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# Serum and Renal Tissue Markers of Nephropathy in Rats Under Immunosuppressive Therapy: Cyclosporine Versus Sirolimus

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ABSTRACT

Cyclosporin (CsA) has been progressively replaced by other drugs with putatively fever side effects, including nephrotoxicity and hypertension. Sirolimus (SRL) is one of the main options for management of kidney transplant patients in the post-CsA era. It shows identical efficacy with apparently less cardiorenal side effects than CsA. However, doubts remain concerning the mechanisms of putative renoprotection by SRL as well as the best serum and/or tissue markers for nephropathy, as assessed in this study employing CsA- and SRL-treated rats. Three groups ($n = 6$) were treated orally during a 6-week protocol: control (vehicle); CsA (5 mg/kg body weight per day Sandimmun Neoral); SRL (1 mg/kg body weight per day Rapamune). Blood pressure and heart rate were assessed with a "tail cuff". Renal dysfunction and morphology were characterized using serum creatinine and blood urea nitrogen (BUN) levels as well as hematoxylin and eosin and periodic acid Schiff staining, respectively. We examined serum concentrations of interleukin (IL)-2, IL-1$\beta$, high-sensitivity C-reactive protein, tumor necrosis factor TNF-$\alpha$, and vascular endothelial growth factor and kidney mRNA expression of interleukin-1$\beta$ (IL-1$\beta$), tumor protein 53 (TP53), mammalian target of rapamycin (mTOR) and proliferating cell nuclear antigen (PCNA), as well as markers of lipid peroxidation in the kidney and serum. Both CsA and SRL induced significant increases in systolic and diastolic blood pressure, but only CsA caused tachycardia. CsA-treated rats also displayed increased serum creatinine and BUN levels, accompanied by mild renal lesions, which were almost absent among SRL-treated rats, which presented hyperlipidemic and hyperglycemic profiles. CsA-induced nephrotoxicity was accompanied by kidney overexpression of inflammatory and proliferative mRNA markers (IL-1$\beta$, mTOR and PCNA), which were absent among SRL group. In conclusion, the antiproliferative and antifibrotic character of SRL may explain its less nephrotoxic profile. Renal over expression of mTOR in the CsA-treated group, associated with renal dysfunction and structural damage, reinforces the potential beneft of SRL as a strategy to reduce CsA-evoked nephrotoxicity.

SHORT-TERM ALLOGRAFT SURVIVAL has steadily improved over the last decades with the use of calcineurin inhibitors (CNIs), in particular due to the efficacy and selectivity of cyclosporine (CsA).[1] Despite this progress, chronic allograft nephropathy (CAN) remains an important cause of renal allograft loss.[2,3] In fact, its clinical use is

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0041-1345/–see front matter
http://dx.doi.org/10.1016/j.transproceed.2013.02.085

associated with undesirable serious side effects, such as nephrotoxicity and arterial hypertension,[4,5] accounting for a large percentage of allograft losses and posttransplant mortalities.[5,6] Thus, long-term graft survival has not been yet achieved with this drug class.

The main strategy to limit the CsA side effects nowadays is the substitution for CNIs with a less toxic immunosuppressive agent. Sirolimus (SRL), an inhibitor of the mammalian target of rapamycin (mTOR), is one of the main options.[7–9] However, other serious adverse effects have been reported such as lipid abnormalities and thrombocytopenia.[10] Moreover, evidence of nephrotoxicity and proteinuria have also been debated recently.[11–14] Although, these adverse side effects occur only in some patients, they could be minimized if we better understood the cellular and molecular effects of SRL on the kidney. Clinical practice per se cannot clarify these aspects, not only because of the short trial periods, but, because of the absence of putative biomolecular markers of renal dysfunction.

We and others have previously reported some components involved in the cardiorenal side effects of CsA, including platelet and vascular hyperreactivity and, oxidative stress as well as renin-angiotensin and sympathetic systems overactivities.[15–18] By augmenting the expression of intrarenal proteins and cytokines CsA has been associated with induction of profibrotic lesions.[19] In contrast to CsA, SRL seems to be beneficial for long-term graft function, which could be due to its antiproliferative activity that results from inhibition of mTOR downregulating associated protein phosphorylation and cell cycle progression.[19–22] However, the precise mechanisms inducting inflammatory cascades and fibrogenic processes remain obscure. This limits our ability to ameliorate or prevention CsA-induced (CAN). Thus, the aim of this experimental study was to compare serum and renal tissue markers of putative nephropathy in rats under CsA versus SRL therapy.

## MATERIALS AND METHODS

Male Wistar rats (Charles River Lab Inc, Barcelona, Spain), 10 weeks of age and weighing 320 g were maintained in an air-conditioned room subjected to 12-hour darklight cycles. They were given standard laboratory rat chow (IPM-R20, Letica, Barcelona, Spain) and free access to tap water. Animal experiments were conducted according to the European Council Directives on Animal Care. The animals were divided into three groups ($n = 6$). The treatments were performed by oral gavage over 6 weeks: The control group received orange juice vehicle; the CsA group, in SandimumNeoral (5 mg/kg body weight/d; Novartis Farma Produtos Farmacêuticos SA, Sintra, Portugal) dissolved in orange juice; SRL group (1 mg/kg BW/d; Sirolimus; Rapamune; Laboratórios Pfizer Lda; Lisbon, Portugal). All the administrations were performed through an esophageal cannula at the same hour of the day (17:00 hours). After animals from the three groups completed the 6-week experimental protocols, we performed the determinations. Body weight was monitored throughout the treatment period. Systolic blood pressure (SBP), diastolic blood pressure (DBP), mean blood pressure (MBP) and heart rate values were obtained using a tail-cuff sphygmomanometer LE 5001 (Letica, Barcelona, Spain) in appropriate contention cages. Blood pressure (BP) values were obtained by averaging 8–10 measurements and registered by the same person, in a quiet environment, between 14:00 and 18:00. BP measurements were performed with special precautions to minimize stress-induced fluctuations.

At the end of treatments the rats were injected with intraperitoneal anesthesia by ketamine (2 mg/kg BW of a 2:1 [v:v] 50 mg/mL) (Ketalar, Parke-Davis; Pfizer Laboratories Lda; Seixal, Portugal) chlorpromazine 2.5% (Largatil, Rhône-Poulenc Rorer, Vitória laboratories, Amadora, Portugal). Blood samples were immediately collected by venipuncture from the jugular vein with either no anticoagulant (for serum samples) or an anticoagulant (EDTA) for blood cell analysis. The rats were sacrificed by cervical dislocation; the heart and kidneys immediately removed, weighed, and stored at −80°C.

Serum creatinine, blood urea nitrogen (BUN), glucose, triglycerides (TGs), total cholesterol (TC), creatine kinase (CK), aspartate and alanine aminotransferases were evaluated by automated validated methods on a Hitachi 717 analyser (Roche Diagnostics Inc, Mass USA). Serum insulin levels were measured using a rat enzyme-linked immunosorbent assay (ELISA) kit from Mercodia (Uppsala, Sweden). Serum levels of interleukin (IL)-2, IL-1$\beta$, tumour necrosis factor alpha (TNF-$\alpha$), and vascular endothelial growth factor (VEGF) were measured using ultrasensitive Quantikine ELISA kits (R&D Systems, Minneapolis, Minn, USA). Serum high-sensitivity C-reactive protein (hsCRP) was measured with an ELISA kit from Alpha Diagnostic Int. (San António, Tex, USA). Kidney mRNA levels of interleukin-1$\beta$ (IL-1$\beta$), tumor protein 53 (TP53), mammalian target of rapamycin (mTOR) and proliferating cell nuclear antigen (PCNA) were measured by reverse transciption-quantitative polymerase chain reaction (RT-qPCR), according to previously described protocols.[23] The thiobarbituric acid reactive-species (TBARs) assay was used to evaluate serum, kidney, and heart tissue lipid peroxidation via malondialdehyde (MDA). Samples were analyzed spectrophotometrically at 532 nm using 1,1,3,3-tetramethoxypropane as an external standard. Serum total antioxidant status (TAS) was measured through the ferric reducing antioxidant potential assay.

T-lymphocyte number and activation status were evaluated in fresh peripheral blood samples that had been collected into EDTA vacutainer tubes. Mononuclear cells were isolated from blood by density gradient centrifugation (Histopaque-1077 and −1119, from Sigma-Aldrich, Sintra, Portugal) followed by 3 washing steps with phosphate-buffered saline solution (PBS, pH = 7.4), supplemented with 3% (v/v) fetal bovine serum (FBS). Antibodies for staining were directed against CD3, CD4, CD8 and CD25 (all from BD Biosciences, San Diego, Calif, USA), conjugated either to fluorescein isothiocyanate (FITC), R-phycoerythin (PE) and peridinin chlorophyll protein (PerCP). Fluorochrome-conjugated isotype-matched antibodies were used as negative controls. For surface staining, mononuclear cells ($\approx 1 \times 10^6$ cells in 100 $\mu$L PBS containing 3% (v/v) fetal bovine serum and 0.1% $NaN_3$) were incubated with 1 $\mu$g antibody in the dark at room temperature for 30 minutes before washing thrice with PBS supplemented with FBS 3% (v/v). Finally we added 400 $\mu$L of PBS supplemented with 3% (v/v) FBS to each tube. The treated samples and controls were analyzed by flow cytometry within 1 hour. Flow cytometric analysis was performed in a FACS Calibur apparatus (San Jose, Calif, USA) based on acquisition of 20000 events. Detectors for forward and side light scatter were set on a linear scale, whereas logarithmic detectors were used for all three fluorescence (FL) channels (FL-1, FL-2, and FL-3). Compensation for spectral overlap between FL