A comparison between the experimental nephrotoxicity of two cyclosporine A microemulsion formulations

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Abstract

Introduction/Objective
The high cost of drugs used in immunosuppressive schedules for transplant recipients has stimulated the search for plausible options in cost reduction. One possible alternative is the use of less expensive generic drugs instead of branded drugs. This option is only viable if the generic drug is bioequivalent, showing an efficacy and toxicity similar to more expensive branded drugs. The aim of this study was to compare the nephrotoxicity of a new generic microemulsion formulation of cyclosporine A (Sigmasporin Microral®, CsA 1) with the reference microemulsion (Sandimmun Neoral®, CsA 2).

Methods
An experimental model, in which salt depletion induces renal interstitial fibrosis and renal failure in CsA-treated rats, similar to that observed in clinical CsA-induced chronic nephrotoxicity was used. The animals were kept on a low salt-diet (0.06% salt) and were divided in three treatment groups: CsA 1, treated with 15 mg/kg/day of Sigmasporin Microral® by gavage; CsA 2, treated with 15 mg/kg/day of Sandimmun Neoral® by gavage; and a control group, receiving 1 ml/kg/day of the active drug vehicle only.

Results
After 3 weeks treatment both CsA formulations caused similar decrease in glomerular filtration rate measured by inulin clearance (0.26±0.06 ml/min/100 g in CsA 1; 0.35±0.05 ml/min/100 g in CsA 2, and 1.00±0.04 ml/min/100 g in the control group, p<0.001 for both CsA groups versus control group). Likewise, both CsA formulations produced similar increase of serum creatinine (0.98±0.06 mg/dl in CsA 1; 0.85±0.05 mg/dl in CsA 2, and 0.59±0.01 mg/dl in control group; CsA 1 vs. CsA 2, NS; CsA 1 vs. control, p<0.001; CsA 2 vs. control, p<0.01). Both CsA microemulsion formulations caused analogous moderate-to-severe renal interstitial fibrosis and tubular atrophy,
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while vehicle-treated animals did not show any histological renal changes. CsA blood levels obtained for the two drug formulations were almost similar (2176±386 ng/ml for CsA 1 and 2137±385 ng/ml for CsA 2).

Conclusions
The new generic CsA microemulsion formulation showed an experimental functional and structural nephrotoxicity profile absolutely similar to the reference microemulsion.

Introduction

Cyclosporine A (CsA) is a cyclic, hydrophobic peptide of 11 amino-acids, soluble in lipids and other organic solvents, a feature that accounts for its great distribution volume and high tissue concentrations. This drug has a powerful immunosuppressive activity, considered unique for its selective effects on lymphocytes. Its clinical use represented a revolution in solid organ transplantation, significantly reducing morbidity and the incidence of acute rejection. It has also been used in bone marrow transplants and in the treatment of several auto-immune diseases, such as psoriasis, lupus, glomerulopathies, diabetes mellitus, rheumatoid arthritis, uveitis and scleroderma.1,4

The main action of CsA is the inhibition of the transduction pathway for the lymphokine synthesis, especially interleukin-2. The drug can inhibit the expression of interleukin-2 receptors in cells which are responsive to this lymphokine. The inhibitory effect on the IL-2 production is due to its relatively selective action on the IL-2 gene transcription. The interaction of the antigen with the T cell receptor leads to an increase of intracellular calcium via inositol triphosphate (InsP3). Calcium, together with calmodulin, stimulates calcineurine, a phosphatase which activates several cytosolic transcription factors, including those of interleukin-2. CsA binds with a cytoplasmatic protein named cyclophilin, and the cyclosporine-cyclophilin complex binds with calcineurine, blocking the activation of the transcription factors and, consequently, the production of interleukin-2.1

The most important side effect of CsA is its nephrotoxicity, that can occur in an acute or chronic form. Acute nephrotoxicity is characterized by a reversible damage of renal function, without significant histologic kidney alterations. Clinically it shows an asymptomatic increase of serum creatinine, delayed recovery of renal function after renal transplantation, acute renal failure, and less frequently by hemolytic-uremic syndrome. This kind of nephrotoxicity is mediated by an intense vasoconstriction of the afferent arteriole.2–4

Chronic nephrotoxicity is characterized by the development of irreversible interstitial fibrosis of the renal parenchyma, tubular atrophy, and hialnosis of the afferent arteriole, together with a progressive decrease of glomerular filtration. Its etiopathogeny seems to be related to the ischemia due to preglomerular vasoconstriction and direct activation by CsA of pro-fibrogenic factors in the renal tissue. This kind of nephrotoxicity is probably the main limiting factor for the clinical use of CsA, related as it is with chronic loss of function in renal grafts, the development of chronic terminal renal failure in heart and liver transplants, and renal fibrosis and functional alterations in patients with auto-immune diseases treated with CsA.5–7

The development of a CsA-induced chronic nephrotoxicity model in rats, by means of dietary salt restriction, allowed to make important progress in the understanding of the possible mechanisms which generate this lesion. This model is characterized by the use of drug doses close to those used in clinical practice, and by the reproduction of the functional and histologic alterations found in patients with CsA-induced chronic nephropathy.8–10

The benefit brought to transplant patients by the introduction of CsA is unquestionable, as the drug increased graft survival during the first year after transplantation from 50% to 80% or 90%. But the cost of the immunosuppressive drugs is high. During the first year after transplantation, the immunosuppression cost corresponds to 15% to 20% of the total amount of the procedure, and in the subsequent years this cost represents 90% of the total amount spent on the
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transplant patient. A cost reduction of the immuno-suppressive agents would decrease the transplantati-
on costs, allowing to extend its benefits to many other patients. One of the alternatives that have been pro-
tosed to this effect is the replacement of generic drugs for reference drugs with a similar pharmacokinetic profile, effectiveness and toxicity, but with a substantially lower cost.11-15
The objective of the present study was to compare the experimental nephrotoxicity of a generic CsA microemulsion (Sigmasporn Microral, Sigma Pharma, Brazil) with the one of the reference drug (Sandimmun Neoral, Novartis, Switzerland), using a consistent experimental model of CsA-induced chronic nephrotoxicity.

**Methods**

**Animals and diet**

Male Munich-Wistar rats, which weighed between 220 g and 230 g, from a colony maintained in the Biote-
rium of the São José do Rio Preto Medical School (FA-
MERP), SP, Brazil were used. The animals received a
low-salt (0.06%) and normoproteic (25%) diet (Teklad, WI, EUA) started a week before treatment and
maintained during the entire study period. The animals
received water at pleasure during the whole study.

**Treatment**

The animals were divided into three groups and
treated during 3 weeks:

- Group CsA 1 received a 15 mg/ml solution of Sig-
masporin Microral at a dosage of 15mg/kg daily, by
gavage;
- Group CsA 2 received a 15 mg/ml solution of San-
dimmun Neoral at a dosage of 15mg/kg daily, by
gavage;
- Control Group received 1 ml/kg of active drug dilu-
ting solution daily, by gavage.

**Basic protocol**

After a week of low-salt diet, the animals were ran-
domly divided into the three groups and treated for 3
weeks. During the entire study period, the animals were
weighed daily.

After drug or diluent administration on the 21st day
of treatment, the rats were placed into metabolic cages
(Nalgene, USA), and their urinary volume was collec-
ted during 24 hours. Once this collection was finished,
the animals were submitted to glomerular filtration
measurements.

**Glomerular filtration rate (inulin clearance)**

The rats were anesthetized with thionembutal (50
mg/kg) by intraperitoneal injection and placed on a
heated surgical bed (Braile Biomédica, Brazil). The
animal’s body temperature was continuously mea-
sured throughout the whole experiment and kept at
37±1°C. Following anesthesia, tracheostomy (PE 90),
jugular vein catheterization (PE 50) for solution infu-
son, and carotid artery catheterization (PE-50) for con-
tinuous blood pressure measurement and blood collec-
tion were performed. Then, a median incision was made
for cistostomy, with a catheter (PE 160) sutured to the
bladder. The urethra was ligated to prevent urine loss.
Following the carotid artery catheterization, 1 ml blood
was collected, replaced by 1 ml NaCl 0.9% solution,
and reserved for ulterior creatinine, sodium, potassium,
and osmolality measurements.

After completion of the surgical preparation, the
animals received 1ml inulin solution (0.3 mg inulin in
12 ml normal saline solution) and 5 ml 0.9% NaCl so-
lution through the jugular vein. At that point, con-
tinuous infusion with inulin solution was initiated (0.5
mg inulin in 12 ml normal saline solution) at a 0.06
ml/min speed (infusion pump Harvard, USA), through
the jugular vein. After a 60 min stabilization period,
urine collection in previously weighed vials was started,
for 3 consecutive periods of 20 minutes each, with
0.3 ml blood being collected at the midpoint of each
urine collection period. After blood collection, the same
volume was immediately replaced by 0.9% NaCl solu-
tion, through the jugular vein. The intracarotid blood
pressure was measured during the entire experiment
by means of a transducer connected to a pressure re-
corder. At the end of the experiment, 3 ml blood were
collected for cyclosporin dosage, and nephrectomy of
the left kidney was performed for histologic analysis.
Once these procedures were finished, the animal was
submitted to euthanasia with an adequate dosis of the
anesthetic used.

The urine volume was determined by the weight
difference of the urine collection vials. The serum and
urine inulin dosage measurements were made by the
anthrone method (Spectrophotometer Biosystems BTS
310, USA). Glomerular filtration was determined by the
usual formula. The values used for each animal correspond to the mean of the three values determined.

Biochemical dosage measurements and osmolality
The 24-hour urine samples and the blood collected before starting the inulin clearance procedure were used to measure the dosage of creatinine (Spectrophotometer Biosystems BTS 310, USA), sodium and potassium (Specific AVL 9180 Electrode, USA), and osmolality (freezing point, osmometer Osmette A, USA). The obtained values were used to calculate the sodium and potassium excretion fractions by the usual formulas.

Blood dosage of cyclosporine
The 3 ml blood collected at the end of the experiment were placed in a vial containing 0.1ml 10% EDTA and stored at -20°C for the determination of whole blood cyclosporine levels by monoclonal antibody (Laboratório Fleury, São Paulo, SP, Brazil).

Histologic study
The material resulting from nephrectomy was immersed in a 4% formal solution, embedded in paraffin and processed into 1 µm to 3 µm thick sections. For the histologic analysis, Schiff’s periodic acid (PAS) staining, hematoxylin-eosin, Masson’s trichromum and PAS were used.

Statistical analysis
Data are presented as mean ± standard error. The comparisons between the three groups were made by ANOVA, followed by Tukey’s post-test, whenever appropriate. The comparisons between the cyclosporine blood levels were made with Student’s bicaudal, unpaired t test. The level of significance was set at p<0.05.

Results
The final weight of the animals was 294 g ±5 g in the control group, 239 g ±13 g in group CsA 1 (p<0.01 vs. control), and 256 g ±10 g in group CsA 2 (p<0.05 vs. control). The analysis of the daily weight gain showed that the animals in the control group gained 18.9 g ±2.5 g, whereas the animals in group CsA 1 lost 19.5 g ±11.9 g (p<0.05 vs. control), and the animals in group CsA 2 lost 8.3 g ±8.3 g (NS vs. control) during the period studied (Figure 1).

Blood pressure
After three weeks treatment, the intracarotid blood pressure in the control group was 126 mmHg ±5 mmHg, as compared to 101 mmHg ±6 mmHg in group CsA 1 (p<0.01 vs. control), and 114 mmHg ±5 mmHg in group CsA 2 (NS vs. control). There was no statistically significant difference between the two groups receiving CsA.

Renal function
Both groups receiving CsA presented a very important GFR decrease with regard to the control group: 0.26±0.06 ml/min/100g in group CsA 1; 0.35±0.05 ml/min/100g in group CsA 2, and 1.00±0.04 ml/min/100g in the control group, with p<0.001 for both groups receiving CsA versus control group. There was no statistically significant difference between the GFR values of groups CsA 1 and CsA 2.

Likewise, the animals receiving CsA presented significant rises of serum creatinine: 0.980 mg/dl ±0.06mg/dl in group CsA 1; 0.85 mg/dl ±0.05 mg/dl in group CsA 2, and 0.59 mg/dl ±0.01 mg/dl in the control group (CsA 1 vs. CsA 2, NS; CsA 1 vs. control, p<0.001; CsA 2 vs. control, p<0.01).

The animals receiving CsA 1 and CsA 2 showed higher serum potassium levels than the animals in the control group (4.94 mEq/L ± 0.4 mEq/L; 4.43 mEq/L ±0.16 mEq/L, and 3.76 mEq/L ±0.25 mEq/L, respectively; p<0.05 for CsA 1 vs. control).

Twenty four-hour diuresis, urinary osmolality, urinary excretion of sodium (U Na V), FeNa, and FeK presented no statistically significant differences between the three studied groups. However, the groups receiving CsA showed a tendency toward greater diuresis and smaller urinary osmolality. The urinary excretion of sodium and the sodium excretion fraction were very reduced in all three groups, attesting to the low salt tenor of the diet given to the animals.
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<td>Parameters of renal function after three weeks of treatment with vehicle (CONTROL), Sigmasporin 15mg/kg (CsA 1) or Sandimmun 15mg/kg (CsA 2).</td>
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<td>GFR (ml/min/100g)</td>
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<td>Scr (mg/dl)</td>
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Mean ± standard error; *p<0.01 vs. control; **p<0.05 vs. control; *** <0.05 vs. control. GFR: glomerular filtration rate; Scr: serum creatinine; UoV: urinary sodium excretion; FeNa: sodium excretion fraction; FeK: potassium excretion fraction; UOsm: urine osmolality.

These results are summarized in the following Table.

Cyclosporine levels in whole blood

The cyclosporine levels were similar in the animals which received CsA 1 (2176 ng/ml ±386 ng/ml) and CsA 2 (2137 ng/ml ±385 ng/ml) (Figure 2).

Histology

The animals receiving CsA presented a moderate to intense interstitial stripe fibrosis and tubular atrophy. There was no significant difference between the degree of injury in groups CsA 1 and CsA 2. The animals in the control group showed no noteworthy histologic alterations.

Discussion

The steady growth of costs involved in health polices has imposed a search for alternatives to maintain the viability of the system without jeopardizing its quality. This aspect is particularly crucial in the field of organ transplants, where the use of immunosuppressive drugs may become mandatory for the patient's whole lifetime. These drugs are expensive, and the options of replacing one drug for a distinct one for a given subject are limited. Considering that renal transplantation has become a relatively routine procedure in many Brazilian medical facilities, that the transplantation frequency of other organs such as the heart, liver, bone marrow, etc. is continuously growing, and that the Brazilian Health System provides the immunosuppressive schedule indefinitely to all transplant patients, the magnitude of the cost involved in the use of such drugs in our society is easy to perceive. Actually, this a universal problem, and even richer countries like the USA are concerned about it. In a renal transplantation program conducted by an American university hospital, the cost of the immunosuppressive drugs accounted for 12% of the whole annual budget of the hospital’s Pharmacy Department. In this setting, the introduction of generic immunosuppressive drugs with bioequivalence and effectiveness similar to those of the more expensive reference drugs would imply an obvious saving. It is estimated that reductions of about 37% to 50% in the expenses of a hospital pharmacy can be achieved by using generic drugs. As a result of this saving, as far as immunosuppressive agents are concerned, a greater number of patients could be offered the opportunity of such treatment, extending the possible benefits of a transplantation to a larger part of the population. However, these modifications can under no circumstances put the patients' safety at risk. To this effect, the bioequivalence and the effectiveness of the generic drug have to be tested, and its toxicity has to be proven similar to that
of the reference drug.\textsuperscript{11-13}

In this study, the toxicity of the generic and reference CsA microemulsion proved to be indistinguishable, as shown by the similar significant decrease of glomerular filtration and development of interstitial fibrosis, caused by both formulations. The findings of this study concerning functional and structural alterations are concordant with those of previous works using the same experimental model.\textsuperscript{9,10,15} The absorption of the two formulations appears to have been similar as well, since the blood levels of CsA were virtually equal for both drugs after three weeks treatment. An interesting finding was that the same nephrotoxicity model was obtained by oral administration of the CsA dosage, whereas previous studies utilized the parenteral way.\textsuperscript{10,15} Studies which used CsA preparations diluted in olive oil given orally to rats needed two to three times higher dosages to obtain significant renal function alterations.\textsuperscript{16,17} Confirming the expression of CsA absorption improvement granted by the introduction of microemulsion formulations of CsA.\textsuperscript{18}

The two CsA formulations caused similar systemic toxicity, reflected by a smaller weight increase of the animals treated with the active drugs, as compared to the animals in the control group. These alterations have been related to a catabolizing effect of CsA.\textsuperscript{19}

The effectiveness of the low-salt diet can be confirmed by the low urinary excretion of sodium found in all groups. The animals treated with CsA also presented a reduced sodium excretion fraction, indirectly demonstrating that the renal tubus maintains its sodium reabsorption capacity intact, in spite of the pronounced pre-glomerular ischemia caused by the drug.\textsuperscript{20} Although there was no statistically significant difference with regard to the control group, a tendency towards greater diuresis and smaller urinary osmolality was noticed on the animals receiving CsA, suggesting that the drug harms the urine concentration mechanisms, which is in agreement with previously published results.\textsuperscript{11} Both CsA formulations induced higher serum potassium levels than those of the control group, although the difference was statistically significant only for CsA 1. In fact, hyperkalemia resulting from tubular alterations has been described in patients receiving CsA.\textsuperscript{21}

The results of this experimental work clearly demonstrate, in a consistent and reproducible chronic CsA nephrotoxicity model, that the functional and structural nephrotoxicity caused by a new generic microemulsion formulation of CsA is absolutely similar to the one produced by the reference CsA microemulsion formulation.\textsuperscript{N}

\section*{References}

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