Kidney function and morphology after short-term combination therapy with cyclosporine A, tacrolimus and sirolimus in the rat

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Abstract
Background. Sirolimus (SRL) may supplement calcineurin inhibitors in clinical organ transplantation. These are nephrotoxic, but SRL seems to act differently displaying only minor nephrotoxic effects, although this question is still open. In a number of treatment protocols where SRL was combined with a calcineurin inhibitor indications of a synergistic nephrotoxic effect were described. The aim of this study was to examine further the renal function, including morphological analysis of the kidneys of male Sprague–Dawley rats treated with either cyclosporine A (CsA), tacrolimus (FK506) or SRL as monotherapies or in different combinations.

Methods. For a period of 2 weeks, CsA 15 mg/kg/day (given orally), FK506 3.0 mg/kg/day (given orally) or SRL 0.4 mg/kg/day (given intraperitoneally) was administered once a day as these doses have earlier been found to achieve a significant immunosuppressive effect in Sprague–Dawley rats. In the ‘conscious catheterized rat’ model, the glomerular filtration rate (GFR) was measured as the clearance of Cr EDTA. The morphological analysis of the kidneys included a semi-quantitative scoring system analysing the degree of striped fibrosis, subcapsular fibrosis and the number of basophilic tubules, plus an additional stereological analysis of the total grade of fibrosis in the cortex stained with Sirius Red.

Results. CsA, FK506 and SRL all significantly decreased the GFR. A further deterioration was seen when CsA was combined with either FK506 or SRL, whereas the GFR remained unchanged in the group treated with FK506 plus SRL when compared with treatment with any of the single substances. The morphological changes presented a similar pattern. The semi-quantitative scoring was significantly worst in the group treated with CsA plus SRL (P<0.001 compared with controls) and the analysis of the total grade of fibrosis also showed the highest proportion in the same group and was significantly different from controls (P<0.02). The FK506 plus SRL combination showed only a marginally higher degree of fibrosis as compared with controls (P=0.05).

Conclusion. This rat study demonstrated a synergistic nephrotoxic effect of CsA plus SRL, whereas FK506 plus SRL was better tolerated.

Keywords: cyclosporine A; immunosuppression; nephrotoxicity; renal morphology; sirolimus; tacrolimus

Introduction
Cyclosporine A (CsA), tacrolimus (FK506) and sirolimus (rapamycin) (SRL) are all potent immunosuppressive and antiproliferative agents, introduced over the last decades as immunoregulative agents in human transplantation, as well as various immunological diseases [1]. The molecular structure of SRL resembles that of FK506, but is very different from that of CsA. SRL, like FK506, binds to FK-binding protein, thus forming a drug–immunophilin complex, but whereas CsA and FK506 inhibit the transcription of interleukin-2, SRL inhibits the P70 S6 kinase and affects more critical biochemical events later in the T-cell cycle [1]. SRL was shown to have an attractive profile, as in humans it was found to be rapidly and dose-proportionally absorbed, well tolerated, safe and with few side-effects [2–5]. Another very encouraging finding was that SRL had no or only a negligible nephrotoxic effect in most animal [1,6,7] and human studies [2]. In contrast to the findings on the calcineurin inhibitors, Golbækadal and co-workers [8] observed a rise in the glomerular filtration rate (GFR) of pigs treated intravenously with SRL, and in our laboratory we found that intravenously injected SRL increased lithium clearance and reduced proximal fractional reabsorption (submitted for publication). Recently, both the European multicentre study [3] and
the Global multicentre study [4] of renal transplant patients treated with SRL showed a significantly higher calculated GFR than did the patients treated with CsA. It was suggested that these findings were the result of diminished proliferation of glomerular mesangial cells, a further protection against the development of glomerulosclerosis [9]. However, other studies revealed a possible nephrotoxic effect, as significant morphological changes were found in the spontaneous hypertensive rat [1], whereas tubule atrophy and interstitial fibrosis were found in the rabbit, although no toxic effects were seen on the GFR, renal blood flow or blood pressure [6].

The effects of CsA and FK506 have been extensively investigated in animal studies and in human allotransplantation studies. Unfortunately, both drugs revealed a similar clinically important nephrotoxic effect, as both reduced the GFR and lithium clearance, and proximal fractional reabsorption increased [10,11]. These changes were considered secondary to afferent glomerular vasconstriction, with glomerulotubular adaptation [10,11]. According to these different modes of action, SRL could theoretically be combined with a calcineurin inhibitor in order to achieve a synergistic immunosuppressive effect, but at the same time, without a synergistic toxic effect. Experimental studies of the immunosuppressive effect have demonstrated that a subtherapeutic SRL dose combined with a subtherapeutic CsA dose significantly prolonged the allograft survival in rats, dogs and pigs, and also demonstrated a synergistic effect in inhibiting rat heart and kidney allograft rejection [12,13]. Combination therapy with FK506 and SRL prolonged graft survival times in rats allotransplanted with hearts [14] or hind limbs [15], in mice allotransplanted with small bowel [16] and in monkeys with a kidney allotransplantation [7].

The present study was conducted in order to examine the effects on glomerular function, as well as the possible morphological changes after treatment with the immunosuppressive agents in different combinations over a 2 week period.

**Subjects and methods**

**Experimental animals**

Male Sprague-Dawley rats of the Møllegaard stock (MolSPRD) initially weighing 140–200 g were used. The rats had free access to tap water and a wet mash diet, which contained 312 mEq sodium/kg dry weight.

**Drug preparation, dosage, administration and treatment period**

CsA was dissolved in vegetable oil to a final concentration of 1.5 mg/ml, FK506 powder was dissolved in sterile water to a concentration of 0.4 mg/ml, and the SRL powder was first dissolved in ethanol:Tween (3:1, v:v) to a concentration of 1.0 mg/ml, then further diluted with saline to a concentration of 0.05 mg/ml. Dosages of the substances earlier found to be relevant in achieving a significant immunosuppressive effect in the Sprague-Dawley rat were: CsA, 15 mg/kg/day [10]; FK506, 3.0 mg/kg/day [11] and SRL, 0.4 mg/kg/day (submitted for publication). Placebo solutions were prepared for each substance, so that the rats in the control groups were given the respective vehicles only. Each day, CsA, FK506 or their placebo was administered orally through a gastric tube; SRL or its vehicle was administered intraperitoneally. The treatment period was 2 weeks for all groups and the routes and methods of administration were chosen to ensure the exact dose. All drugs were administered as a single dose in the mornings, and the dose of each substance was adjusted according to the weight increase of the individual rat.

**Clearance studies in conscious catheterized rats**

The rats were anaesthetized with pentobarbital sodium 36–40 mg/kg intraperitoneally and N2O-O2 50%. One catheter was placed in the femoral vein for i.v. infusion, and another was placed in the femoral artery for measurement of the blood pressure. The bladder was catheterized through a small suprapubic incision, which was closed by suture, and the urethra was ligated. About 25 min after the pentobarbital administration, the rats were placed in a restraining cage for recovery. Peroperative fluid loss was restored by i.v. infusion of 10 ml/kg body weight of isotonic saline during the operation. A priming dose of C\textsubscript{\text{EDTA}} was followed by a continuous i.v. infusion of 25 μl/min of C\textsubscript{\text{EDTA}} in isotonic saline. The clearance determination started 2 h later when the rats were conscious. Urine was collected in precalibrated Eppendorf tubes for 30 min, extended, if necessary, until a minimum of 100 μl urine had been produced. Blood samples were taken from the tail tip before and after urine sampling.

**Blood concentrations of the immunosuppressive drugs**

The trough concentrations in whole blood were measured for CsA (TDx, TDxFLx cyclosporine monoclonal whole blood assay, Abbott Laboratories, IL, USA) and FK506 (Imx Tacrolimus II assay, Abbott Laboratories). Blood samples were harvested from the i.v. catheter immediately after the end of the clearance period, that is 12–15 h after the last administration of the particular drug.

**Blood pressure**

During the clearance period both the systolic and the diastolic blood pressures were continually measured intraarterially through the catheter placed in the femoral artery and connected through a transducer to a computer.

**Analytical method and calculation of the GFR**

C\textsubscript{\text{EDTA}} in plasma and urine was analysed by an immediately performed radiation count for a 20 min period, which was repeated within 30 min, as the mean values were used for statistical analysis. The clearance of C\textsubscript{\text{EDTA}} was taken as a measure of the GFR and therefore represents the mean values for the whole nephron population of the individual rat.

**Morphological evaluation**

After the clearance period both kidneys were removed, drained and weighed. The right kidney was bisected in the
frontal plane and a central slice was immersed in 4% formaldehyde buffered to pH 7.0 and embedded in paraffin wax.

Four-micrometre sections from each kidney were stained with PAS. The morphological findings were summarized and graded semi-quantitatively (scores: 0, 0.5, 1, 2 or 3), according to 'basophilic tubules', a descriptive term designating collapsed and atrophic tubules in which the cells showed a deeper degree of basophilia in the cytoplasm than in normal tubular epithelium (tubular atrophy was visualized by thickening of the tubular basement membrane), and 'interstitial fibrosis', either as subcapsular or striped interstitial fibrosis. Subcapsular fibrosis is a term designating triangular groups of five to 10 basophilic, sometimes also atrophic, tubules surrounded by an increased amount of interstitial tissue. Striped interstitial fibrosis consisted of narrow or broader areas as described for the subcapsular fibrosis, but extending from the subcapsular cortex towards the medulla; mostly, however, confined to the cortex. References to this method were published earlier [10].

Transverse sections through the middle part of the kidney were used for stereological investigations. Paraffin sections were stained with Sirius Red and the Olympus CASTgrid system (Computer Assisted Stereological Toolbox, Olympus, Denmark) was used for the analysis. The CASTgrid system is a light microscope with a video camera connected to a computer equipped with a frame grabber. Different overlays are electronically superimposed on a digital live picture, Ordinary point counting with a point density of 9323 μm−2 and a total number of 12 points per field at a final magnification of 3900× was performed. The microscope had a step motor connected and a step length of 1500 μm was used to scan each section systematically and this resulted in a total number of 44–70 fields. Only the cortex, defined as the area containing proximal tubules, was analysed. The number of points lying above the red stained interstitium, including the peritubular basement membranes, was counted and the volume fraction of this area calculated with the total area of the cortex as reference volume.

Statistical methods

For comparison of data collected from more than two groups, the Kruskal–Wallis ANOVA was applied first. When significant variation was indicated by the ANOVA, group-to-group comparison with the Mann–Whitney test was performed. The P-value given is for a two-tailed test and a value < 5% was considered statistically significant. When a Gaussian approximation was applied, the standard deviation (SD) from a parametric t-test is shown as ±SD.

Experimental groups

These are shown in Table 1.

Results

Effect on renal function

The GFR, as measured by the clearance of Cr̄EDTA, was significantly lower in all treatment groups than in the control group (Figure 1). The mean GFR of the controls was 1126 μl/min/g kidney weight (KW). The greatest deterioration in GFR was seen in the two groups where CsA was combined with either FK506 (572 μl/min/g KW, P < 0.001) or with SRL (497 μl/min/g KW, P < 0.001), and lowest with CsA treatment alone (673 ± 391 μl/min/g KW, P < 0.01)—P-values as when compared with controls.

Interestingly, in the group treated with a combination of SRL and FK506, the GFR (817 μl/min/g KW, Table 1. Dosage regimens for mono- or combination therapy with CsA, FK506 and SRL

<table>
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<tr>
<th>n</th>
<th>CsA mg/kg/day</th>
<th>FK506 mg/kg/day</th>
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<tr>
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<tr>
<td>FK506</td>
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<tr>
<td>FK506 + SRL</td>
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Fig. 1. Glomerular filtration rate as measured by chromium–EDTA clearance after 2 weeks of immunosuppressive treatment.


$P<0.02$) was only reduced to a degree similar to that seen in the groups treated with either drug alone: FK506 (820 $\mu$g/min KW, $P<0.02$) or SRL (819 $\mu$g/min KW, $P<0.02$). In the three groups where only a single drug was used, the reduction in GFR agreed with earlier findings for the calcineurin inhibitors [10,11].

**Whole blood concentrations of the immunosuppressive agents, CsA and FK506**

Whole blood trough concentrations of CsA did not differ significantly between the group receiving CsA alone (843 ± 343 nmol/l), the group receiving CsA plus SRL (700 ± 329 nmol/l), and the group receiving CsA plus FK506 (722 ± 275 nmol/l) (the Kruskal–Wallis ANOVA was 0.966, $P=0.62$). Nor did the whole blood concentrations of FK506 vary significantly between the groups: the group receiving FK506 alone (1.3 ± 1.0 ng/ml), the group receiving FK506 plus SRL (1.5 ± 1.4 ng/ml), and the group receiving CsA plus FK506 (2.0 ± 0.7 ng/ml) (the Kruskal–Wallis ANOVA was 3.70, $P=0.16$).

**Effect on blood pressure**

The results of the blood pressure measurements are given in Figure 2. Neither the intra-arterial systolic blood pressure nor the diastolic blood pressure displayed significant changes, as the Kruskal–Wallis ANOVA was 9.71 ($P=0.13$) for the systolic blood pressure and 12.05 ($P=0.07$) for the diastolic blood pressure.

**Effect on renal morphology**

A total of 83 kidney biopsies were examined. The biopsies were stratified according to the treatment protocol and blind for the analysing pathologist. The results of the semi-quantified scoring are shown in Figure 3. A considerable variation in the total scores was seen between the treatment groups—the Kruskal–Wallis one-way ANOVA was 41.99, $P<0.0001$. The scores for basophilic tubules varied slightly in the treatment groups, whereas the scores for both subcapsular fibrosis and striped fibrosis were minor and consistent within groups. Substantially higher scores for all markers were seen in the group treated with a combination of CsA and SRL ($P<0.0001$, when compared with controls), but those receiving the combination of CsA plus FK506 also displayed a significantly higher score ($P<0.05$, when compared with controls), whereas the FK506 plus SRL group only showed a minor, non-significant, higher score ($P=0.09$, when compared with controls) (Figure 3).

When the kidneys were analysed according to the quantitative grade of fibrosis, indications of a similar pattern were seen, although the difference between groups was not overall statistically significant (Kruskal–Wallis ANOVA 8.65, $P=0.19$) (Figure 4). Again, although, the groups treated with a combination of CsA plus SRL or CsA plus FK506 had the highest scores, significantly higher than those of the controls ($P<0.05$ for both groups), whereas the group treated with SRL plus FK506 only had a marginally higher score ($P=0.05$) (Figure 4).

**Discussion**

A potential approach to minimizing the nephrotoxicity of calcineurin inhibitors would be to give them in a smaller dose and simultaneously use the synergistic immunosuppressant, SRL, which has a distinctive mechanism of action. Initial clinical studies of

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**Fig. 2.** Blood pressure after 2 weeks of immunosuppressive treatment.
combined treatment with CsA and SRL in humans revealed no specific interactions [5].

The aim of this study was to analyse further the glomerular function and possible morphological changes after combined therapy with CsA, FK506 and SRL. As we wanted to ensure that the immunosuppressive potential of the individual drugs was fully exploited, rather high doses were used which were earlier found to have significant immunosuppressive effects when used as monotherapy: CsA 15 mg/kg/day [10], FK506 3.0 mg/kg/day [11] and SRL (data submitted for publication). The bioavailability of CsA and FK506 was ensured as whole blood trough values were measured at the end of the treatment period when the treatment was assumed to have reached the steady state. The whole blood values were rather low for both CsA and FK506, as compared with the usual human trough level values in most clinical settings, but consistent between groups for the different treatment modalities. Another study found that trough levels of both SRL and CsA and AUC concentrations were significantly higher when the drugs were administered concomitantly [17], whereas others found no change in either CsA blood levels or the AUC during combination therapy [5, 17]. It should be emphasized that the study setting varies from the usual clinical procedures, as in our study CsA and FK506 were administered in a dissolved form through a gastric tube, and SRL was administered intra-peritoneally. Furthermore, the doses were given as a single administration once a day, and were adjusted according to the weight increase of the individual rat.

Despite the encouraging initial observations, Kahan and co-workers [18] demonstrated that combination therapy with CsA and SRL in renal transplant patients after 6 and 12 months resulted in significantly higher

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Fig. 3. Subcapsular fibrosis, striped fibrosis and basophilic tubules in rat kidneys after 2 weeks of immunosuppressive treatment—semi-quantitative scoring.

Fig. 4. Grade of fibrosis in rat kidneys after 2 weeks of immunosuppressive treatment—qualitative scoring.
serum creatinine concentrations and lower creatinine clearance than in patients treated with SRL alone. A rat study showed that after 2 weeks of treatment SRL induced significant kidney lesions consisting of tubular collapse, vacuolization, and nephrocalcinosis, but the SRL dosage in that study was very high, 3 mg/kg/day administered orally, and the rats were put on a low-salt diet [19].

Conversely, results of combination therapy with FK506 and SRL indicate that these drugs might act in a complementary way, as neither additive or synergistic drug-associated toxicities, nor pharmacological antagonism was seen [2,7]. Furthermore, a higher creatinine clearance was found in humans treated with SRL plus FK506 than in those given CsA plus mycophenolate mofetil [20]. Our study indicates that SRL has a pronounced synergistic nephrotoxic effect on the GFR and a number of morphological fibrotic changes when combined with CsA, but in combination with FK506 it only showed minor changes on a level similar to those seen with treatment with the individual substances. The aggravated nephrotoxic effect might be due to the fact that SRL and CsA share a common biotransformation site, the cytochrome 450-3A subfamily, but at present there is no evidence to suggest the pathophysiological mechanisms behind this synergistic nephrotoxic effect. In short-term studies SRL probably has a tubulotoxic effect, as the increase in end proximal delivery seen during SRL treatment results in increased sodium delivery to the distal tubule. However, as this finding seems to disappear after a period of time (data submitted for publication) it might be due to a haemodynamic effect of SRL rather than to a tubulotoxic effect. Whereas different haemodynamic effects of the calcineurin inhibitors from those of SRL may be an advantage, combination therapy might alternatively result in further deterioration of renal function, owing to uncompliant haemodynamic mechanisms. Thus, this study demonstrates a potential synergistic nephrotoxic effect of CsA in combination with SRL. Whether this additive effect observed in rats is relevant in the dosages used in clinical transplantation remains to be elucidated.

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