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SEX DIFFERENCES IN RESPONSE TO CYCLOSPORINE IMMUNOSUPPRESSION IN EXPERIMENTAL KIDNEY TRANSPLANTATION

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SUMMARY

1. Female donors and recipients have increased risk of acute rejection and subsequent chronic allograft nephropathy (CAN), especially when cyclosporine A (CsA) is used. Decreased renal nitric oxide (NO) production is associated with chronic kidney disease. In the present study, we investigated the impact of gender, CsA dose and renal NO synthase (NOS) on CAN.

2. Kidneys from male and female F344 rats were transplanted into same-sex Lewis allograft or F344 isograft recipients and recipient rats were treated with 1.5 or 3 mg/kg per day CsA for 10 days. Grafts were removed at 22 weeks post-transplantation. Normal two-kidney F344 rats were investigated as age-matched controls.

3. Low-dose CsA was associated with accelerated CAN in female rats compared with male rats; however, with high-dose CsA, allograft females had similar pathology/function to allograft males. Isograft females (similar to isograft males) had no graft failure and only slightly, albeit significantly, greater injury than age-matched controls. Isograft females had higher renal cortical neuronal (n) NOS but lower medullary endothelial (e) NOS than isograft males. There was no difference in renal eNOS and nNOS between allograft groups.

4. In conclusion, 1.5 mg/kg per day CsA is not sufficient to prevent early graft loss in females. When the dose of CsA is doubled, allograft females and males have similar post-transplant survival. Renal NOS expression was unremarkable in any transplant group.

Keywords

cyclosporine; immunosuppression; kidney transplantation; sex difference

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INTRODUCTION

Despite better immunosuppressive regimens and improvement in 1 year renal allograft survival rates, kidney grafts are lost over the long term due to chronic rejection. Histologically, chronic allograft nephropathy (CAN) is characterized by glomerulosclerosis, tubular atrophy, interstitial fibrosis and vasculopathy. In addition to alloantigen-dependent factors, several alloantigen-independent factors have also been implicated in the development of chronic rejection. Ischaemic injury plays a key role in the process by enhancing the expression of donor antigens and inducing and maintaining severe immunological destructive mechanisms against the transplanted organ, thus aggravating acute rejection.[1] In addition, ischaemia–reperfusion injury *per se*, without transplantation (Tx), leads to long-term (50 weeks) exacerbation of normal ageing kidney injury.[2] During the chronic phase after Tx, ongoing inflammatory processes lead to exacerbation of functional deterioration and structural damage to the renal allograft.[3]

The most widely used animal model of CAN is the Fisher 344 to Lewis rat Tx model. In male rats, the first signs of CAN develop between 12 and 16 weeks after Tx, depending on the surgical procedure and immunosuppressive protocol. Very few experimental organ transplantation studies use females, although there is evidence that female gender may predispose to more severe acute rejection, as noted in skin grafts.[4] We previously reported that when ovariectomized female rats receive renal transplants from female donors, they develop accelerated early CAN compared with male rats.[5] Clinical data show an increased risk of acute rejection in women receiving kidney Tx, indicating that the female kidney is more antigenic, is more susceptible to acute rejection and/or cyclosporine A (CsA) nephrotoxicity and/or requires a higher CsA dose because of increased metabolism.[6,7] In contrast, slower progression of CAN has been reported in female allograft recipients.[8] The slower progression of CAN is in accordance with the observed slower progression of renal disease in females with other forms of chronic kidney disease (CKD).[9,10]

Clinical evidence suggests that nitric oxide (NO) deficiency occurs as a result of CKD and may contribute to further injury progression.[11,12] In animals, L-arginine (an NO synthase (NOS) substrate) supplementation is renoprotective, whereas chronic inhibition of NOS causes CKD and, when superimposed on a second model, accelerates the progression of CKD, indicating that NO attenuates the injury process.[13,14] In experimental CKD models induced by glomerulonephritis, renal ablation/infarction, chronic puromycin aminonucleoside nephrosis and normal ageing, renal injury correlated with the decrease in abundance of renal neuronal (n) NOS, suggesting a critical threshold for renal NO production below which renal injury occurs.[15,16] There is little information on the impact of CAN on renal nNOS.

In the present study, we investigated sex differences in long-term (22 weeks) outcome of renal Tx using different surgical (ischaemia time) procedures and two different doses of CsA. By performing studies in both allografts and isografts of both sexes we were able to separate primary immunological influences (acute rejection) from ischaemia–reperfusion injury and CsA toxicity. The primary hypothesis tested here was that early loss of graft function in females treated with CsA results from inadequate immunosuppression rather than CsA-induced nephrotoxicity. We also investigated the relationship between CAN and renal NOS abundance.

METHODS

Inbred female and male Fisher 344 (F344, RT1v1; Harlan, Indianapolis, IN, USA) animals served as donors. Lewis (LEW, RT1; Harlan) female and male rats were used as recipients in the allograft groups, whereas F344 female and male rats were used as recipients in the isograft groups. Donors and recipients were always of the same gender in every Tx performed. All rats

were aged 9–14 weeks at the time of Tx. All animals were maintained under a 12 h light–dark cycle with free access to standard rat chow (except prior to and during metabolic cage studies; see below) and water *ad libitum*.

Orthotopic renal Tx was performed using a full sterile technique. Rats were anaesthetized by intraperitoneal injection of a mixture of pentobarbital sodium (32.5 mg/kg; Sigma, St Louis, MO, USA) and methohexital sodium (25 mg/kg; Eli Lilly and Co., Indianapolis, IN, USA). After median laparatomy, the left renal vessels and ureter of the recipient were isolated, clamped and the native kidney removed. The left donor kidney was perfused with cold (4°C) lactated Ringer's solution, removed and positioned orthotopically into the recipient. Donor and recipient renal artery, vein and ureter were end-to-end anastomized with 10–0 prolene sutures. Cold ischaemic time ranged from 7 to 10 min; the warm ischaemic time varied according to the time of cold ischaemia so that there was a total of 45 min (Series I) or 30 min (Series II and III) total ischaemic time. Details of the surgery have been published by us previously.[5]

All transplant recipients (both allograft and isograft) were treated for the first 10 days after surgery with one of the following immunosuppressive regimens: (i) low-dose (1.5 mg/kg per day, s.c.) CsA (Series I and II); or (ii) high-dose (3 mg/kg per day, s.c.) CsA (Series III). The CsA (Sandimmune) was obtained from Novartis (Basle, Switzerland). Additional normal, untreated F344 male and female age-matched rats were followed for 22 weeks (Series IV) as age-matched controls for Series III.

Treatment protocols and groups in Series I–IV are given in Table 1. Because most allografted female rats died early in Series I, total ischaemia time was reduced to 30 min in Series II and only female rats, both allografts and isografts, were studied. Owing to rapid injury progression in Series II allograft females, the experiment was terminated 7 weeks after Tx. Concerns about the possible superimposition of infections during the period of immunosupression caused us to add the antibiotic ceftriaxone sodium, 10 mg/kg per day, i.m. (Rocephin; Roche, Nutley, NJ, USA), to all rats in Series III, in which both sexes were analysed at 22 weeks after Tx.

Twenty-four hour urine samples were collected in metabolic cages before Tx, before nephrectomy at 7 days post-Tx and at Weeks 4, 8, 12, 16, 20 and 22 after Tx for determination of total protein and creatinine excretion. Just prior to rats being killed, blood pressure (BP) and heart rate (HR) were measured under general anaesthesia (as used for Tx). Although this method gives an indication of any BP differences between groups, it is not likely to reflect true conscious BP in these animals. A blood sample was withdrawn (from the abdominal aorta) for later analysis of plasma creatinine and blood urea nitrogen (BUN). All analyses were performed as described previously.[17] Transplanted kidneys were then perfused, until blood free, through the abdominal aorta with cold (4°C) phosphate-buffered saline (PBS), decapsulated, removed and weighed. A thin section of kidney, including the cortex and medulla, was fixed for histology and the remaining cortex and medulla were separated, flash frozen in liquid nitrogen and stored at −80°C for later analysis.

Kidney sections were fixed in 10% buffered formalin, blocked in paraffin and 5 μm sections were stained with periodic acid-Schiff and haematoxylin–eosin. Slides were examined in a blinded manner by one author (AS). Glomerular sclerosis and interstitial, tubular and vascular lesions were graded according to the Banff classification.[17]

Renal abundance of nNOS and endothelial (e) NOS was determined in Series III rats. Western blot analysis was performed as described previously.[18] Briefly, measurements were conducted on kidney cortex (200 μg total protein) and kidney medulla (100 μg total protein) for nNOS and eNOS. For nNOS detection, we used a rabbit monoclonal antibody (1 : 5000 dilution; 1 h incubation at room temperature) and a secondary goat anti-rabbit IgG–horseradish peroxidase (HRP) antidody (1 : 3000 dilution; 1 h incubation; Bio-Rad, Richmond, CA, USA). Membranes

were stripped and reprobed for eNOS (mouse monoclonal antibody, 1 : 250 dilution, 1 h incubation; secondary antibody goat anti-mouse IgG–HRP conjugate, 1 : 2000 dilution, 1 h incubation; Transduction Laboratories, Lexington, KY, USA). The abundance of NOS protein was calculated as integrated optical density (IOD) of nNOS or eNOS factored for Ponceau red stain (total protein loaded) and for a positive control and expressed as a percentage change from the respective control value. This allowed quantitative comparisons between different membranes. Protein abundance is represented as 'IOD/+control/ponceau'.

Results are presented throughout as the mean±SEM. Parametric data were analysed by *t*-test and anova followed by multiple pairwise comparisons by the Newman–Keuls' test. Non-parametric data were tested using the Mann–Whitney test or Kruskal–Wallis one-way analysis of ranks followed by Dunns post hoc test. $P < 0.05$ was considered significant.

RESULTS

In Series I, with the low-dose CsA regimen and long (45 min) ischaemia time, five of seven of the allograft females died 4–6 weeks after Tx and only two survived the 22 weeks of follow up. Therefore, there are no data included for the Series I allograft females. All female isograft and male isograft and allograft rats survived for 22 weeks post-Tx. The female isograft group developed significant proteinuria that was not different to isograft and allograft males. In contrast, histological analyses revealed severe signs of CAN in male allograft rats, whereas similar mild injury was seen in female and male isograft groups (Table 2).

In Series II (females only), the total ischaemia time was reduced to 30 min in an effort to prolong survival. Four weeks after Tx there was already a difference in protein excretion between allograft and isograft groups (10 ± 1 *vs* 5 ± 3 mg/day; $P < 0.05$). By 6 weeks post-Tx, significant proteinuria developed in allograft compared with isograft rats (37 ± 14 *vs* 4 ± 1 mg/day, respectively; $P < 0.05$), with a further increase at Week 7 (Table 2) resulting in early termination of the experiment. Histological analyses revealed signs of accelerated severe CAN in allograft females, with only mild changes in the isograft group (Table 2).

The Series III rats received 3 mg/kg per day CsA with a 30 min total ischaemia time and all transplanted animals survived 22 weeks follow up. The functional parameters (serum creatinine, BUN, clearance of creatinine (Ccr)) demonstrated better graft function in isograft compared with allograft females and, remarkably, the one-kidney isograft females showed similar 24 h Ccr to the two-kidney age-matched female controls. In males, the 24 h Ccr was similarly reduced in both isograft and allograft Tx compared with two-kidney controls (Table 3). The allograft females developed significant proteinuria, whereas isograft females were at control values; of note, there was no age-dependent proteinuria in normal female age-matched controls (Table 3). In males, an age-dependent increase in urinary protein excretion developed in the control two-kidney normal rats that was similar to isograft males and higher than allograft males at 22 weeks (Table 3).

Moderate glomerular sclerosis developed in Series III male and female isograft rats given high-dose CsA that was slightly greater than that seen in age-matched controls (Fig. 1a). Male and female allograft rats exhibited similar and significantly more sclerotic glomeruli than the respective isograft groups. The Banff injury scale was similarly elevated in allograft males and females compared with isografts of both sexes (Fig. 1b).

Blood pressure was similar to normal age-matched controls in isograft females, but lower values were measured in female allograft rats. In the males, BP was lower in all Tx groups compared with time controls and this was particularly marked in the allograft groups (note that in the allograft groups the recipient was a Lewis rat). In all Tx groups of both sexes, there was hypertrophy of the single transplanted kidney compared with a normal kidney from age-

matched controls (Table 3), with no difference between isografts and allografts of each sex. The sex difference (male kidney larger) was preserved in all groups.

Renal constitutive NOS abundance was assessed in Series III and the changes were unremarkable. Following CsA treatment, renal cortical eNOS abundance was similar in male and female and isograft and allograft groups (Fig. 2). In kidney medulla, CsA-treated male kidneys exhibited higher abundance of eNOS than the females, although the difference between allograft females and males did not reach statistical significance ($P = 0.057$); no differences between isografts and allografts of the same sex were noted (Fig. 2). Cortical nNOS abundance was lower in male isograft kidneys than the other groups following CsA treatment. There was no difference in the abundance of medullary nNOS among the four groups (Fig. 2).

DISCUSSION

The main findings of the present study are that, in female allograft recipients, high-dose immunosuppressive therapy is required to prevent early graft loss. Our data demonstrate that the 1.5 mg/kg dose of CsA routinely used in this kidney Tx model for male rats as initial immunosuppression is not sufficient to adequately suppress acute rejection in females. Whether this is due to a requirement for higher absolute CsA levels in female tissue or due to enhanced CsA breakdown is not clear. Decreasing the ischaemia time prolonged survival in female rats given 1.5 mg/kg CsA, but accelerated CAN still developed 7 weeks post-Tx. Only when the CsA dose was doubled to 3 mg/kg did we see kidney survival in females and equivalent structural damage in allograft males and females at 22 weeks after Tx. The preserved structure in the isograft females on high-dose CsA suggests that the female kidney is not inherently more susceptible to the nephrotoxic actions of CsA.

Gender differences in kidney transplantation have largely been ignored. Our previous work using ovariectomized female recipients demonstrated a more rapid decline and earlier manifestation of CAN, especially when female donor kidneys were used.[5] Although female kidneys are smaller, the nephron number is not different between genders and the smaller glomerular volume of the females is likely to be beneficial.[8,10] In addition, the female kidney is more resistant to ischaemic injury,[19] confirmed in the present study, where female isograft groups showed similar or less marked functional/ structural impairement compared with isograft males. The reduction in ischaemia time in Series II was insufficient to achieve long-term kidney survival, suggesting that factors other than ischaemia–reperfusion contribute to the greater vulnerability observed in female allografts. Recent clinical analysis revealed a significant risk for chronic transplant failure when female kidney or heart grafts were used, indicating gender differences in graft immunogenicity.[20] Meier-Kriesche *et al*. found an increased risk of acute rejection in female kidney allograft recipients, suggesting more aggressive post-transplant immunological reactions of the host.[8] Using female donors and recipients in our present studies likely led to potentiation of both donor and recipient sex-dependent risk factors for CAN development. However, it should be emphasized that in many settings female sex and the presence of oestrogens is beneficial and reduces the rate of progression of CKD.[8-10] Thus, there is no overall increased risk of CAN in women who receive renal transplants.

Cyclosporine A is a known nephrotoxin and decreases renal blood flow and glomerular filtration rate.[21] In addition, CsA tissue distribution is gender and race dependent and female kidneys are reportedly more vulnerable to CsA nephrotoxicity.[7] However, the similar minimal injury seen in the isograft CsA-treated female and male rats argues against a greater susceptibility of the female kidney to CsA toxicity, which is also supported by the improved outcome of the female allograft in response to high- versus low-dose CsA. Cytochrome P450 3A4, responsible for the metabolism of CsA, exhibits higher activity in women than in men;

thus, pharmacokinetic differences between genders may explain why female allograft recipients require high-dose CsA.[22] Plasma CsA levels were not determined during the 10 day CsA treatment period in the present study and we cannot determine whether faster metabolism or a requirement for a greater absolute CsA level causes the higher dose requirement in females. The poor outcome in Series I and II females was likely due mainly to inadequate post-Tx immunosuppression (due either to accelerated CsA metabolism or a higher concentration requirement), corrected by increasing the CsA dose in Series III.

Significant experimental and clinical data support the hypothesis that progressive CKD is associated with decreased renal NO synthesis.[11-16] With regard to renal Tx, experimental NOS inhibition worsens injury, whereas L-arginine supplementation decreases renal damage.[13,23] The changes in renal NOS isoforms are dependent on the CKD model used. The impact on eNOS is highly variable, whereas nNOS is consistently reduced in 5/6 renal mass ablation/ infarction, chronic glomerulonephritis, chronic puromycin aminonucleoside nephrosis, the ageing male kidney and in the Zucker obese model of Type II diabetes.[15,16] Further, in the 5/6 renal mass ablation/ infarction model, we have observed a significant negative correlation between the severity of renal damage and decreased renal nNOS abundance.[16]

In the present study, we investigated the impact of CAN on renal eNOS and nNOS abundance in Tx rats studied in Series III. There was no consistent change in cortical or medullary nNOS abundance, except a slight decrease noted in isograft males. Thus, in this model, there was no relationship between nNOS abundance and glomerular injury. Of note, however, the most severely damaged kidneys in this group (female allograft) only exhibited approximately 20% glomerulosclerosis, the ‘cut-off’ to see declines in renal nNOS abundance in the 5/6 renal mass ablation/infarction model.[16]

In summary, female gender is a risk factor for post-Tx acute rejection and subsequent development of CAN. An increased dose of CsA was effective in females to suppress initial post-Tx immune reactions.

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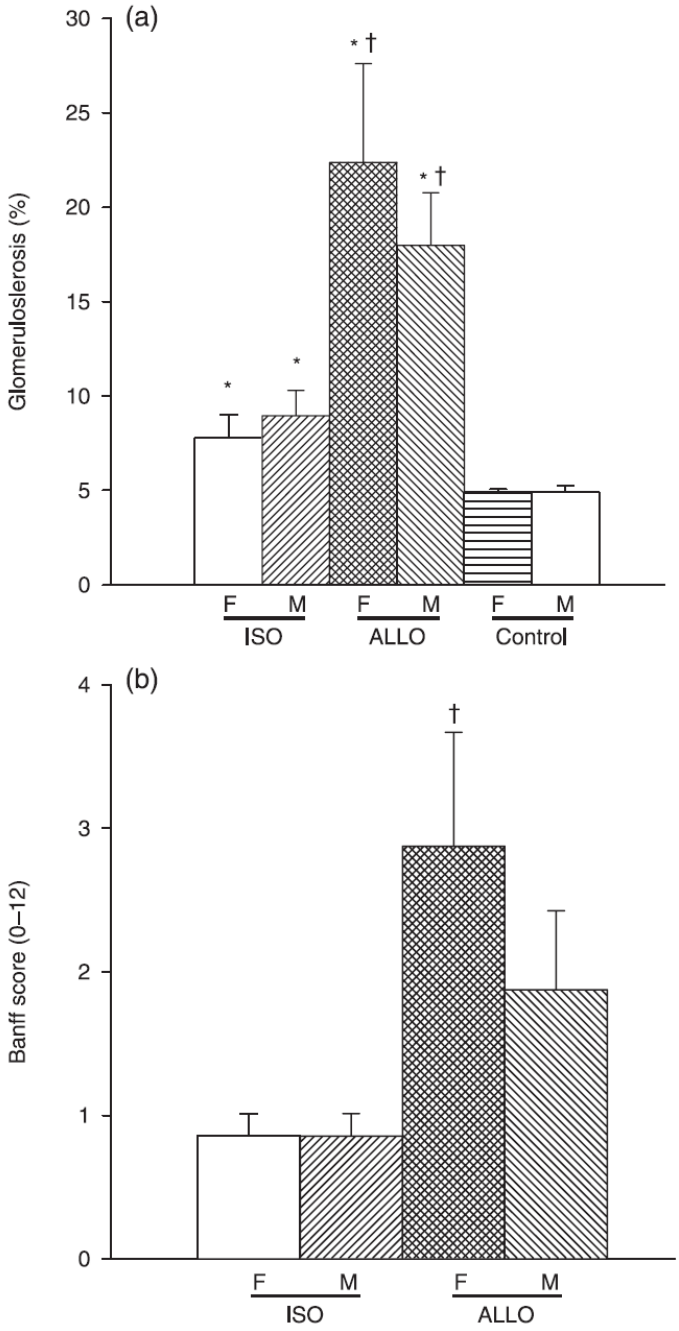

Fig. 1.

(a) Glomerulosclerois in isografts and allografts 22 weeks after transplantation in the cyclosporine A (CsA)-treated and control groups. (b) Banff score summarising glomerular, vascular, interstitial and tubular changes in isografts and allografts 22 weeks after transplantation in the CsA-treated groups. ISO, isograft; ALLO, allograft; F, female; M, male. Data are the mean±SEM. *$P < 0.05$ compared with the respective control; $^{\dagger}P < 0.05$ compared with the respective ISO group.

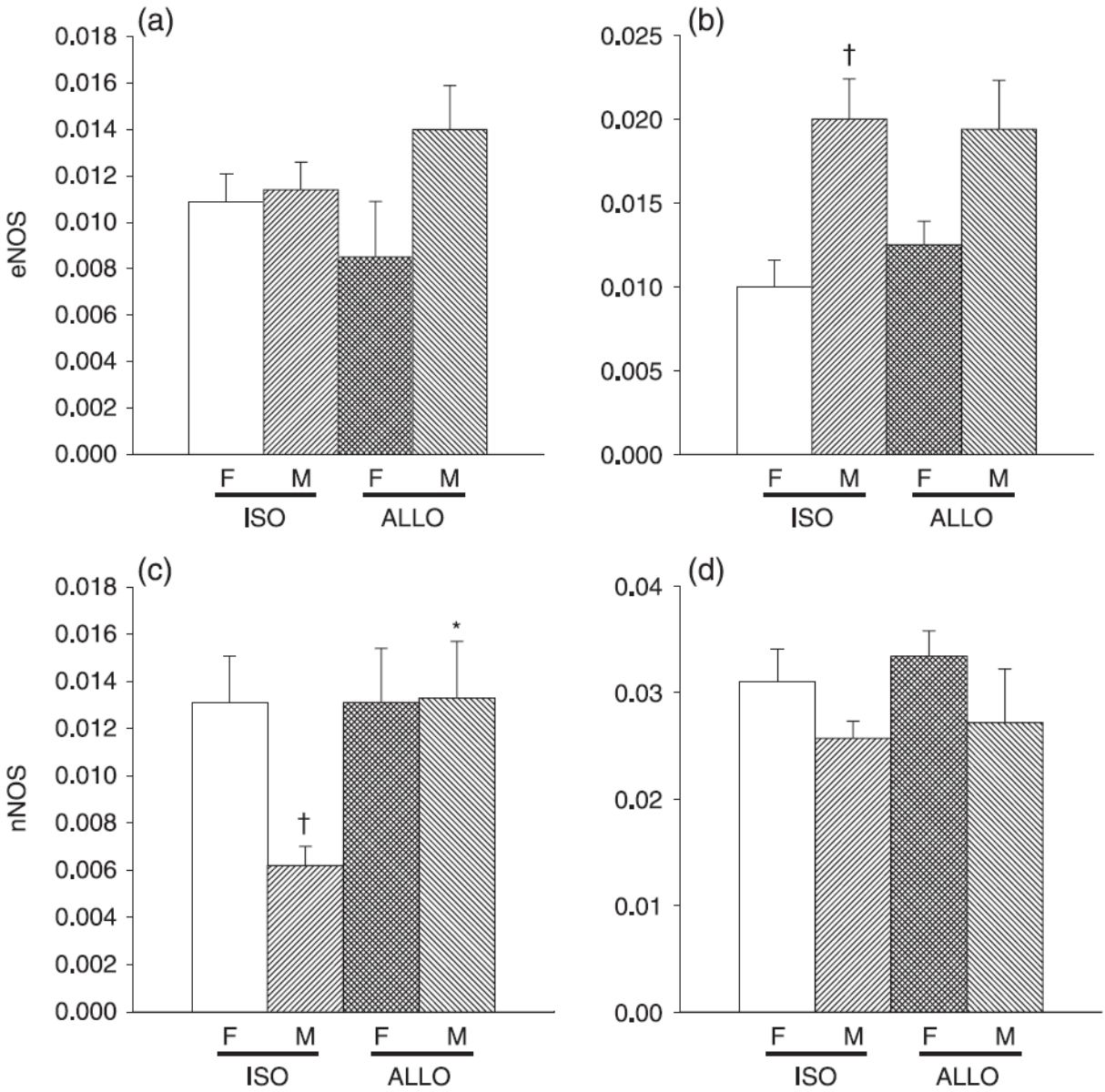

Fig. 2.

Renal (a,c) cortical and (b,d) medullary abundance of (a,b) endothelial nitric oxide synthase (eNOS) and (c,d) neuronal nitric oxide synthase (nNOS) protein in isografts and allografts 22 weeks after transplantation. ISO, isograft; ALLO, allograft; F, female; M, male. The abundance of NOS protein was calculated as integrated optical density (IOD) of nNOS or eNOS factored for Ponceau red stain (total protein loaded) and for a positive control and expressed as a percentage change from the respective control value. Protein abundance is represented as 'IOD/+control/ponceau'. Data are the mean±SEM. *$P < 0.05$ compared with the respective ISO group; †$P < 0.05$ compared with the respective female group.

Table 1

Experimental design

Series	No. ISO F	No. ISO M	No. ALLO F	No. ALLO M	Ischaemia time (min)	Immunosuppressive therapy	Follow up (weeks)
I	5	6	2†	6	45	1.5 mg/kg per day CsA	22
II	6	–	6	–	30	1.5 mg/kg per day CsA	7
III	7	7	8	8	30	3 mg/kg per day CsA	22
IV (controls)	F344 F (n = 7)		F344 M (n = 7)		–	–	22

†Only two rats of seven transplanted survived the 22 weeks follow up.

ISO, isograft; ALLO, allograft; F, female; M, male; CsA, cyclosporine A.

Table 2

Proteinuria and glomerulosclerosis in low-dose (1.5 mg/kg per day) cyclosporine A-treated groups

	Baseline proteinuria (mg/day)	Proteinuria (mg/day)	Banff score (0–12)
Series I			
ISO F	1.3 ± 0.3*	44.8 ± 24.1†	1.6 ± 0.4
ISO M	10.2 ± 1.9	67.5 ± 11.1†	1.7 ± 0.2
ALLO M	8.7 ± 0.7	65.5 ± 16.1†	7.7 ± 0.9*
Series II			
ISO F	2.0 ± 0.2	3.8 ± 0.9.	2.7 ± 1.0
ALLO F	2.7 ± 0.3	76.2 ± 33.0*†	8.7 ± 0.3‡

In Series I, an allograft female group was not included because only two of seven transplanted rats survived the follow up.

Data are the mean±SEM.

*$P < 0.05$ compared with ISO M;

†$P < 0.05$ compared with respective baseline values;

‡$P < 0.05$ compared with ISO F.

ISO, isograft; ALLO, allograft; F, female; M, male; CsA, cyclosporine A.

Table 3

Functional parameters in cyclosporine A-treated groups and controls 22 weeks after transplantation

Series III and IV	BW (g)	$U_{prot}V$ (mg/day)	PCr (mg/dL)	BUN (mg/dL)	Ccr (mL/min per kg BW)	BP (mmHg)	Tx (or left) weight (g)
ISO F	202 ± 2§	3.8 ± 0.5§	0.36 ± 0.01	22 ± 1*	6.9 ± 0.4	90 ± 5	1.12 ± 0.03*§
ISO M	418 ± 6*	43.7 ± 7.2	0.40 ± 0.01*	23 ± 1*	5.9 ± 0.5*	97 ± 3*	2.37 ± 0.12*
ALLO F	218 ± 2§	24.1 ± 9.3*†	0.44 ± 0.02*†	31 ± 1*†	5.1 ± 0.3*†	78 ± 3*†‡	1.31 ± 0.05*‡
ALLO M	451 ± 12†	15.5 ± 1.9*†	0.45 ± 0.04*	30 ± 1*†	5.4 ± 0.3*	63 ± 3*†	2.18 ± 0.10*
Control F	222 ± 4‡	3.3 ± 0.2‡	0.29 ± 0.01	16 ± 1	6.8 ± 0.5	99 ± 2‡	0.81 ± 0.02‡
Control M	456 ± 8	35.8 ± 4.3	0.30 ± 0.02	18 ± 2	7.3 ± 0.5	113 ± 3	1.84 ± 0.14

ISO, isograft; ALLO, allograft; F, female; M, male; BW, bodyweight; BP, blood pressure; $U_{prot}V$, total urine protein excretion; PCr, plasma creatinine level; Ccr, 24 h creatinine clearance; Tx, transplant kidney.

Data are the mean±SEM.

$^{*}P < 0.05$ compared with control F344 of respective gender;

$^{\dagger}P < 0.05$ compared with respective gender identical ISO;

$^{\ddagger}P < 0.05$ compared with respective male group.