INTRODUCTION

Glitazones are oral antidiabetic agents. They selectively stimulate the peroxisome proliferator-activated gamma (PPAR-γ) receptor and reduce insulin resistance. The only glitazone actually in use in most European countries is pioglitazone (administered at 15–45 mg per day). It has been documented that patients with diabetes mellitus (DM) exhibit a higher incidence of bone fractures (1). There is also accumulating evidence that glitazones may increase fracture risk in diabetic patients, as demonstrated by a recent meta-analysis of 10 controlled trials (2).

Many experimental studies investigated the potential mechanisms of this glitazone effect. In vitro, PPAR-gamma agonists were found to promote adipocyte over osteoblast differentiation (3-5). Decrease in bone mineral density as assessed by dual-energy X-ray absorptiometry (DXA) has been documented in ovariectomized rats receiving pioglitazone (6) or rosiglitazone (7), and in male mice receiving rosiglitazone (8). These findings were not confirmed in female rats receiving high dose pioglitazone (9).

Several markers of bone formation and resorption have also been used in order to clarify the underlying pathophysiological mechanisms. Significantly lower plasma osteocalcin levels have been described in ovariectomized rats receiving pioglitazone, but not in female rats receiving high-dose pioglitazone (6). Other markers (urinary calcium excretion, alkaline phosphatase (ALP), urine deoxypyridinoline, aminoterminal propeptide of type I collagen, C-terminal collagen cross-linked peptide) have been used in human studies with contradictory results (10-14).

Our study aims to investigate the effect of a low-dose pioglitazone regimen on bone mineral density and bone formation-resorption markers in control and diabetic rats.

MATERIAL AND METHODS

Animal model

Animals used in this study were treated according to the Directive by the Council of the European Communities (86/609/CEE) and the Directive by the European Parliament (2003/65/CE) on the protection of animals used for experimental and other scientific purposes.

Twenty ten-week-old male Wistar rats were used as described elsewhere (15, 16). In summary, diabetes was induced after an overnight fast by a single intraperitoneal injection of streptozocin (50 mg/kg dissolved in 0.9% sterile sodium chloride). Rats were considered diabetic if blood glucose was >300 mg/dl three days after streptozocin injection.

Our animal model is a type 1 diabetes model created by streptozocin induced destruction of pancreatic β-cells. This model was chosen to study the bone effects of pioglitazone regardless of its glucose lowering effect because pioglitazone is not supposed to affect glucose levels in an insulinopenic model.

Key words: bone mineral density, diabetes mellitus, osteocalcin, pioglitazone, streptozocin
was used to avoid total destruction of pancreatic β-cells that occurs with higher streptozocin doses. Therefore, insulin administration was not necessary; however, the established diabetes was not mild, most rats having plasma glucose levels that exceeded 500 mg/dl.

**Study design**

The rats rendered diabetic by streptozocin were randomized into 2 groups of 5 rats each: (i) no treatment (D); (ii) pioglitazone (Actos®, Lilly) 3 mg/kg per day (17) incorporated in chow (D+P). Ten age-matched male rats that had been injected with saline alone served as non-diabetic controls. They were also randomized into 2 groups of 5 rats each: (i) no treatment (N); (ii) pioglitazone 3 mg/kg per day (N+P). Daily food intake was checked at regular intervals to affirm the dose of the administered drug by weight.

At the end of the study (9 weeks after diabetes induction and having completed 8 weeks of treatment), rats were placed into individual metabolic cages to obtain 24-hour urine samples that were quantified and frozen at −80°C for later analysis of calcium and creatinine levels. Then, they were anesthetized with an intraperitoneal injection of thiopental (40 mg/kg) and totally centrifuged to obtain serum, which was stored at −80°C until assayed.

**Assays**

Urine creatinine concentration was determined by the modified Jaffe method, ALP by the chromatometric method, and urine calcium levels by the Schwarzenbach method using the Cobas Integra 400 analyzer (Roche Diagnostics GmbH, Indianapolis, Ind., USA). Serum osteocalcin concentration was determined by electrochemiluminescence (sandwich method) using the Elecsys analyzer (Roche Diagnostics GmbH, Indianapolis, Ind., USA). For the urinary levels of C-telopeptide (CTX), we used the specific competitive EIA assay kit (Urine CrossLaps® EIA, Immunodiagnostic Systems (IDS), Indianapolis, Ind., USA). We also measured CTX concentration in the urine. The median CTX concentration was 84.7 µg/mmol creatinine in diabetic rats and 78.9 µg/mmol creatinine in control rats. Pioglitazone treatment did not have a significant effect on CTX levels. Differences among the four studied groups were not significant (p=0.19). Pioglitazone treatment had no effect on CTX levels. Differences among the four studied groups were not significant (p=0.19).

**Bone mineral density assessment**

At least three rats in each group had their bone mineral density measured by DXA in the 2 months following sacrifice. Small animal high-resolution scan was performed (line spacing 0.3 mm) using a HOLOGIC Discovery (Bedford, MA, USA). A mean value of bone mineral density (BMD, gram/cm²) for the whole left femur and two sub regions, the diaphysis and proximal metaphysis was measured.

**Statistical analysis**

Values are expressed as means ±S.E.M. The Shapiro-Wilk test was used to assess normality and logarithmic transformation was applied when data were skewed. Differences were considered to be significant when p <0.05. The statistical significance of the difference between group means was determined by one-way analysis of variance followed by Tukey’s post-hoc test for multiple comparisons. For data that were not normally distributed, we used the non-parametric Kruskal-Wallis test to compare means.

**RESULTS**

Fig. 1 shows the biochemical parameters for the four studied groups. The control group gained weight during the eight weeks of the study (25±12 g). The diabetic rats lost weight (−37±15 g), and this loss was not prevented by pioglitazone.

Two bone formation markers were studied, osteocalcin and alkaline phosphatase. Osteocalcin was significantly lower in the diabetic (5.4±1.6 ng/ml) than in control rats (22±6.0 ng/ml) (p<0.01). Pioglitazone did not have a significant effect on osteocalcin levels in diabetic and control rats (Fig. 1). On the contrary, average ALP was 203±28 U/l in the control group and significantly higher in the diabetic group (1482±164 U/l) (p<0.01). Although pioglitazone treatment did not have a significant effect on ALP levels in control rats, it did reduce ALP concentration in diabetic rats (915±126 U/l) (p=0.01) (Fig. 1). Calciuria, expressed by the urine calcium to creatinine ratio, was used as a bone resorption marker. Calciuria increased in diabetic rats (0.86±0.21) compared with the control rats (0.20±0.08) (p=0.038). Pioglitazone administration did not affect calciuria levels in both groups (Fig. 1).

We also measured CTX concentration in the urine. The median CTX concentration was 84.7 µg/mmol creatinine in diabetic rats and 78.9 µg/mmol creatinine in control rats. Pioglitazone treatment had no effect on CTX levels. Differences among the four studied groups were not significant (p=0.19).

Bone mineral density was evaluated for whole femur, in proximal metaphysis and in diaphysis. Regardless of the site, there was no significant difference between the four studied groups (Table 1).

**DISCUSSION**

Diabetes induction in our rats was associated with weight loss, increased urinary calcium and diminished plasma osteocalcin levels. Our findings on bone markers confirm previous studies showing reduced osteocalcin levels (18) and hypercalciuria in diabetic rats (19). It is well documented that diabetes promotes an adipocyte-like phenotype suppressing osteoblast differentiation that explains lower levels of bone formation markers. Osteoclast activity is also increased by glucose leading to increased levels of bone resorption markers (20).

We found that diabetes mellitus induction did not affect bone mineral density. A recent study using the streptozocin model also reported no significant decrease in bone mineral density in diabetic rats (21). The short duration of the experiment in both studies has to be taken into account. Rats were also maintained on treatment only for eight weeks, which may not be sufficient for the development of bone complications.

Biomechanical testing (three point bending of the femur mid-shaft, testing of femoral diaphysis in torsion) is the gold standard for assessing bone fracture resistance. A recent study

**Table 1. Bone mineral density (g/cm²) in experimental animals. Values are expressed as means ±S.E.M.**

<table>
<thead>
<tr>
<th>BMD</th>
<th>N</th>
<th>N+P</th>
<th>D</th>
<th>D+P</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole femur</td>
<td>0.26±0.015</td>
<td>0.26±0.009</td>
<td>0.25±0.003</td>
<td>0.22±0.009</td>
<td>0.09</td>
</tr>
<tr>
<td>Proximal metaphysis</td>
<td>0.26±0.015</td>
<td>0.26±0.012</td>
<td>0.25±0.005</td>
<td>0.23±0.013</td>
<td>0.22</td>
</tr>
<tr>
<td>Diaphysis</td>
<td>0.23±0.018</td>
<td>0.24±0.010</td>
<td>0.23±0.005</td>
<td>0.22±0.009</td>
<td>0.51</td>
</tr>
</tbody>
</table>
demonstrated decreased mechanical properties in ovariectomized rats with glucose intolerance treated with rosiglitazone (22). High-resolution micro-computed tomography also yields more reproducible results in small animals. However, both techniques were not available in our laboratory.

We are the first to examine the effect of a low-dose pioglitazone regimen in male rats. We chose this dose (3 mg/kg) because it is closer to doses used in clinical practice (<1 mg/kg). We found that pioglitazone treatment did not affect bone formation and resorption marker levels and did not modify bone mineral density in the four studied groups. These results are in contradiction with previous studies examining the effects of glitazones on bones. However, these previous studies were conducted on female rats (6, 9) or used a higher pioglitazone dose in the 30-40 mg/kg range (9, 23).

Our results concerning the alkaline phosphatase and the CTX levels should be interpreted with caution. The ALP is a much less specific marker of bone formation than osteocalcin. Our CTX values are urine not plasma levels, and they were considerably skewed with many outliers.

This study is clearly underpowered given the small number of rats in each group. The high mortality rate in this animal model due to the sustained severe hyperglycaemia considerably limits the total number of rats in the studied groups. Nevertheless, animal studies are frequently underpowered because of technical limitations and concerns about animal welfare and commonly search only for trends. Their results usually need to be confirmed in larger clinical trials.

In conclusion, pioglitazone at the 3 mg/kg dose was not associated with significant skeletal complications in our experimental model. However, its negative impact on bones has been well documented in large human studies, mainly on older female patients (2). Glitazone use has recently been limited or restricted in many countries by concerns about adverse cardiovascular effects and increased bladder cancer risk. Therefore, pioglitazone should be used with caution in diabetic patients and alternative treatments considered when possible.

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