

Hypertension Induced by Immunosuppressive Drugs: A Comparative Analysis Between Sirolimus and Cyclosporine

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ABSTRACT

The purpose of this study was to compare the effects of sirolimus (SRL) vs cyclosporine (CsA) concerning the cardiovascular mechanisms hypothetically contributing to hypertension development. Three rat groups were studied: control (vehicle), CsA (5 mg/kg/d), and SRL (1 mg/kg/d). The following parameters were evaluated after 7 weeks of treatment: blood pressured (BP) and heart rate (HR; tail cuff), lipid profile, hematology, plasma and platelet 5-HT and catecholamines (HPLC-ECD), and oxidative equilibrium (serum malondialdehyde [MDA] and total antioxidant status [TAS]). Systolic (SBP) and diastolic blood pressure (DBP) values were higher ($P < .001$) in both the CsA (146.2 ± 4.5 and 124.9 ± 4.5 mm Hg) and SRL (148.9 ± 4.8 and 126.4 ± 6.0 mm Hg) groups vs the controls (115.9 ± 3.3 and 99.1 ± 2.0 mm Hg). However, HR values were elevated in CsA but not SRL animals. The dyslipidemic pattern of CsA was even more enhanced in the SRL group, with significantly higher low-density lipoprotein cholesterol (LDL-c) and triglyceride (TG) levels vs CsA ($P < .05$); red blood cells, hematocrit, hemoglobin concentration, mean platelet volume, and platelet distribution width were significantly ($P < .05$) higher in the SRL vs CsA group. The pro-oxidative profile (increased MDA/TAS) in the CsA group was not reproduced in the SRL cohort. While plasma and platelet 5-HT were elevated in SRL rats, catecholamine content was higher in CsA animals. In conclusion, this study demonstrated that CsA and SRL produce identical hypertensive effects. However, while CsA promotes oxidative stress and sympathetic activation, SRL mainly interferes with lipid profile and hematological parameters. Thus, the hypertensive effects of CsA, a calcineurin inhibitor, and of SRL, an mTOR inhibitor, are associated with impairment of distinct cardiovascular pathways.

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IMMUNOSUPPRESSIVE THERAPY has greatly improved morbidity and mortality among transplanted patients over the last decades, particularly due to more efficient prevention of acute rejection. However, despite the high efficacy demonstrated by some of the immunosuppressants, such as cyclosporine (CsA), their clinical use is commonly associated with serious side effects, such as nephrotoxicity and arterial hypertension (HTN).¹⁻³ In a rat model of CsA-induced HTN, previous studies from our group have already demonstrated the main components of the cardiovascular side effects of this calcineurin inhibitor (CNI), which seems to include vascular impairment, to be mainly due to reduced nitric oxide (NO) content, oxidative stress, platelet hyperactivation, and sympathetic overactivity.⁴⁻⁷

Sirolimus (SRL), another key immunosuppressive agent for prevention of rejection in organ transplantation, acts through a different intracellular mechanism: the inhibition of the mammalian target of rapamycin (mTOR). Considering the advantages of this drug compared with CsA, some therapeutic protocols have been based on the replacement of CNIs by the mTOR inhibitor, with positive results on renal function and histology in heart and renal transplantation.⁸⁻¹¹ However, SRL-based immunotherapy is associated with other serious adverse effects, such as lipid abnormalities and thrombocytopenia.^{12,13} Even the benefits previously reported have been recently questioned by several studies, because of evidence of nephrotoxicity and proteinuria.¹⁴⁻¹⁸

In contrast, chronic allograft nephropathy (CAN) is the main cause of renal graft loss, while death due to cardiovascular complications in patients with functioning allografts accounts for a huge percentage of graft losses and posttransplant mortality.¹⁹⁻²¹ The cardiovascular side effects of SRL seem to be less evident than those with CsA, but the exact mechanisms underlying the distinct patterns remain to be elucidated. One of the key hypotheses has purposed that there may be a link between distinct intracellular mechanisms underlying immunosuppression and the pattern of side effects displayed by the 2 types of agents.^{22,23} As long as the main cause for HTN remains to be fully explained, the choice of the most appropriate antihypertensive drug will essentially consider its efficiency to control blood pressure (BP) for a specific type of transplant, as well as the hypothetical side effects and potential interferences with immunosuppression.²⁴

The purpose of this study was to evaluate the effects of CsA and SRL on BP and on some cardiovascular mechanisms hypothetically underlying HTN development in the rat.

MATERIALS AND METHODS

Male Wistar rats (Charles River Laboratories Inc, Barcelona, Spain) of 280 to 300 g were maintained in an air conditioned room, subjected to 12-hour dark/light cycles, given standard laboratory rat chow (IPM-R20, Letica, Barcelona, Spain), and allowed free access to tap water. Animal experiments were conducted according to the European Communities Council Directives on Animal Care. The rats divided into 3 groups (each with 8 rats) were treated with

the following diets for 7 weeks: the control group ingested orange juice (vehicle), and the CsA group (5 mg/kg body weight [BW]/d; Sandimmune Neoral, Novartis Pharma, Lisbon, Portugal) and SRL group (1 mg/kg BW/d; Rapamune, Wyett Europe Ltd, Berkshire, United Kingdom), both dissolved in orange juice. All the administrations were performed through an appropriate esophageal cannula, at about the same hour of the day (17:00 hours). All the animals from the 3 groups completed the 7-week experimental protocol. Systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate (HR) values were obtained using a tail-cuff sphygmomanometer LE 5001 (Letica, Barcelona, Spain) in appropriate restriction cages. BP values, obtained by averaging 8 to 10 measurements, were registered by the same person, in a similar peaceful environment, between 14:00 and 18:00 hours. Measurements were performed with special precautions to minimize stress-induced fluctuations in BP, as previously described.²⁵

At the end of the treatments the rats were subjected to intraperitoneal anesthesia with 2 mg/kg BW of a 2:1 (v:v) solution of 50 mg/mL ketamine (Ketalar, Parke-Davis, Laboratórios Pfeizer, Seixal, Portugal) in 2.5% chlorpromazine (Largactil, Rhône-Poulenc Rorer, Laboratórios Vitória, Amadora, Portugal), followed by sacrifice using cervical dislocation. Blood samples were immediately collected from the jugular vein into syringes with no anticoagulant (for serum sample collection) or the appropriate anticoagulant: heparin or a solution of acid citrate-dextrose (ACD; 0.1 mL/mL blood), containing citric acid (71 mmol/L), sodium citrate (85 mmol/L), and D-glucose (111 mmol/L). Blood was centrifuged (160g for 10 minutes at 20°C) to obtain the platelet-rich plasma (PRP), which was then centrifuged (730g for 10 minutes at 20°C) to obtain the platelet pellet and the platelet-poor plasma (PPP).

Serum total cholesterol (Total-c), high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), and triglycerides (TG) were analyzed on a Hitachi 717 (Roche Diagnostics) using standard laboratory methods. Total-c reagents and TG kits were obtained from bioMérieux (Lyon, France); HDL-c Plus and LDL-c Plus tests were obtained from F. Hoffmann-La Roche Ltd (Roche Diagnostics Division, Basel, Switzerland). The main relationships between the Total-c, HDL-c, and LDL-c serum concentrations were calculated as atherogenic and cardiovascular risk indices (LDL-c/Total-c, LDL-c/HDL-c, and Total-c/HDL-c).

Several hematological parameters, from platelet and red blood cells (RBC), were measured in heparinized whole blood using a Coulter counter (Beckman Coulter, Inc, Fullerton, Calif, United States): platelet count (PLT), plateletcrit (PCT), platelet distribution width (PDW), mean platelet volume (MPV), RBC number, hematocrit (HCT), hemoglobin concentration (Hb), RBC distribution width (RDW), and mean corpuscular volume (MCV).

Products of lipid peroxidation, namely malondialdehyde (MDA), were evaluated by the thiobarbituric acid (TBA) assay.²⁶ The assay mixture consisted of 0.1 mL serum, 0.4 mL 0.9% NaCl, 0.5 mL 3% sodium dodecylsulfate (SDS), and 3 mL TBA reagent containing equal parts of 0.8% aqueous TBA and acetic acid. After the mixture was heated for 75 minutes at 95°C, 1 mL cold 0.9% NaCl was added for extraction with 5 mL *n*-butanol. After centrifugation at 730g for 15 minutes at 4°C, the organic phase was analyzed spectrophotometrically at 532 nm, using 1,1,3,3-tetramethoxypropane as an external standard. The results were expressed as $\mu\text{mol/L}$ of MDA. Total antioxidant status (TAS) was evaluated in serum samples using commercially available kits (Total Antioxidant Status, Randox Lab Ltd, Crumlin, United Kingdom)²⁷; the results were expressed as mmol/L.

Concentrations of plasma and platelet serotonin (5-HT) and cat-

Table 1. Blood Pressure, Heart Rate, and Lipidic, Hematological, and Oxidative Profiles

	Control	CsA	SRL
Blood pressure and heart rate			
SBP (mm Hg)	115.9 ± 3.31	146.2 ± 4.5***	148.9 ± 4.8***
DBP (mm Hg)	99.1 ± 2.0	124.9 ± 4.5***	126.4 ± 6.0***
HR (beats/min)	339.1 ± 6.3	375.5 ± 6.9**	346.4 ± 12.2†
Lipid profile and atherogenic ratios			
Total-c (mg/dL)	51.0 ± 4.1	63.2 ± 3.6*	76.4 ± 7.4*
TG (mg/dL)	100.5 ± 4.9	172.8 ± 28.0*	275.3 ± 17.6***,††
LDL-c (mg/dL)	3.8 ± 0.5	8.4 ± 1.2*	11.1 ± 1.0***,†
HDL-c (mg/dL)	34.6 ± 1.3	38.2 ± 1.4	53.8 ± 5.3*,†
LDL-c/Total-c	0.08 ± 0.01	0.13 ± 0.12	0.16 ± 0.02*
LDL-c/HDL-c	0.11 ± 0.01	0.22 ± 0.03*	0.23 ± 0.03*
Total-c/HDL-c	1.54 ± 0.04	1.65 ± 0.05	1.43 ± 0.04
Hematological parameters			
PLT (×10 ⁹ /L)	733.0 ± 17.9	560.0 ± 32.8***	525.2 ± 22.9***
PCT (%)	0.39 ± 0.01	0.30 ± 0.02*	0.31 ± 0.03*
PDW (%)	16.4 ± 0.1	16.7 ± 0.2	43.4 ± 1.2***,†††
MPV (fl)	5.3 ± 0.1	5.5 ± 0.2	7.6 ± 0.1***,†††
RBC (×10 ¹² /L)	6.7 ± 0.1	6.2 ± 0.2*	7.8 ± 0.1***,†††
HCT (%)	36.4 ± 0.6	32.2 ± 0.9**	40.0 ± 0.9***,†††
Hb (g/L)	12.5 ± 0.2	11.4 ± 0.3**	14.0 ± 0.2***,†††
RDW (%)	15.0 ± 0.5	15.9 ± 0.9	21.5 ± 1.1***,†††
MCV (fl)	54.2 ± 0.8	52.3 ± 0.4	51.7 ± 0.9*
Oxidative profile			
MDA (μmol/L)	1.80 ± 0.59	5.60 ± 2.43*	0.55 ± 0.08***,†††
TAS (mmol/L)	0.77 ± 0.02	0.79 ± 0.02	0.45 ± 0.03***,†††
MDA/TAS (×10 ⁻³)	2.37 ± 0.80	6.98 ± 2.82*	1.23 ± 0.10***,†††

Data are expressed as mean values ± SEM of 8 separate values (rats).

Significant differences between the groups: * $P < .05$, † $P < .05$, ** $P < .01$, and †† $P < .01$, and *** $P < .001$ vs the control group; ††† $P < .001$, SRL group vs CsA group.

echolamines (CA), norepinephrine (NE) and epinephrine (E), were assessed by high-performance liquid chromatography (HPLC)-electrochemical detection, using 3,4-dihydroxybenzylamine (DHBA) as internal standard, according to the extraction protocols and chromatographic conditions previously described in detail.²⁵ Levels were calculated using a known concentration of the corresponding standard, which was obtained from Sigma Chemical Co (St Louis, Mo, United States). Chromatograms were obtained using the appropriate Gilson 710 software. Values were expressed in ng/mL.

Results are expressed as mean values ± standard errors of the mean (SEM). Differences between groups were tested by performing analysis of variance (ANOVA), followed by the Fisher protected least significant difference (PLSD) test, using the Statview 4.53 software from Abacus Concepts Inc (Berkeley, Calif, United States). Differences were considered to be significant at $P < .05$.

RESULTS

The CsA-treated rats displayed significantly higher values of SBP and DBP ($P < .001$) compared with the control rats (Table 1). The same profile was obtained for the SRL group ($P < .001$). However, while HR was increased in the CsA group ($P < .01$) vs the control, no significant change was observed in the SRL-treated rats (Table 1).

The rats of the CsA group demonstrated significantly higher values of Total-c, TG, and LDL-c ($P < .05$) versus the control rats (Table 1). In the SRL-treated rats the dyslipidemic pattern was even more pronounced than that of the CsA group, presenting even higher values of Total-c

($P < .05$), TG ($P < .001$), and LDL-c ($P < .001$). The ratios between the Total-c, HDL-c, and LDL-c blood concentrations were calculated to be used as possible atherogenic and cardiovascular risk indices. According to Table 1, the LDL-c/Total-c and LDL-c/HDL-c ratios were higher among the CsA-treated rats compared with the controls, and even higher ($P < .05$) with the SRL animals.

The PLT count and PCT were significantly ($P < .001$ and $P < .05$, respectively) reduced in both groups under immunosuppressive treatment vs the control group. However, while the PDW and MPV values were unchanged in the CsA-treated rats compared with the controls, the SRL rats displayed levels substantially higher ($P < .001$ vs both the control and CsA groups; Table 1). The RBC profile was also distinct between the 2 groups of rats. CsA-treated rats showed a noticeable decrease in RBC count ($P < .05$), HCT and Hb ($P < .01$), together with a nonsignificant trend toward an increment in RDW, compared with the control group; SRL-treated animals displayed a significant increment in RBC count ($P < .001$), HCT and Hb ($P < .01$), accompanied by increased RDW ($P < .01$; Table 1).

The serum MDA content was elevated in CsA-treated ($P < .05$) versus control rats, together with unchanged serum TAS levels (Table 1). In the SRL rats these behaviors were not observed; the MDA values were significantly lower than those of the CsA group ($P < .001$). Accordingly, while

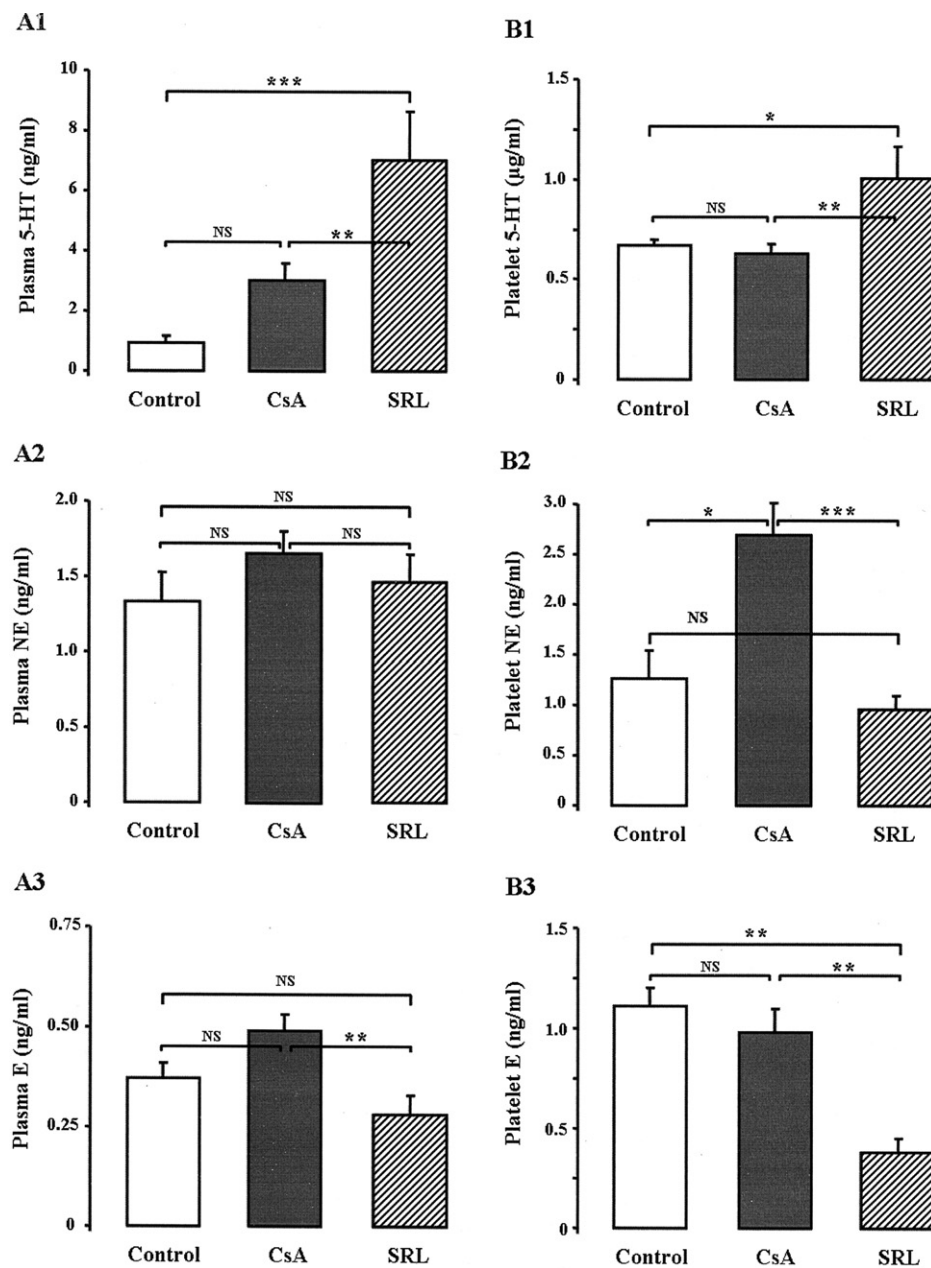


Fig 1. Plasma (A) and platelet (B) serotonin (1), norepinephrine (2), and epinephrine (3) concentrations in the 3 rat groups at the end of treatments. Data are expressed as mean values \pm SEM of 8 separate values (rats). Significant differences between the groups are expressed: * $P < .05$, ** $P < .01$ and *** $P < .001$.

in the CsA-treated rats the MDA/TAS index was substantially elevated ($P < .05$), compared with controls, in the SRL group the ratio was even lower than the control value.

Plasma and platelet 5-HT contents were statistically unchanged between the CsA and control groups (Fig 1A1 and 1B1, respectively). However, among SRL-treated rats there was a noticeable increment in both plasma ($P < .001$) and platelet ($P < .05$) 5-HT compared with the other groups (Fig 1A1 and 1B1). In the CsA-treated rats there was a nonsignificant trend toward higher plasma norepinephrine (NE) and epinephrine (E) concentrations than those of the control animals (Fig 1A2 and 1A3); platelet NE

increment was substantially higher ($P < .05$) compared with the controls (Fig 1B2). In the SRL-treated rats the plasma and platelet catecholamine contents were invariably lower compared with the CsA group (Fig 1).

DISCUSSION

Important changes in therapeutic protocols for transplantation have led to improved renal function and histology. A reduction in acute rejection episodes also has been observed due to advances in the maintenance phase of immunotherapy,^{8-11,28} namely, reducing or replacing CNIs by

mTOR inhibitors, such as SRL. Efficient prevention of acute, and even chronic, rejection episodes remains the most valuable function of CsA immunosuppressive action. However, its clinically-serious side effects, namely, nephrotoxicity and arterial HTN, have recommended the growing use of new drugs. The mechanisms of CsA-induced HTN have been extensively researched for more than 2 decades. SRL side effects are now recognized to be even more widespread than those of CsA, including dyslipidemia and myelosuppression (thrombocytopenia, leukopenia), and even the repeatedly reported improvement in renal function is now more controversial.^{14–18}

Since cardiovascular disease is the main cause of death in transplantation, including renal transplantation, optimal control of cardiovascular risk factors is essential for the long-term management of those patients. HTN development has been reported to be less evident for mTOR inhibitors than the CNIs.^{29,30} However, in our study, using doses that reproduce those used in patients under maintenance therapy, there was a similar increment in BP for SRL and CsA animals, which has encouraged us to perform further research on the mechanisms that hypothetically cause the BP rise. This was reinforced by the previous remarkable suggestion from Sander et al^{22,23} that the distinct mechanisms underlying immunosuppression, namely, intracellular receptors and signals, might be linked with the different patterns of side effects by the 2 drug classes.

The dyslipidemia obtained in CsA-treated rats was even more evident in the SRL group, with a notorious profile of hypercholesterolemia and hypertriglyceridemia, which is a well-known mark of rapamycin.^{12,13,31} Thus, the SRL group showed an increased risk for atherosclerosis than the CsA-treated rats.

Concerning hematological parameters, there was a reduction in PLT count and PCT in both groups under immunosuppressive treatment, which agrees with literature reports of thrombocytopenia.^{12,13,32} This finding might also be related to increased platelet activation, which was previously documented by us for CsA.³³ However, RBC count and HCT were increased in SRL-treated rats, which is opposite to almost all other reports,^{12,13} thus deserving further confirmation.

CsA-induced reactive oxygen species (ROS) formation, the reduction of both NO availability and effects, due to its reaction with the overproduced superoxide anion,^{34,35} have been reported to be key explanations for its cardiorenal toxic effects. According to some authors, calcineurin is a modulator of NO synthase activity,³⁶ which might be due to its influence on ROS formation. In our study, MDA/TAS data corroborated the pro-oxidative profile of CsA, while SRL seemed to have no deleterious effects on oxidative equilibrium. This finding agrees with the previously mentioned hypothesis of a specific modulation of NOS by calcineurin inhibition.³⁶

The biological monoamines 5-HT, NE, and E have several cardiovascular actions, affecting platelet, vascular, and cardiac muscle reactivity and thus influencing hemostasis and BP control. Plasma and platelet serotonergic measures were particularly augmented in the SRL group and

unaffected in the CsA cohort. In contrast, the results showed a trend toward an increment in plasma and a significant increase in platelet NE in the CsA-treated rats, which agrees with the tachycardic effects of this CNI, in contrast to SRL animals, in which the levels were invariably lower and the HR normal, consistent with previous reports in rats.³⁷ This distinct effect seems to be in agreement with the suggestion of a specific CNI-mediated sympathetic overactivation and HTN, in which rapamycin, an mTOR inhibitor, is logically not implicated.³⁸

Even though CAN is the main cause of renal allograft loss, deaths from cardiovascular complications account for a huge percentage of posttransplantation mortality.^{19–21} Because posttransplantation HTN, a major cardiovascular complication, can jeopardize a long-lasting, successful organ transplant, antihypertensive therapy is advised.^{24,39} However, as long as the causes for HTN remain to be fully explained, the choice of the most appropriate antihypertensive drug must essentially consider its efficiency to control BP values for a specific type of transplant, as well as the hypothetical side effects and the potential interference with immunosuppressive ability,^{24,39} thus excluding a pivotal factor, the pathophysiological mechanisms underlying immunosuppressive drug-induced HT.

This study demonstrated that both CsA and SRL have hypertensive effects. However, while CsA significantly promotes tachycardia and oxidative stress, SRL seems to mainly interfere with lipid profile, hemorheology, and 5-HT levels, without the same influence on catecholamine contents and lipid peroxidation. Thus, the cardiovascular disturbances underlying HTN development might be associated with distinct molecular/cellular mechanisms hypothetically explained by differences in the mechanism of action of immunosuppressants. These distinct effects should be taken into consideration for the therapeutic protocol decision according to the type of transplant and the patient's previous clinical history, as well as for the choice of antihypertensive therapy. According to our data, which will be further evaluated, beta-blockers or modulators of the renin-angiotensin axis might be effective therapy for CNI-induced HTN, but not for the SRL-evoked types, in which a 5-HT antagonist such as ketanserin might produce better results.

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