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Sirolimus and tacrolimus rather than cyclosporine A cause bone loss in healthy adult male rats

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ABSTRACT

The aim of this work was to study the effects of cyclosporine (CsA), tacrolimus (FK-506), and rapamycin (RAPA) on bone mass, femoral microstructure, femoral biomechanical properties, and bone remodeling in healthy adult male rats.

Forty-eight 5-month-old male Wistar rats were used. CsA (2 mg/kg/day), FK-506 (3 mg/kg/day), RAPA (1.25 mg/kg/day), or water (0.5 ml/rat/day, control group) were administered orally for 3 months.

After sacrifice, mean values of immunosuppressants in blood were: CsA (670.4 ng/ml), FK-506 (19.2 ng/ml), and RAPA (4.8 ng/ml). Levels of biochemical parameters were normal in all groups. Femoral BMD was decreased in FK-506 and RAPA groups and lumbar BMD in FK-506 group. Trabecular volume fraction (BV/TV) decreased only in FK-506 group. RAPA and CsA affected femoral cortical structure, but FK-506 did not. FK-506 produced an increase in bone remodeling, and CsA a decrease. FK-506 group showed a decrease in biomechanical parameters relative to all groups. RAPA group showed a decrease in ultimate stress vs control group, and CsA group presented an increase in biomechanical parameters versus control group.

We found that administration of both RAPA and FK-506 as monotherapy for healthy rats produced osteopenia. CsA treatment only produces slight damages in the cortical zone of the femur.

1. Introduction

With the use of new and more powerful immunosuppressive drugs, survival rates in patients receiving solid-organ transplantation have improved significantly due to effective control of acute rejection episodes. However, a substantial number of works describe the development of osteoporosis among patients who have received transplantation of organs such as the kidney, heart, or liver (Monegal et al., 2001; Al-Gabri et al., 2005; Maalouf and Shane, 2005; Kulak et al., 2012; Wang et al., 2013; Monegal et al., 2013). These patients have increased risk of vertebral and nonvertebral fracture (Ramsey-Goldman et al., 1999). The rate of bone loss increases during the first year after kidney or heart transplantation but experiences a stabilization at the lumbar level. However, in parts of the skeleton where cortical bone is predominant, like the hip, an accelerated rhythm of bone loss is maintained over the years following transplantation (Delmas, 2001). Osteoporosis and increased risk of fracture have a negative impact on the quality of life of patients. In view of the morbidity and mortality associated with hip and vertebral fractures, such a pattern will influence the quality of life of these patients.

The many factors contributing to the pathogenesis of osteoporosis after transplantation include the underlying disease (e.g., primary biliary cirrhosis), concurrent disorders, hypogonadism, postoperative malnutrition and immobilization, and treatment with glucorticoids. The negative effects of glucocorticoids on bone are known (Delmas, 2001). In recent years, however, the accepted dose of glucocorticoids after transplantation has been significantly reduced, and the action of immunosuppressants themselves on bone cannot be overlooked as a potential negative factor. Cyclosporin (CsA), FK-506 (tacrolimus), or rapamycin (RAPA) are immunosuppressants used in many transplantation patients. In experimental models, CsA has been shown to induce bone loss through an increase in bone resorption (Tsuruoka et al., 2005). Indeed, a correlation has been observed between CsA serum concentrations and increased bone remodeling in patients with heart (Thiébaud et al., 1996) or liver (Giannini et al., 2000) transplantation. While it is currently accepted that FK-506 presents minor secondary effects when compared to CsA, there are also works that show that this immunosuppressant increases bone remodeling and brings about a significant decrease in bone

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mineral density (BMD) in human trabecular bone (Stempfle et al., 1998) and in experimental models (Kirino et al., 2004). RAPA, on the other hand, is a more novel treatment. According to several reports, its effects on bone are lower than those of other immunosuppressants (Romero et al., 1995; Campistol et al., 2005). In spite of the importance of the problem, there are few works in the literature that study the causes of bone loss in patients who have undergone solid-organ transplantation.

The importance of this question led us to study the actions of 3 commonly used immunosuppressants on bone quality: CsA, FK-506, and RAPA. Due to the difficulty of performing this study in humans as a result of heterogeneity in age, transplanted organ, sex, and initial patient status, we decided to use male adult rats as a homogenous experimental model. Rats have been previously used by many investigators to study bone loss and the positive and negative effects of various drugs on loss of bone (Zhu, 2010).

The aim of this work was: 1) to establish an experimental model of administration of immunosuppressants to healthy male rats in which the level of blood immunosuppressants was similar to the levels clinically accepted for human patients treated with these drugs; and 2) to use this experimental model to comparatively study the effects produced by CsA, FK-506, and RAPA on BMD, trabecular and cortical bone microstructure, biomechanical properties, and bone remodeling through the levels of biochemical markers of bone turnover.

2. Materials and methods

2.1. Animals

Forty-eight five-month-old male Wistar rats weighing 405 ± 32 g (mean ± SD) were used. The animals were kept under constant living conditions (22 °C, 12 h per day of light–dark cycles), and food (standard laboratory chow) and water were available ad libitum.

The animals were randomized to 4 different groups: SHAM (n = 12), in which each rat received 300 μl/day of distilled water by oral gavage; CsA (n = 12), in which each rat received 2 mg/kg/day of CsA by oral gavage; RAPA, in which each rat received 1.25 mg/kg/day of RAPA by oral gavage; FK-506, in which each rat received 3 mg/kg/day of FK-506 by oral gavage. Treatment was maintained for 3 months.

2.2. Immunosuppressant preparation

Doses of immunosuppressants were prepared according to a medium weight of the experimental rats of 400 g. Cyclosporine A (Sandimmun, 100mg, Novartis) was diluted in distilled water (150 μl of CsA in 5475 μl of distilled water). 300 μl of this dilution was administered daily by oral gavage to each rat (2 mg/kg/day). FK-506 (Tacrolimus, Prograf 5mg, capsules). Each day, the content of 4 capsules (20 mg) was diluted in 2 ml of ethanol to solubilize the product and 3 ml of distilled water were added. 300 μl of this dilution was administered daily by oral gavage to each rat (3 mg/kg/ day). Rapamicine (Rapamune, 1 mg/ml, oral solution, Wyeth) was administered directly to rats from pharmaceutical preparation, 500 μl/rat by oral gavage daily (1.25 mg/kg/day). Each rat of the SHAM group received daily 300 μl of distilled water by oral gavage.

The day following the last treatment, the experimental animals were weighed and sacrificed by exsanguination while under ether anesthesia. Blood samples were obtained by cardiac puncture (2 ml with EDTA for determination of immunosuppressant levels in total blood) and 7 ml without any additive for serum determinations. Total blood was immediately used to determine immunosuppressant levels, and serum aliquots were frozen at −80 °C until determination of biochemical markers of bone turnover or other biochemical determinations. Once the blood was collected, the animals were frozen at −20 °C until determination of BMD in previously thawed animals. Prior to BMD analyses, the left femurs were excised and cleaned of adjacent tissue. The right femur was also excised and cleaned for computerized microtomographic analysis (μCT) and biomechanical testing. Lumbar spine BMD was determined in situ. Repeated freeze–thaw cycles have been shown to have no influence on the mechanical properties of bone (Borchers et al., 1995). All the experiments with animals were carried out according to Spanish law regarding the use, protection, and care of experimental animals (Royal Decree 53/2013).

2.3. Blood immunosuppressants

2.3.1. CsA

The automated Dimension® CSA method uses an immunoassay technique in which free and CsA-bound antibody-enzyme species are separated using magnetic particles. CsA was measured in whole blood samples (EDTA tubes) that were previously mixed and lysed. Sensitivity of the methods was 25 ng/ml and inter- and intra-assay variation coefficients were <11% and <15.9%, respectively.

2.3.2. FK-506

FK-506 levels were measured in an IMx analyzer (Abbott, Germany), which performs microparticle enzyme analysis (MEIA). FK-506 was measured in whole blood samples (EDTA tubes) previously extracted using a precipitation reagent. The sensitivity of the method was 4.1 ng/ml and inter- and intra-assay variation coefficients were <4% and <10%, respectively.

2.3.3. RAPA

RAPA levels were measured in an IMx analyzer (Abbott, Germany). This autoanalyzer also performs MEIA. RAPA was measured in whole blood samples (EDTA tubes) previously extracted using a precipitation reagent. The sensitivity of the method was 1 ng/ml and inter- and intra-assay variation coefficients were <7% and <9%, respectively.

2.4. BMD

BMD was determined in situ in the lumbar spine (L2, L3, and L4) and in the whole left femur by dual energy X-ray densitometry (DEXA) using a HOLOGIC QDR-1000 TM (S/N 277) (Hologic, Inc., Waltham, MA, USA) with small-animal software (Gala Paniagua et al., 1998). Intra-assay and inter-assay variation coefficients were <0.53% and <1.2%, respectively. The femur scans were analyzed for BMD of the whole femur. The scans of the L2, L3, and L4 vertebrae were analyzed for BMD of the whole 3 vertebrae and the results were expressed as the mean of the values obtained.

2.5. Trabecular and cortical microarchitecture analysis of femur by microCT

The distal region of the right femur was analyzed by μCT (Skyscan N.V., Aartselaar, Belgium) imaged with an X-ray tube voltage of 100 kV and a current of 100 μA, and with a 1.0 mm aluminum filter. The trabecular and cortical bone femur region were studied. For more details, see reference De La Piedra et al. (2011).

2.6. Biomechanical testing

The femora were subjected to mechanical testing with a Microtest EM1/10/FR/m testing machine (Microtest, S.A., Madrid, Spain) and 3-point bending strength was measured. The extrinsic biomechanical properties of bone include ultimate force, stiffness, and work to failure. The intrinsic biomechanical properties include ultimate stress, apparent young modulus, and toughness. For more details, see reference De La Piedra et al. (2011).

2.7. Biochemical markers of bone turnover

Serum bone Gla protein (Osteocalcin) (BGP) was determined by ELISA for the specific quantitative determination of rat osteocalcin levels