the bone surface¹³. A higher value of Tb.Pf the trabecular network shows a poorer connectivity, which also implies a reduction in mechanical resistance. The DA is a measure of the alignment of the trabeculae in a determined direction, calculated in such a way that 0 is complete isotropy and 1 is complete anisotropy.

In the case of the cortical region, the parameters calculated included: cortical thickness (Ct.Th), the average transverse area of the bone (B.Ar), the average polar inertial moment (I) and the eccentricity (Ecc). I is a basic index of mechanical resistance which indicates the resistance to rotation of a transverse section in a determined axis (assuming uniform biomechanical properties). Ecc is a parameter which indicates the difference in elongation of a transverse section with respect to a circular form (a circle is considered to be an ellipse with zero eccentricity).

Determination of volumetric bone mineral density

Using the images obtained through micro-CT, the volumetric bone mineral density (BMDv) was

determined, both in the cortical and trabecular regions. Direct calibration was used with attenuation coefficients of calcium hydroxyapatite models of known density (250 and 750 mg/cm³). The method of calculating the BMDv differed slightly in different areas of the bone, since in the case of the trabecular region it refers to a volume of bone and medullar tissue, whilst in the case of the cortical region it was limited to a volume occupied solely by calcified lamellar bone.

Statistical analysis

The data obtained were put into a text database which was exported to the statistical software package IBM SPSS Statistics 19 (IBM Corporation, Somers, NY, US) for their subsequent statistical analysis. The individual results were reviewed in order to avoid loss of data and unusual values. Then the descriptive analysis of the variables of the study was proceeded with. The descriptive statistics of the numerical variables were expressed as an average \pm standard deviation, maximum value and minimum value.

The comparative statistical study of the numerical data was carried out by means of an single factor variance analysis (ANOVA) and the Tukey HSD test for multiple comparatives. In those cases which did not comply with homogeneity of variance criteria, the Broen-Forsythe test was applied for the variance analysis and the Games-Howell test for the multiple comparatives.

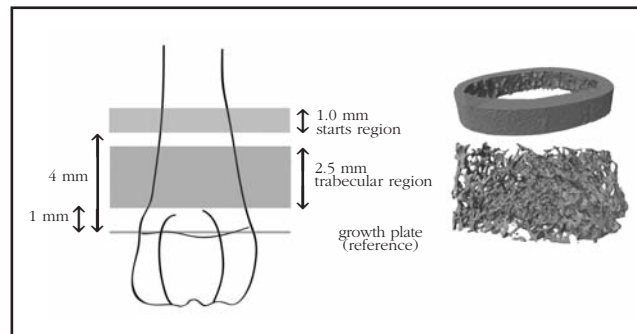
The level of statistical significance was established at values of $p < 0.05$ for all the variables analysed.

Results

The GK rats had a weight of 385 ± 23 g and glycaemia of 195 ± 84 mg/dl, which confirms the presence of diabetes in this group, while the Wistar rats had a weight of 395 ± 35 g and glycaemia of 124 ± 15 mg/dL ($p < 0.01$). The results of the bone mineral density volumetry are reflected in Table 1. BMDv did not show differences between the two groups, neither in the cortical section, nor the trabecular. Although there appears to be a loss of trabecular bone mass in the diabetic rats, this did not reach significant levels, probably due to the sample size used, or the development time of the diabetes.

Table 2 shows the results of the microstructural variables in the trabecular region. Statistically significant differences were observed in the volumetric bone fraction (BV/TV), indicating a loss of trabecular bone in the diabetic rats as compared with the control group. The considerable increase in Tb.Pf in the GK rats confirms, also, a significant loss of trabecular connectivity in diabetes. On the other hand, the increase in SMI shows a prevalence of tubular trabeculae in diabetic rats in comparison with the control group, in which plate-like trabeculae predominate. Although the BS/TV, the Tb.Th and the Tb.N are reduced in diabetic rats, at the same time the Tb.Sp is increased, together

Figure 1. Scheme of a distal rat femur in which are indicated the regions analysed using micro-CT, indicating its dimensions and distances from the reference (growth plate). The trabecular region starts at 1 mm from the growth plate to avoid the primary spongy tissue, while the cortical region starts at 4 mm to select a zone with few or no trabeculae



indicating a deterioration in the trabecular micro-architecture (Figure 2), their values not reaching statistically significant levels.

In the cortical region, although the diabetic group appears to have a reduction in the thickness of the cortical wall, this being determined through the transverse sections (Cs.Th) or assumed in the three-dimensional model (Ct.Th), this does not reach statistically significant levels (Table 3). The variables B.Ar, I and Ecc show very similar values in the two groups. The cortical regions of two representative samples can be seen in Figure 3.

Discussion

The study carried out shows that diabetic rats have a volumetric bone density similar to non-diabetic rats. However, differences are observed in the trabecular bone in the structural parameters. There is a reduction in BV/TV, a lower trabecular connectivity and a predominance of cylindrical trabeculae in the GK rats. The trabecular structural variables in which there are significant differences between the diabetic and control rats are those related to bone resistance. The lower quantity of bone indicated by the reduction in BV/TV, and the great loss of trabecular connectivity revealed by the increase in Tb.TP in the diabetic group, result in an evident reduction in biomechanical resistance. In addition, it has been shown that the tube-like trabeculae, which predominate in the diabetic group, are less resistant to mechanical load than the plate-like trabeculae¹², more abundant in the control group, from which is deduced the SMI value. All these data indicate that, although their bone density is normal, the GK rats would have a lower biomechanical resistance, which could suggest that the higher prevalence of fractures which occurs in type 2 diabetes would be related to alterations in bone quality.

There are few studies carried out with this experimental model. Zhang et al. measured, in a group of GK rats, the bone mineral density using

Table 1. Volumetric bone mineral density in the trabecular and cortical regions of the distal femur of control and diabetic rats

	Rats WI control (average ± SD)	Rats GK diabetics (average ± SD)	Value of p
BMDv trabecular (mg/cm ³)	306.63 ± 48.78	261.23 ± 45.54	NS
BMDv cortical (mg/cm ³)	1,490.97 ± 227.57	1,727.13 ± 133.95	NS

SD: standard deviation. BMDv: volumetric bone mineral density. NS: not significant

Table 2. Results of the microstructural variables of the trabecular region of the distal femurs of control and diabetic rats

	Rats WI control (average ± SD)	Rats GK diabetics (average ± SD)	Value of p
BV/TV (%)	20.68 ± 2.87	15.51 ± 2.90	0.034
BS/BV (mm ⁻¹)	44.50 ± 9.21	47.76 ± 5.21	NS
BS/TV (mm ⁻¹)	9.10 ± 1.71	7.31 ± 0.88	NS
Tb.Th (µm)	84.15 ± 11.72	77.38 ± 5.83	NS
Tb.Sp (µm)	385.28 ± 103.73	332.18 ± 54.31	NS
Tb.N (mm ⁻¹)	2.47 ± 0.34	2.00 ± 0.30	NS
Tb.Pf (mm ⁻¹)	2.98 ± 2.58	10.01 ± 2.45	0.004
SMI	1.24 ± 0.04	1.66 ± 0.18	0.001
DA	0.58 ± 0.06	0.55 ± 0.02	NS

SD: standard deviation. BV/TV: bone volumetric fraction. BS/BV: bone specific surface. BS/TV: bone specific density. Tb.Th: trabecular thickness. Tb.Sp: trabecular separation. Tb.N: trabecular number. Tb.Pf: trabecular pattern factor. SMI: structural model index. DA: degree of anisotropy

Table 3. Results of the microstructural variables of the cortical region of the distal femurs of control and diabetic rats

	Rats WI control (average ± SD)	Rats GK diabetics (average ± SD)	Value of p
B.Ar (mm ²)	5.78 ± 0.50	5.79 ± 0.32	NS
I (mm ⁴)	21.87 ± 5.09	21.11 ± 2.38	NS
Cs.Th (µm)	396.92 ± 23.23	373.61 ± 22.74	NS
Ct.Th (µm)	458.23 ± 15.40	433.07 ± 22.99	NS
Ecc	0.74 ± 0.03	0.79 ± 0.02	NS

SD: standard deviation. B.Ar: average transverse area of the bone. I: average polar inertial moment. CsTh: thickness of transverse section. CtTh: cortical thickness. Ecc: eccentricity

Figure 2. Three-dimensional representation of the trabecular region analysed in the distal femur of a representative sample of the control group and the diabetic group

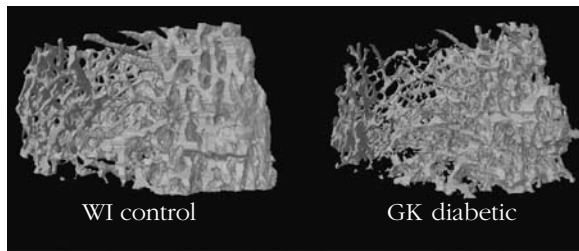
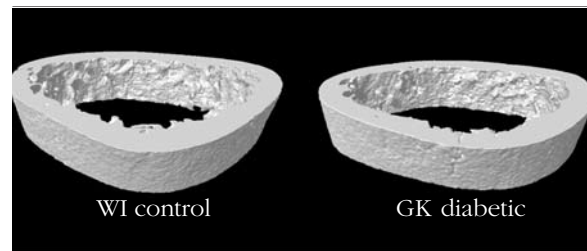


Figure 3. Three-dimensional representation of the cortical region analysed in the distal femur of a representative sample of the control group and the diabetic group



DXA and the microstructure with classic histomorphometric techniques in 2D⁷. These authors found a reduction in bone mass and a change in the histomorphometry with a decrease in BV/TV, similar to that found in our study. Ahmad et al., using peripheral quantitative computerised tomography (pQCT), observed a decrease in volumetric bone mineral density predominantly in the trabecular section¹⁴. The measurements were taken in the humerus, tibia and metatarsals of female rats, which could explain the differences observed from our results. In none of the regions analysed in this work were there found differences in the cortex. However, another work, using radiographic techniques in a group of 10 GK rats observed a decrease in cortical thickness in the metatarsal and humerus¹⁵. The data observed are heterogeneous, probably due to the different techniques used and the different places where measurements were taken. However, in all of them, trabecular affection predominates and, when it was determined, a reduction in biomechanical bone resistance. Another model of non-obese diabetic rat is that of Zucker rats, developing the disease progressively until the serious complications of the disease appear. These rats show a decrease in bone mass, both cortical and trabecular, smaller sized large bones, and a deterioration in the biomechanical and microstructural properties of the cortical and trabecular bone^{16,17}.

Various mechanisms may explain this change. Glucose represents the principal source of energy to the osteoclasts, with hyperglycemia being responsible for an increase in osteoclast activity, with an increase in bone remodelling and a decrease in the quantity and quality of bone². On the other hand, hyperglycemia provokes the non-enzymatic glycosylation of the bone proteins, damaging bone quality¹⁸. In turn, glycosuria increases hypercalciuria with changes to the PTH/vitamin D system. These deleterious effects on bone quality may be partially compensated for by an increase in bone mass associated with obesity¹⁹. An alteration in calcium metabolism may also contribute to the deterioration of bone quality. In diabetics an increase in calciuria has been described, which has been related to hyperglycemia and glycosuria. This provokes secondary hyperparathyroidism

which exerts a prejudicial effect on the bone, especially on the trabecular section²⁰. The alteration of the vitamin D and parathormone metabolism is particularly prominent in patients with reduced renal function. Microangiopathy can alter endothelial function²¹, and macroangiopathy with arteriosclerosis can cause a reduction in blood supply to the bone^{22,23}. On the other hand, in patients with neuropathy a change in the load on the bone can also contribute to the loss of bone mass. We can say, therefore, that there are multiple mechanisms which exert a deleterious effect on bone in experimental animals with type 2 diabetes which can explain the alteration in bone quality in these models.

In conclusion, we can say that Goto-Kakizaki rats are a valid model for the study of type 2 diabetes, since they eliminate a significant confusion factor, overweight. Although the sample size is small, we found a deterioration in the microstructure in the femoral trabeculae, with, however, the volumetric bone mineral density being preserved.

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