Randomized trial

Randomized clinical trial of the effect of microemulsion cyclosporin and tacrolimus on renal allograft fibrosis

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Background: The aim of this study was to compare the effect of Neoral® cyclosporin- and tacrolimus-based therapy on the development of renal allograft fibrosis (chronic allograft nephropathy; CAN) in a prospective randomized trial.

Methods: A total of 102 patients undergoing renal transplantation were randomized to immunosuppression with either microemulsion cyclosporin (Neoral®; 15 mg per kg per day adjusted to whole-blood trough concentrations of 200–300 ng/ml) or tacrolimus (0·2 mg per kg per day adjusted to whole-blood trough levels of 8–15 ng/ml) in conjunction with steroids, or at a lower dose (7 mg per kg per day and 0·1 mg per kg per day respectively) with the addition of azathioprine for non-heart-beating renal transplant recipients. Renal transplant interstitial fibrosis was quantified using computerized histomorphometric measurement of picrosirius red-stained 1-year protocol renal transplant biopsies. Levels of interstitial fibrosis were compared in relation to observed efficacy and toxicity profiles of the two drugs.

Results: There was a significant increase in allograft interstitial fibrosis in the patients treated with Neoral® compared with those given tacrolimus. There was no significant difference in the demographic characteristics between the patient groups or in the incidence of acute rejection (Neoral® 36 per cent *versus* tacrolimus 35 per cent) or steroid-resistant rejection (both 10 per cent) between the two drugs. There was a higher incidence of insulin resistance in the tacrolimus group (post-transplant diabetes mellitus, glucose tolerance testing) but this was not statistically significant. Neoral® was associated with a significant increase in total cholesterol ($P = 0{\cdot}030$) and low-density lipoprotein ($P = 0{\cdot}021$) levels, which persisted throughout the study period.

Conclusion: Despite equivalent efficacy and pretransplantation risk factors for CAN, Neoral® was associated with increased allograft fibrosis and significantly higher serum low-density lipoprotein cholesterol levels compared with tacrolimus.

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Introduction

Chronic allograft nephropathy (CAN) remains a leading cause of graft loss after the first year[1]. CAN occurs, in part, as a consequence of excessive deposition of extracellular matrix (ECM) proteins (fibrosis) that is characterized histologically by glomerulosclerosis, interstitial fibrosis and allograft vasculopathy[2]. These changes represent a complex process involving ongoing injury, inflammation and repair that lead to an irreversible decline in graft function and organ failure. It is unclear whether the progression of graft dysfunction is affected by the choice of calcineurin inhibitor. Primary immunosuppression with the calcineurin inhibitors tacrolimus and microemulsion cyclosporin (Neoral®; Sandoz Pharmaceutical, Basle, Switzerland) requires a balance of drug efficacy and toxicity, as both cellular rejection and side-effects such as hyperlipidaemia, hyperglycaemia and direct nephrotoxicity can contribute to the progression of CAN[2]. Randomized trials comparing the effect of microemulsion cyclosporin and tacrolimus on graft survival following renal transplantation have produced conflicting results[3–6]. Their effects on renal allograft

fibrosis have yet to be compared in a randomized trial.

Histomorphometric measurement of ECM protein accumulation in protocol human renal allograft biopsies as early as 6 months after transplantation accurately predicts a decline in transplant function and long-term kidney transplant survival, and can be used as a validated surrogate *ad interim* marker of CAN[7,8]. The present study was a single-centre randomized prospective comparison of the effect of tacrolimus *versus* Neoral®-based primary immunosuppression on the development of CAN, using histomorphometric quantitation of allograft interstitial ECM accumulation from 1-year protocol biopsies. The results are discussed in relation to the observed efficacy and adverse effect profiles of the two drugs.

Patients and methods

Study design

This was an open-label randomized prospective clinical trial. The trial was powered to detect a 10 per cent difference in collagen III accumulation of 1-year renal transplant biopsies. Previous studies in this department[7,8] indicated that 80 patients were required to achieve an α value of 0·05 and a power of 0·8. The study was conducted in accordance with the Declaration of Helsinki and had received local ethics committee approval.

Study medication and therapeutic drug monitoring

One hundred and two consecutive patients who underwent renal transplantation were randomized before operation to receive either tacrolimus (Prograf®; Fujisawa, Munich, Germany) (0·1 mg/kg twice daily) or Neoral® cyclosporin (15 mg per kg per day in two divided doses, reduced by 2 mg per kg per day each week to a baseline level of 5 mg per kg per day at week 6). Patients who received kidneys from non-heart-beating cadaveric donors received either tacrolimus 0·05 mg/kg twice daily or Neoral® 7 mg per kg per day in divided doses, and in addition non-heart beating recipients received azathioprine 1 mg/kg as a single daily dose. The dosages of tacrolimus and Neoral® were adjusted to achieve whole-blood 12-h trough concentrations of 8–15 ng/ml and 200–300 ng/ml respectively over the first 3 months following transplantation. Subsequently target trough levels were reduced to 5–10 ng/ml and 100–200 ng/ml.

Each patient received prednisolone 20 mg/day for the first 3 months after transplantation, after which the steroid dosage was tapered in a linear fashion to a dosage of 10 mg on alternate days (or 5 mg/day for diabetic patients) at 6 months. In addition to standard immunosuppression, all patients received oral aspirin 150 mg once daily for the first 3 months, co-trimoxazole 480 mg once daily for the first 6 months and long-term nifedipine 20 mg twice daily from the day of transplantation, blood pressure permitting.

Morphometric quantitation of extracellular matrix content

One-year protocol day-case needle-core biopsies were performed under real-time ultrasonographic guidance using a 16-G Tru-Cut needle (Travenol Laboratories, Deerfield, Illinois, USA) mounted on a spring-loaded biopsy gun. Biopsies were fixed in formalin, embedded in paraffin, sectioned serially at 4 μm thick and stained with picrosirius red. The picrosirius polarization method was used to quantify interstitial ECM content[9]. Picrosirius red-stained sections were examined on a Nikon E800 microscope with the use of circularly polarized light. Field images were observed on a Photonic Science Coolpix Monochrome cooled charge coupled device integrating mounted video camera on the vertical tube of the microscope along with a $\times 1$ relay lens. The images were digitized by a video frame grabber in a Power Mackintosh G3 computer Scion CG-7® (Meyer Instruments Inc., Houston, Texas, USA) in monochrome mode, displayed on a high-resolution monitor and analysed by the use of the Java video analysis software NIH Image (courtesy of Wayne Rasband, National Institutes of Health, Bethesda, Maryland, USA) with macros written by P.N.F. To eliminate everything except ECM from the vessel image, a blue-filtered, monochrome, bright-field image of the picrosirius red-stained sections was subtracted from a circularly polarized image. This produced an image composed of bright ECM fibres on a black background. A histogram of the brightness of each pixel in the image was plotted. All of the non-ECM area was assigned a grey scale area of zero, using a scale from 0 (black) to 255 (white). Thus any pixel with a grey scale level of more than zero represented ECM. The ECM content in each section was expressed as the percentage area fraction of pixels with a grey scale value of more than zero, and the total relative ECM was content expressed as the product of the field area and percentage area fraction.

Evaluation of efficacy, safety and toxicity

Renal function was measured in serum blood samples and the glomerular filtration rate was calculated at 3, 6 and 12 months by measuring the clearance of a bolus of ^{51}Cr-labelled EDTA. Drug efficacy was determined by rejection-free survival, the absolute incidence of

acute cellular rejection and frequency of steroid-resistant acute rejection. Histologically proven acute rejection was graded according to the Banff working classification of kidney transplant pathology[10] and treated with intravenous methylprednisolone (500 mg/day for 3 days). Both steroid-resistant rejection and severe acute rejection were treated with pulsed antithymocyte globulin (Imtix Sangstat, Windsor, UK) therapy (5–2·5 mg per kg per day). Other adverse events including delayed graft function (need for dialysis in the first post-transplant week), primary non-function (failure of the transplanted kidney without previous graft function) and graft failure were recorded. Premature withdrawal from the study for whatever reason was considered as treatment failure. A switch to the alternative drug treatment regimen owing to refractory rejection or adverse events relating to study medication resulted in patient withdrawal. Risk factors for CAN and acute coronary events were also recorded. These included serum lipid profiles, results of glucose tolerance testing, fasting glucose and glycated haemoglobin (HbA1c) A1c levels and hypertensive control.

Statistical analysis

Analysis was by intention to treat. All data were assessed for normality of distribution and equality of variance. Student *t* test and ANOVA were used to compare normally distributed data, and the Mann–Whitney *U* test or Kruskall–Wallis test was used to compare non-normally distributed data. Data are presented throughout as mean(s.d.). General linear model repeated-measures ANOVA (MANOVA) was used for analysis of variance when the same measurement was made several times on each subject. Categorical data were compared using the Pearson χ^2 test or Fisher exact test in the case of two categories per treatment group. Kaplan–Meier estimates of acute rejection, and patient and graft survival were obtained for the two treatment groups and compared with the log rank test. All data analysis was performed using Statistical Package for the Social Sciences for Windows™, version 9.0 (SPSS, Chicago, Illinois, USA).

Results

Patient characteristics

Fifty-two patients were randomized to the tacrolimus group and 50 to the Neoral® group. There were no differences between the treatment groups with respect to patient demographics, or donor and recipient characteristics (*Tables 1* and *2*).

Table 1 Demographic and clinical characteristics of renal allograft recipients

	Neoral® ($n = 50$)	Tacrolimus ($n = 52$)
Mean(s.d.) age (years)	45(12)	45(14)
Sex ratio (F : M)	15 : 35	20 : 32
Primary diagnosis		
Glomerulonephritis	6 (12)	7 (13)
Hypertension	3 (6)	3 (6)
Diabetes	2 (4)	7 (13)
Hereditary nephropathy	9 (18)	16 (31)
Systemic autoimmune disease	9 (18)	3 (6)
Interstitial nephritis	6 (12)	9 (17)
Glomerulosclerosis	3 (6)	1 (2)
Other	1 (2)	1 (2)
Unknown	11 (22)	5 (10)
No. of previous transplants		
0	44 (88)	46 (88)
1	4 (8)	5 (10)
2	1 (2)	1 (2)
3	1 (2)	0 (0)

Values in parentheses are percentages unless indicated otherwise. There were no significant differences in patient demographics.

Table 2 Renal transplant donor and recipient characteristics

	Neoral® ($n = 50$)	Tacrolimus ($n = 52$)
Donor sex ratio (F : M)	18 : 32	20 : 32
Donor type*		
LD	8 (16)	9 (17)
CAD	21 (42)	22 (42)
NHBD	21 (42)	21 (40)
Donor age by type (years)†		
LD	49(10)	45(12)
CAD	44(17)	39(17)
NHBD	48(10)	49(9)
Cold ischaemia time (h)†		
LD	1·7(1·6)	2·2(2·1)
CAD	19·0(8·0)	18·7(7·9)
NHBD	15·5(5·0)	15·1(5·6)
Warm ischaemia time (min)†		
NHBD (min)	21·2(11·0)	24·0(12·7)
Anastomosis (min)	30·8(5·8)	32·1(6·7)
HLA mismatch (A, B, DR)*		
0	4 (8)	7 (13)
1	4 (8)	2 (4)
2	10 (20)	8 (15)
3	16 (32)	16 (31)
4	13 (26)	16 (31)
5	3 (6)	3 (6)
6	0 (0)	0 (0)

*Values in parentheses are percentages; †values are mean(s.d.). LD, living donor; CAD, cadaveric donor; NHBD, non-heart-beating donor.

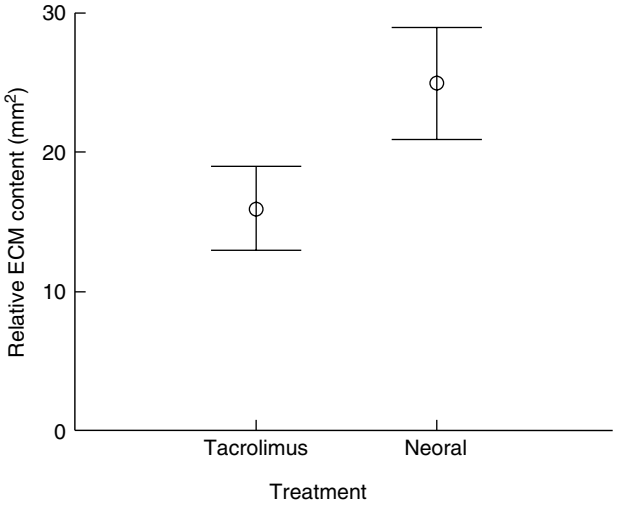

Fig. 1 Mean(s.d.) relative interstitial extracellular matrix (ECM) content in protocol 1-year renal transplant biopsies. There was a significantly higher level of interstitial collagen in patients randomized to Neoral® ($P = 0{\cdot}002$, Student *t* test)

Computerized histomorphometry of picrosirius red staining

There was a significantly higher area fraction of sirius red staining in the Neoral® group (24·9(11·1) mm^2) compared with the tacrolimus group (15·9(6·5) mm^2) ($P = 0{\cdot}002$, Student *t* test; *Fig. 1*), demonstrating a significantly higher level of interstitial ECM density (fibrosis) in Neoral®-treated patients.

Efficacy and graft loss

There were no differences in acute rejection rate, 90-day rejection free survival or requirements for antithymocyte globulin (ATG) therapy, or in the incidence of other adverse events, between the two groups (*Table 3* and *Fig. 2*). There was no significant difference between overall mean serum creatinine level (Neoral® 170(90) mmol/l *versus* tacrolimus 157(61) mmol/l; $P = 0{\cdot}564$, Student *t* test) or glomerular filtration rate at 1 year (47(14) *versus* 47(18) ml/min respectively) or at any other time point, or when stratified by donor type. Whole-blood trough Neoral® concentrations were supratherapeutic at 7 and 14 days (500(201) and 409(165) ng/ml respectively) but mean levels were within therapeutic limits after 6 weeks (data not shown). Mean trough levels of tacrolimus were 16·4(7·7) and 13·3(6·2) ng/ml at 7 and 14 days respectively.

Table 3 Summary of acute rejection rates, graft function and failure in renal allograft recipients

Outcome	Neoral® ($n = 50$)	Tacrolimus ($n = 52$)
Acute rejection*	18 (36)	18 (35)
90-day rejection-free survival‡	65 (7)	63 (7)
ATG therapy*	5 (10)	5 (10)
Delayed graft function*	20 (40)	18 (35)
Time to graft function (days)†	22(10)	18(11)
Primary non-function*	3 (6)	1 (2)
Death*	0 (0)	2 (4)
Graft failure*	5 (10)	2 (4)

*Values in parentheses are percentages; †values are mean(s.d.); ‡values are cumulative survival (S.E.) expressed as percentages. ATG, antithymocyte globulin.

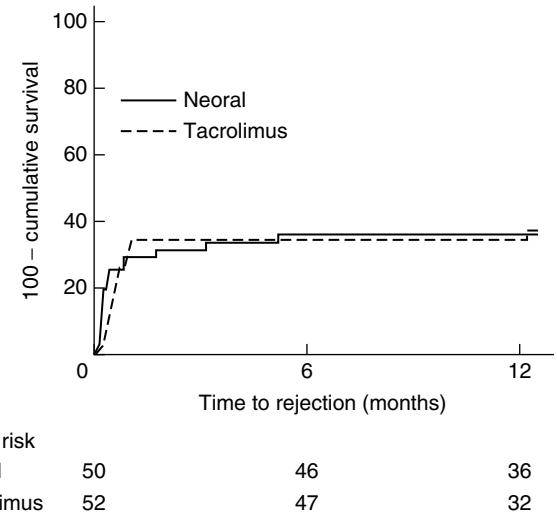

Fig. 2 Cumulative rates of first biopsy-proven rejection in patients randomized to Neoral® or tacrolimus (Kaplan–Meier analysis)

There were two deaths during the study period, both in the tacrolimus group. Both patients died from myocardial infarction in the perioperative period. Five grafts failed in the Neoral® group and two in the tacrolimus group ($P = 0{\cdot}217$, Fisher exact test). Five patients were withdrawn from the study, including three patients in the Neoral® group who were converted to tacrolimus therapy because of refractory rejection, one Neoral®-treated patient who experienced a second rejection episode but was unwilling to consider methylprednisolone or ATG therapy and was converted to tacrolimus-based immunosuppression, and one patient in the tacrolimus group who developed post-transplantation diabetes mellitus (PTDM) and was converted to Neoral® treatment.

Safety

Glucose intolerance

Four patients in the tacrolimus group and two in the Neoral® group developed PTDM, and required long-term treatment for glucose intolerance during the study ($P = 0{\cdot}358$, Fisher's exact test). Glucose tolerance test results and the proportion of patients with abnormal or borderline test results were not significantly different at any time (*Table 4*). There was no significant difference between the two groups in HbA1c or serum fasting glucose levels measured at 1 week, 1, 3 and 6 months, and 1 year (*Table 5*).

Serum lipid profiles

Serum lipid profiles were evaluated on days 0 and 7, and then at 1, 3, 6 and 12 months after operation. Serum levels of total cholesterol, triglycerides, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) were not significantly different between the two groups on the day of operation. MANOVA revealed a significant difference between the two groups in total cholesterol ($P = 0{\cdot}030$) and LDL cholesterol ($P = 0{\cdot}021$) (*Figs 3* and *4*). Serum triglyceride and HDL levels showed no significant difference at any time point (data not shown).

Table 4 Summary of glucose tolerance test results

Time after transplantation	Neoral®		Tacrolimus		
	Abnormal	Borderline	Abnormal	Borderline	*P**
90 days	5 of 18	5 of 18	7 of 20	7 of 20	0·654
180 days	0 of 7	2 of 7	3 of 14	8 of 14	0·068
1 year	0 of 6	2 of 6	0 of 10	4 of 10	0·790

*χ^2 test.

Table 5 Fasting glucose and glycated haemoglobin (HbA1c) measurements in renal allograft recipients

Time after transplantation	Neoral®		Tacrolimus	
	Glucose (mmol/l)	HbA1c (%)	Glucose (mmol/l)	HbA1c (%)
7 days	6·0(3·1)	7·1(1·3)	6·8(3·6)	7·5(1·6)
30 days	5·6(3·2)	6·7(1·7)	6·3(2·6)	7·3(1·4)
90 days	5·7(4·5)	6·7(1·6)	5·9(2·7)	7·2(1·5)
180 days	5·6(2·2)	7·0(1·4)	6·0(2·9)	7·4(1·8)
1 year	5·9(4·2)	7·3(1·9)	6·8(5·1)	7·0(1·5)

Values are mean(s.d.). Hb, haemoglobin. There was no significant difference between the two groups in fasting glucose ($P = 0{\cdot}120$) and HbA1c ($P = 0{\cdot}786$) (repeated-measures ANOVA).

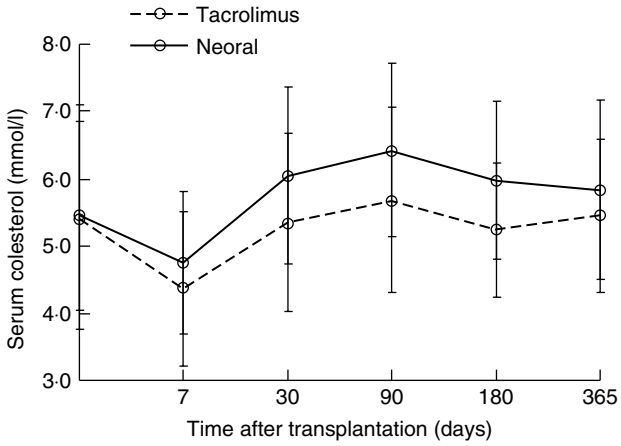

Fig. 3 Mean(s.d.) total serum cholesterol levels in allograft recipients with time after transplantation in patients randomized to Neoral® or tacrolimus. There was a significantly higher level of total cholesterol in the Neoral® group ($P = 0{\cdot}030$, repeated-measures ANOVA)

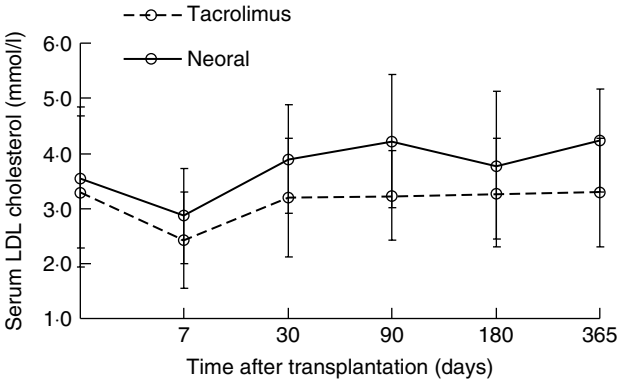

Fig. 4 Mean(s.d.) serum low-density lipoprotein (LDL) cholesterol in allograft recipients with time after transplantation in patients randomized to Neoral® or tacrolimus. There was a significantly higher level of LDL cholesterol in the Neoral® group ($P = 0{\cdot}021$, repeated-measures ANOVA)

Blood pressure

There was no difference in the overall systolic and diastolic blood pressure, and requirements for treatment between the groups (number of antihypertensives at 1 year 1·8(0·9) in Neoral® group *versus* 1·7(0·7) in tacrolimus group; $P = 0{\cdot}151$, Student *t* test).

Discussion

The most clinically relevant finding of this study was a statistically significant increase in renal allograft fibrosis at 1 year in patients treated with microemulsion

cyclosporin compared with those receiving tacrolimus-based immunosuppression. There were no differences between the groups in pretransplant donor or recipient characteristics or in efficacy. There was a significant increase in serum LDL cholesterol levels in cyclosporin-treated patients and a non-significant trend towards increased glucose intolerance with tacrolimus. These data suggest that the toxicity profiles of the two drugs in this study are central to the observed changes in graft fibrosis.

The equivalent efficacy of tacrolimus and Neoral® in the present study contrasts to the results of a similar randomized comparison of tacrolimus–azathioprine and Neoral®–azathioprine in which tacrolimus was associated with superior efficacy and better graft survival at 3 years[6]. Although target whole-blood trough levels were similar, the starting dose of Neoral® was much higher in the majority of patients in the present study (15 *versus* 8 mg per kg per day[5,6]) and this was reflected in the high initial Neoral® whole-blood trough levels at 7 and 14 days. Equivalent efficacy in the Neoral® group at this higher dose was not associated with a reduction in CAN. This suggests that cyclosporin toxicity may be a more important determinant of subsequent allograft fibrosis in these patients. This is further supported by the observation that in randomized trials of the two drugs in combination with mycophenolate mofetil, with Neoral® at a lower starting dose (Neoral® 8 mg per kg per day *versus* tacrolimus 0·16 mg per kg per day), equivalent efficacy was also reported but there was no difference in graft survival[3,4].

Studies in humans and animals have demonstrated that both tacrolimus and cyclosporin exert a direct toxic effect on the kidney. In the early post-transplant period cyclosporin produces higher levels of intrarenal vasoconstriction and profibrotic growth factor production than tacrolimus[11]; this, in combination with the high starting dose of Neoral® in the present study, may have contributed to the increased incidence of intragraft fibrosis in this group. However, there were no significant differences between the groups in the frequency of delayed graft function or in duration of dialysis dependence in those with delayed graft function. It is possible that an increased direct nephrotoxic effect of Neoral® may be detected in a larger study, as demonstrated in the European multicentre randomized trial of Sandimmun® (Novartis, Basle, Switzerland) cyclosporin and tacrolimus in renal transplantation[12].

Neoral® was associated with significantly higher serum levels of total and LDL cholesterol than tacrolimus. This difference did not reduce over the 1-year study interval and its effect on graft fibrosis was therefore independent of the higher initial whole-blood trough levels of Neoral®. Oxidative modification of LDL has an important pathophysiological role in the development of CAN. Oxidised LDL acts as a potent proinflammatory mediator, and is implicated in macrophage activation as well as vascular smooth muscle cell and mesangial proliferation[13,14]. Pre-existing hypercholesterolaemia, uraemia, genetic predisposition and steroid administration all contribute to hyperlipidaemia in renal transplant patients; however, cyclosporin is thought to have its own independent effect. Cyclosporin inhibits bile acid synthesis in animal models, reducing cholesterol gut loss and thereby increasing total cholesterol[13]. Lipoprotein-bound cyclosporin may alter LDL clearance by interfering with the LDL receptor molecule[13,14]. In addition, cyclosporin is thought to increase the toxicity of a high serum cholesterol concentration by promoting oxidation of LDL at high doses[15,16].

There was no significant difference in the level of glucose intolerance between the two groups, although there appeared to be a trend towards increased intolerance in the tacrolimus group in the early post-transplant period. The glucose intolerance associated with tacrolimus (8 per cent) was less marked than in previous studies comparing Sandimmun cyclosporin and tacrolimus (16–31 per cent)[12,17–19]. More recent trials of Neoral® *versus* tacrolimus in combination with mycophenolate mofetil[3,4] also reported no significant difference in the incidence of PTDM. This lower rate of PTDM undoubtedly reflects greater experience with the use of tacrolimus. The incidence of hyperglycaemia, as well as other adverse effects, is dependent on the dose of tacrolimus[20,21] and recent lower rates of PTDM (7–9 per cent) have been attributed to lower starting dosages and more rapid dose reduction[20,21]. There was, however, a high incidence of abnormal glucose tolerance test results at 180 days in the tacrolimus group, and another possible explanation for the low incidence of PTDM is a tendency to undertreat hyperglycaemia with insulin in this study.

This study suggests that tacrolimus is associated with less renal interstitial ECM accumulation than Neoral® in *de novo* renal transplants. At doses with similar efficacy the safety profile of tacrolimus was superior to that of Neoral®. In particular, Neoral®-associated hyperlipidaemia was implicated in the increased renal transplant fibrosis seen in these patients.

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