see commentary on page 583

Amitriptyline attenuates interstitial inflammation and ameliorates the progression of renal fibrosis

Eduardo Achar[1,2,4], Thiago T. Maciel[1,4], Carlos F. Collares[2,3], Vicente P.C. Teixeira[1] and Nestor Schor[1]

[1]Nephrology Division, Departament of Medicine, Federal University of São Paulo (UNIFESP), São Paulo, Brazil; [2]Departament of Medicine, São Paulo City University (UNICID), São Paulo, Brazil and [3]São Paulo Poison Control Center, São Paulo, Brazil

Amitriptyline is a pleiotropic tricyclic antidepressant, which has anti-oxidant and anti-inflammatory properties. We tested whether amitriptyline might be useful in the treatment of chronic renal disease using the mouse model of unilateral ureteral obstruction. Amitriptyline caused a significant reduction of interstitial fibrosis, determined by Masson's staining, with minimal myofibroblast formation and macrophage infiltration following ureteral obstruction. Using quantitative PCR we found that this treatment significantly reduced the expression of key molecular markers of progressive tubulointerstitial injury such as osteopontin, MCP-1, ICAM-1, and TGF-β1 compared to their level in a saline-treated control group. Sublethal X-irradiation or mycophenolate mofetil, treatments that reduce inflammation, were comparable to amitriptyline in the reduction of interstitial fibrosis and macrophage infiltration. These studies in animals suggest that amitriptyline is worth testing as a therapeutic agent that might preserve renal function by blocking inflammation and renal fibrosis.

Kidney International (2009) **75,** 596–604; doi:10.1038/ki.2008.578; published online 26 November 2008

KEYWORDS: amitriptyline; inflammation; tubulointerstitial fibrosis; unilateral ureteral obstruction

Correspondence: *Nestor Schor, Nephrology Division, Department of Medicine, Federal University of São Paulo, Rua Botucatu, 740, São Paulo, 04023-900 SP, Brazil. E-mail: nestor@nefro.epm.br*

[4]These authors contributed equally to this work.

Received 6 February 2008; revised 16 September 2008; accepted 9 October 2008; published online 26 November 2008

Renal tubulointerstitial fibrosis is a common feature of chronic renal disease,[1,2] characterized by epithelial–mesenchymal transdifferentiation, accumulation of extracellular matrix, and mononuclear infiltration, leading to tubular cell loss and inflammation.[2,3] Unilateral ureteral obstruction (UUO) is a representative model of tubule interstitial renal fibrosis and inflammation, characterized by cellular and molecular events,[4,5] such as significant interstitial inflammatory cell infiltration, tubulointerstitial fibrosis, release of transforming growth factor-β1 (TGF-β1), upregulation of monocyte chemoattractant peptide (MCP-1),[6,7] osteopontin,[8] and intercellular adhesion molecule 1 (ICAM-1).[9]

Several drugs have been studied so far to ameliorate or even abolish interstitial fibrosis and inflammation during the progression of renal disease.[5,10–12] Attempts to avoid tubulointerstitial inflammation by imunosupression were successful to inhibit renal fibrosis. Rapamycin and mycophenolate mofetil (MMF), immunosuppressive agents, were described to improve the progression of injury elicited by UUO. However, cost and adverse effects caused difficulty in the establishment of an efficient therapy based on that approach.

Data from our group demonstrated that amitriptyline (AMT), a drug commonly used for many years for the treatment of depression and other conditions, facilitates the elimination of urinary calculi in felines and significantly ameliorates their renal function.[13] Although AMT is classified as a tricyclic antidepressant, its full range of actions is not fully known and not attributable solely to its antidepressant actions. AMT treatment reduces the pain caused by peripheral-nerve disease[14] and is also a potent H-1 receptor blocker that inhibits the release of histamine from mast cells *in vitro*.[15] In humans, analgesic effects of AMT were also associated to inhibition of noradrenaline and serotonin reuptake.[16] Additional benefits of AMT have been described in interstitial cystitis[17,18] and idiopathic cystitis.[19] Its role in inflammation has been addressed[20] and Obuchowicz *et al.*[21] showed that AMT alters cytokine activity, inhibiting the release of proinflamatory interleukin-1, whereas others described its effect increasing the release of immunosuppressive and anti-inflammatory interleukin-10.[22] AMT exhibits antioxidant properties *per se*,[23] inhibits natural killer cell activity[24] as well as nitric oxide, prostaglandin E_2, and

hialuronic acid production.[25] Taken together, these studies clearly demonstrate that AMT may have pleiotropic effects.

This study is the first attempt to examine the pathophysiological significance of AMT administration in a model of kidney disease. Based on literature data, we hypothesized that administration of AMT *in vivo* might ameliorates renal tubulointerstitial fibrosis after UUO.

RESULTS

Amitriptyline reduces interstitial fibrosis and macrophage influx after UUO

Nonobstructed contralateral kidneys from mice of both groups (UUO and UUO + AMT) did not show any morphological, cellular, or molecular alteration (data not shown). Kidneys from mice subjected to ureteral obstruction developed a severe tubulointerstitial injury consisting of tubular dilatation and atrophy, interstitial inflammation, and a marked interstitial fibrosis, characterized by interstitial extracellular matrix deposition. Glomeruli and vessels were well preserved. Analysis of interstitium by Masson's trichrome staining showed that UUO promoted intense deposition of extracellular matrix and consequently changes in renal morphology due to interstitial fibrosis. AMT administration attenuated interstitial fibrosis induced by UUO, preserving tubular morphology (Figure 1).

We also analyzed expression of key markers during the course of renal damaged induced by UUO. It is well described that following ureteral obstruction, tubular epithelial cells undergo epithelial–mesenchymal transdifferentiation, acquiring myofibroblast phenotype. This was assessed by immunohistochemical detection of αSMA, a marker of interstitial myofibroblast differentiation. Contralateral kidneys normally express αSMA of renal arterioles and mesangial cells inside glomeruli, and that was not influenced by UUO. Ureteral obstruction promoted an increase in the expression of αSMA starting 1 day after the procedure. Strong expression was observed 3 days after ureteral obstruction, maintained at high levels during the course of 10 days after the procedure. AMT administration significantly attenuated epithelial–mesenchymal transdifferentiation after UUO (Figure 2), with minimal αSMA expression observed after 1 and 3 days of intervention. Although an increased signal was observed 5 days after UUO, that was totally reversed at the end of 10 days, when αSMA expression and renal morphology was similar to contralateral kidneys and localized mainly in glomeruli and vessels (Figure 2).

Another key feature of ureteral obstruction injury is intense inflammatory response due to macrophage infiltration. Contralateral kidneys did not present any significant signal for macrophage infiltration, assessed by CD68 immunohistochemistry. As expected, ureteral obstruction in nontreated mice induced a severe interstitial inflammation due to macrophage infiltration in periglomerular and peritubular interstitium, which presented maximum signal at 5 days after the ureteral ligation, and was maintained intense after 10 days (Figure 3). Surprisingly, AMT administration promoted a significant effect, reducing macrophage infiltration and preventing interstitial inflammation (Figure 3). We observed CD68 staining only in a few periglomerular areas 5 days after ligation. However, the net effect of AMT administration was a significant inhibition of interstitial inflammation and preservation of renal morphology after ureteral obstruction.

Renal expression of TGF-β1, osteopontin, MCP-1, and ICAM-1 was attenuated by amitriptyline after UUO

As we observed inhibition of inflammatory response after AMT administration in UUO model, we proposed that AMT could attenuate renal injury induced by ureteral obstruction through inhibition of genes involved in development and progression of interstitial fibrosis. As demonstrated in Figure 4a, obstructed kidneys significantly express high levels of TGF-β1 (11.5 ± 1.0 fold change at day 5, $N = 6$). Administration of AMT reduced the expression of that profibrogenic cytokine (3.1 ± 1.1 fold change at day 5, $N = 6$, $P < 0.05$).

Several mechanisms seemed to be related to the reduction of macrophage infiltration. To better understand the mechanisms of AMT blockade on interstitial inflammation, we examined the mRNA levels of the typical macrophage chemokines osteopontin, MCP-1, and ICAM-1 by quantitative real-time PCR (qPCR).

As seen in Figure 4b, UUO promoted a significant time-dependent increase in osteopontin mRNA expression (maximum expression at day 5, 34.6 ± 2.0 fold change, $N = 6$) and that was suppressed by AMT treatment (6.7 ± 0.9 fold change at day 5, $N = 6$, $P < 0.01$). In our experimental model, UUO promoted a 40.5 ± 1.5 and 60.1 ± 2.8 fold change increase at days 5 and 10 ($N = 6$), respectively. AMT treatment decreased MCP-1 expression at day 5 (15.3 ± 1.1 fold change, $N = 6$, $P < 0.05$) and almost abolished it at day 10 (5.0 ± 0.5 fold change, $N = 6$, $P < 0.01$), which could explain the effect of AMT in the inhibition of macrophage infiltration (Figure 3). AMT treatment also promoted a significant inhibition of ICAM-1 gene expression after ureteral obstruction (18.5 ± 0.5 fold change in UUO group vs 4.1 ± 1.2 fold change in AMT group at day 5, $N = 6$, $P < 0.05$).

Comparison of AMT, mycophenolate mofetil, or X-irradiation on progression of renal injury triggered by UUO

To compare the effects of AMT treatment with other therapies suitable to suppress the inflammatory response observed during renal injury triggered by UUO, we employed the imunossupressor MMF and whole body X-irradiation (Xi). As demonstrated in Figure 5 and Table 1, MMF and Xi were able to inhibit (SMA expression 5 days after UUO. These results demonstrate the efficacy of both therapies to prevent epithelial-mesenchymal transition (EMT) and consequently interstitial fibrosis during the injury. Data from MMF and Xi therapy are also comparable to that acquired after administration of AMT, which reinforces the possible use of AMT to treat fibrotic renal injury. When we analyze the inflammatory process triggered UUO, due to macrophage

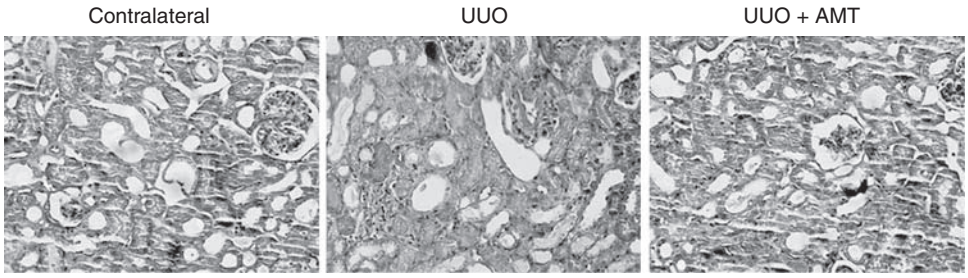

Figure 1 | Interstitial renal fibrosis. Sections of contralateral kidneys, nontreated obstructed kidneys (UUO), and amitriptyline-treated obstructed kidneys (UUO + AMT), stained with the Masson's trichrome stain, 5 days after the procedure. Each image was randomly acquired from the cortex area and is representative for 5 animals. Original magnification × 200.

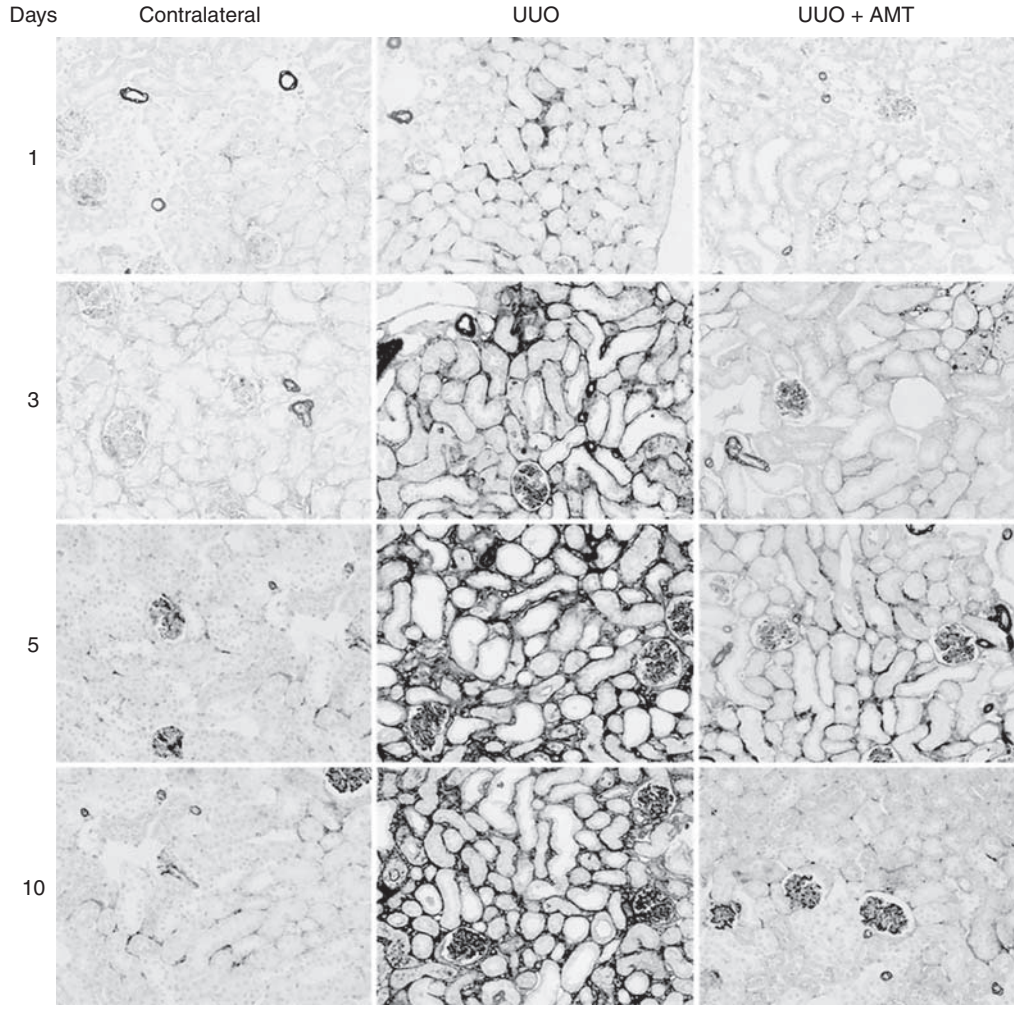

Figure 2 | α-Smooth muscle actin expression. Representative interstitial α-smooth muscle actin expression in contralateral kidneys, nontreated obstructed kidneys (UUO), and amitriptyline-treated obstructed kidneys (UUO + AMT) after 1, 3, 5, or 10 days of the procedure. Each image was randomly acquired from the cortex area and is representative for five animals. Original magnification × 200.

infiltration, the suppression induced by MMF and Xi were effective to avoid immune cell migration to injury site (Figure 6 and Table 2). None or few anti-CD68 stains can be observed in samples from UUO + MMF or UUO + Xi after 5 days. A weak signal is observed in AMT-treated obstructed kidneys, demonstrating that all therapies employed were efficient in avoiding tubulointerstitial fibrosis and inflammation.

We also tested the effects of all therapies (AMT, MMF, or Xi) on expression of genes involved in fibrosis development and tubulointerstitial inflammation in the UUO model, as described above. As demonstrated in Figure 7a, MMF was

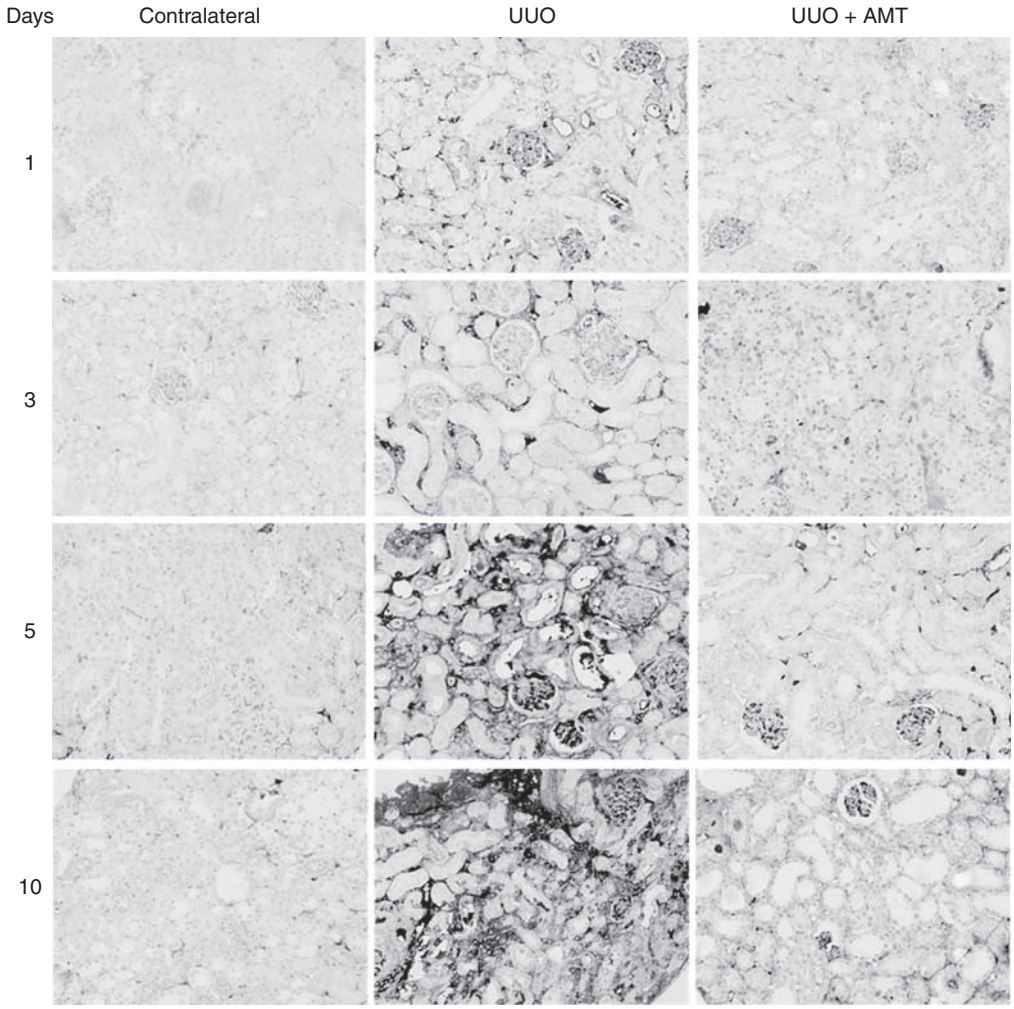

Figure 3 | Macrophage infiltration. Representative macrophage (CD-68 positive) infiltration in contralateral kidneys, nontreated obstructed kidneys (UUO), and amitriptyline-treated obstructed kidneys (UUO + AMT) after 1, 3, 5, or 10 days of the procedure. Each image was randomly acquired from the cortex area and is representative for five animals. Original magnification × 200.

able to significantly inhibit TGF-β1 expression 5 days after the procedure (3.9 ± 0.4 fold change, $N = 5$, $P < 0.05$). Similar results were obtained in Xi-obstructed kidneys (6.6 ± 0.2 fold change at day 5, $N = 5$, $P < 0.05$). When compared to AMT-treated obstructed kidneys data, TGF-β1 expression was lower than other therapies, suggesting that maybe AMT could present the best result in an attempt to inhibit TGF-β1. However, no statistically significant differences were observed between treatments ($P = 0.292$). Analysis of osteopontin expression (Figure 7b) revealed the same pattern obtained for TGF-β1: MMF and Xi-inhibited osteopontin mRNA levels (21.6 ± 0.9 and 13.4 ± 1.6 fold change at day 5, respectively, $N = 5$, $P < 0.05$). In that context, AMT gave the best results, with 69 and 51% reduction when compared to MMF or Xi, respectively ($P = 0.004$ between groups). qPCR data for MCP-1 mRNA levels revealed that MMF and Xi-treated mice presented low levels of MCP-1 24 h after the procedure (2.3 ± 1.6 and 3.7 ± 1.2 fold change, respectively, $N = 5$, $P < 0.05$), due to effective immunosuppression, versus 12.9 ± 0.9 fold change in AMT-treated obstructed kidneys and 13.0 ± 1.9 fold change in nontreated obstructed kidneys. Of note, during the progression of renal injury induced by UUO, MMF and Xi groups presented an increase in MCP-1 levels. This increment on MCP-1 expression was still significantly reduced in MMF-treated mice (20.1 ± 2.8 fold change at day 5, $P < 0.05$; Figure 7c). Xi mice did not show any difference between nontreated group ($P = 0.811$), whereas AMT group showed the lowest level of that gene, however, without significance when compared to MMF and Xi ($P = 0.375$). Analysis of ICAM-1 gene expression (Figure 7d) demonstrated a blockade induced by MMF and Xi (7.3 ± 1.3 and 8.6 ± 1.4 fold change at day 5, respectively, $N = 5$, $P = 0.01$), without statistical differences between the treatments ($P = 0.318$).

DISCUSSION

Progression of renal fibrosis induced by ureteral obstruction is characterized by a significant increase in expression of several molecules, including TGF-β1. It is well described that TGF-β1 enhances extracellular matrix protein synthesis

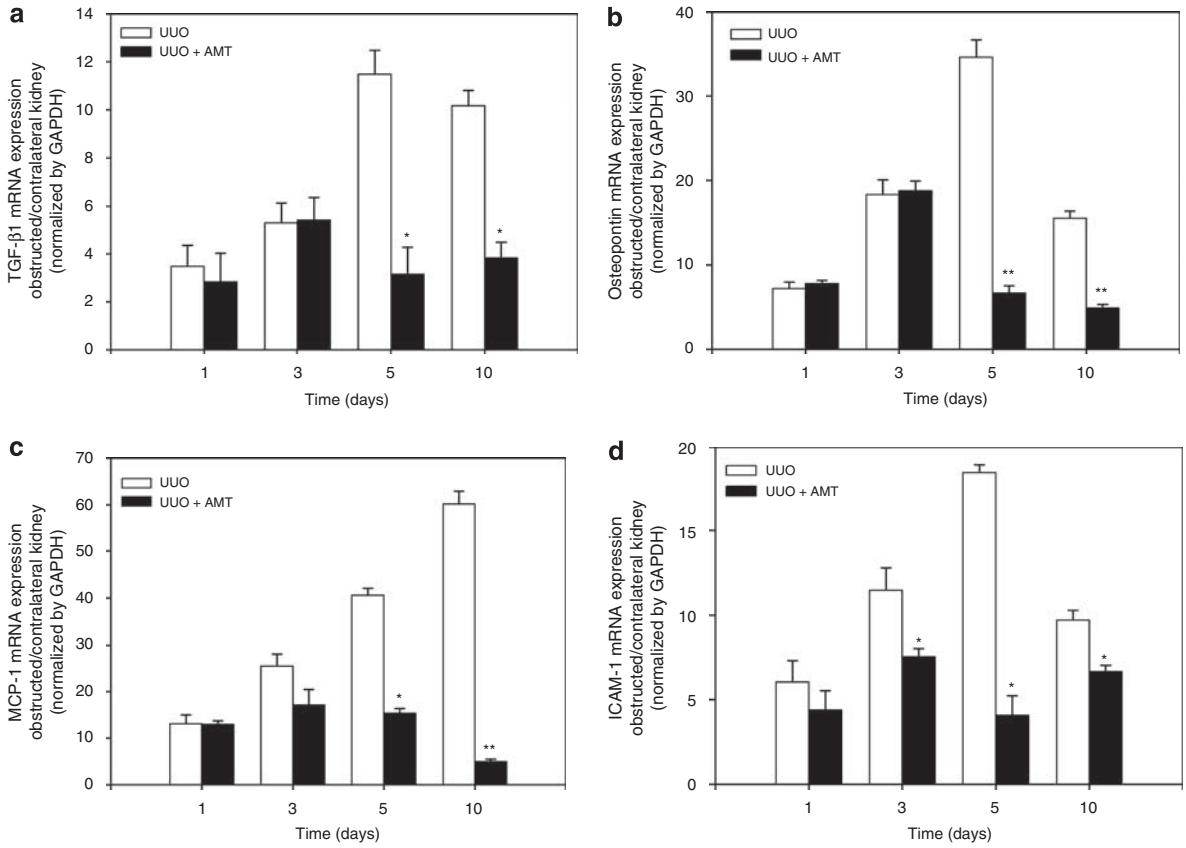

Figure 4 | Gene expression analysis. qPCR for TGF-β1 (**a**), osteopontin (**b**), MCP-1 (**c**), and ICAM-1 (**d**) in nontreated obstructed kidneys (UUO) and amitriptyline-treated obstructed kidneys (UUO + AMT). Data expressed as mRNA expression relation between obstructed/contralateral kidneys. Each bar represents the mean ± s.e.m. for at least five mice (*$P < 0.05$, **$P < 0.01$).

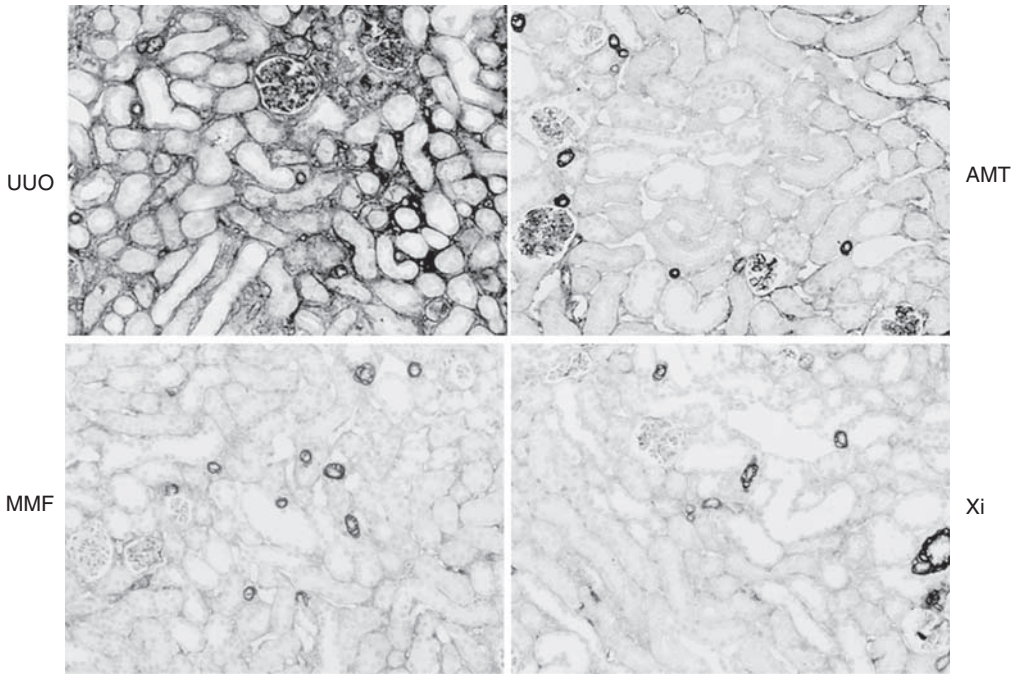

Figure 5 | α-Smooth muscle actin expression. Representative interstitial α-smooth muscle actin expression in nontreated obstructed kidneys (UUO), amitriptyline-treated obstructed kidneys (UUO + AMT), mycophenolate mofetil-treated obstructed kidneys (UUO + MMF), and X-irradiated obstructed kidneys (UUO + XI), after 5 days of the procedure. Each image was randomly acquired from the cortex area and is representative for five animals. Original magnification × 200.

Table 1 | Semiquantitative analysis of α-smooth muscle actin expression

	Myofibroblast (α-smooth muscle actin)					
	Obstructed kidney (days)			Contralateral (days)		
	1	3	5	1	3	5
UUO	+/++	++	++/+++	+	+	+
UUO+AMT	+	+	+	+	+	+
UUO+MMF	+	+	0/+	+	+	0/+
UUO+Xi	+	+	+	+	+	0/+

AMT, amitriptyline; MMF, mycophenolate mofetil; UUO, urinary unilateral obstruction; Xi, whole body X-irradiation.
Semi-quantitative analysis was performed by an independent pathologist. Expression of α-smooth muscle actin was scored as: rare (0), minimal (+), moderate (++) and intense (+++). At least 5 samples were processed for analysis.

Table 2 | Semiquantitative analysis of macrophage infiltration

	Macrophage infiltration (CD68+)					
	Obstructed kidney (days)			Contralateral (days)		
	1	3	5	1	3	5
UUO	++	++/+++	+++	0/+	+	0/+
UUO+AMT	+	+	+	+	+	+
UUO+MMF	+	+	+	+	0/+	+
UUO+Xi	+	+	+	+	+	+

AMT, amitriptyline; MMF, mycophenolate mofetil; UUO, urinary unilateral obstruction; Xi, whole body X-irradiation.
Semi-quantitative analysis was performed by an independent pathologist. Expression of CD-68 was scored as: rare (0), minimal (+), moderate (++), and intense (+++). At least five samples were processed for analysis.

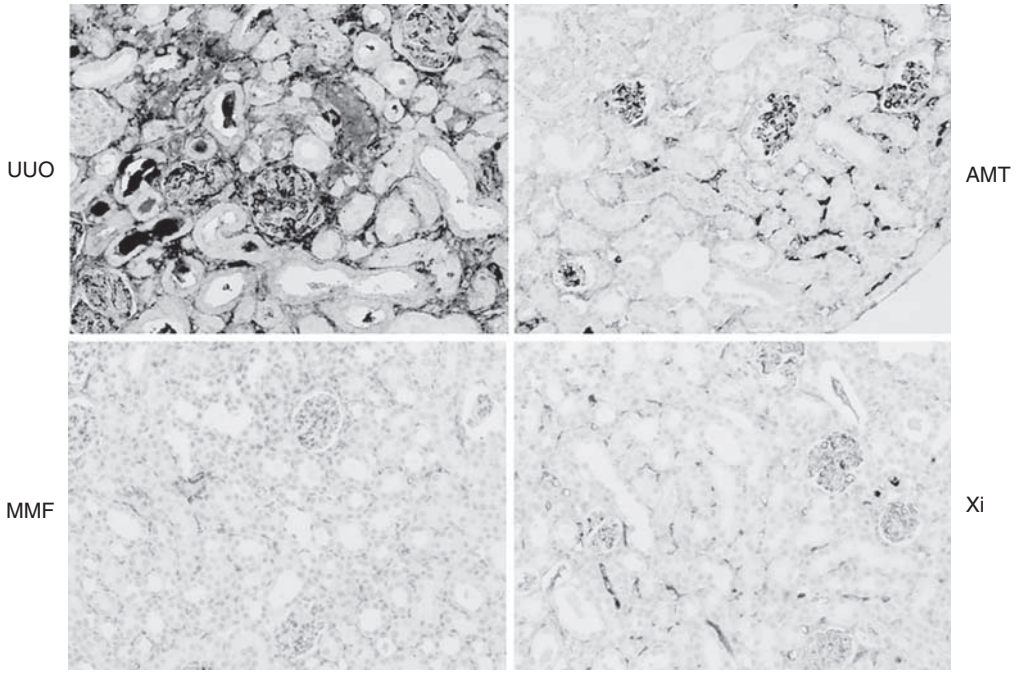

Figure 6 | Macrophage infiltration. Representative macrophage (CD-68 positive) infiltration in nontreated obstructed kidneys (UUO), amitriptyline-treated obstructed kidneys (UUO + AMT), mycophenolate mofetil-treated obstructed kidneys (UUO + MMF), and X-irradiated obstructed kidneys (UUO + XI), after 5 days of the procedure. Each image was randomly acquired from the cortex area and is representative for five animals. Original magnification × 200.

and deposition within interstitium.[26] TGF-β1 is also involved in epithelial–mesenchymal transdifferentiation, increasing expression of αSMA.[27,28] It is well established that UUO induce the expression of the mentioned inflammatory mediators. As we observed inhibition of inflammatory response after AMT administration in UUO model, we proposed that AMT could attenuate renal injury induced by ureteral obstruction through inhibition of genes involved in development and progression of interstitial fibrosis. As demonstrated in Figure 4a, obstructed kidneys significantly express high levels of TGF-β1 and administration of AMT reduced the expression of that profibrogenic cytokine. However, during the first days of injury AMT did not change TGF-β1 expression induced by UUO, which might explain the slight expression of αSMA observed after AMT treatment (Figure 2). We also tested the hypothesis that AMT could modulate epithelial–mesenchymal transdifferentiation. We employed human proximal tubular cells culture and AMT administration did not change αSMA expression induced by TGF-β1 (data not shown). Of note, in the unilateral ureteral ligation model, TGF-β1 is released mainly by tubular epithelial cells[29] and directly affects macrophages infiltration within interstitium.[30] As we observed, AMT significantly downregulated TGF-β1 and that could explain the inhibition of macrophage infiltration in this experimental model.

Several mechanisms seemed to be related to the reduction of macrophage infiltration. To better understand the mechanisms of AMT blockade on interstitial inflammation,

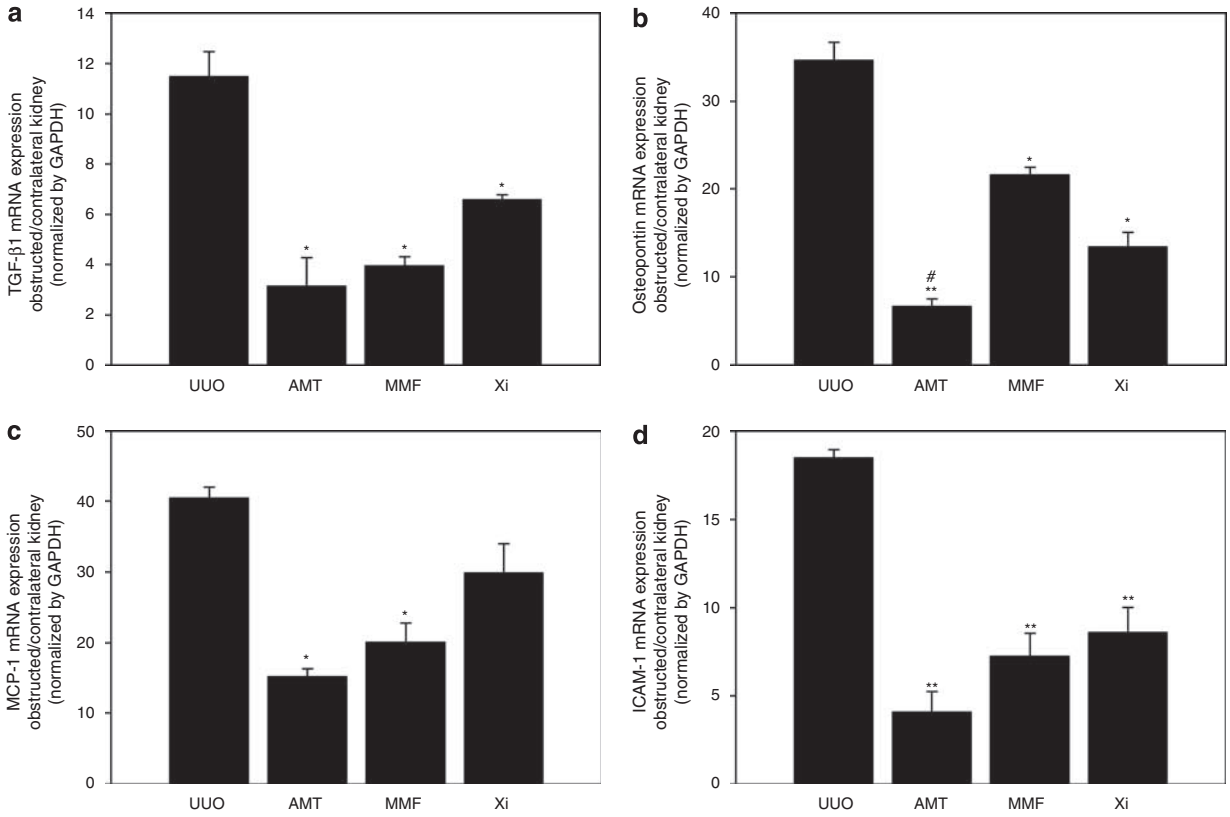

Figure 7 | Gene expression analysis. qPCR for TGF-β1 (**a**), osteopontin (**b**), MCP-1 (**c**), and ICAM-1 (**d**) in contralateral kidneys, nontreated obstructed kidneys (UUO), amitriptyline-treated obstructed kidneys (AMT), mycophenolate mofetil-treated obstructed kidneys (MMF), and X-irradiated obstructed kidneys (Xi), after 5 days of the procedure. Data expressed as mRNA expression relation between obstructed/contralateral kidneys. Each bar represents the mean ± s.e.m. for at least five mice (*$P<0.05$ and **$P<0.01$ vs nontreated obstructed kidneys, #$P<0.01$ between treatments).

we examined the mRNA levels of the typical macrophage chemokines osteopontin, MCP-1, and ICAM-1 by qPCR. Osteopontin is a phosphoprotein produced by the kidney that mediates cell adhesion and migration. Knockout mice studies demonstrated that osteopontin is a critical factor for interstitial macrophage accumulation.[31] As seen in Figure 4b, UUO promoted a significant time-dependent increase in osteopontin mRNA expression and that was suppressed by AMT treatment.

The most impressive data were obtained when we analyzed MCP-1 gene expression. Kitagawa *et al.*[7] demonstrated that MCP-1-deficient mice in CCR2 have attenuated tubular injury with a reduction in the expression of MCP-1, TGF-β, and collagen, and less infiltration of macrophages in the region and interstitial glomerular. In our experimental model, UUO promoted a significant increase of MCP-1 levels. Of interest, AMT treatment decreased MCP-1 expression at day 5 and almost abolished it at day 10, which could explain the effect of AMT in the inhibition of macrophage infiltration (Figure 3). It also deserves consideration the fact that Wolf *et al.*[32] have proposed a relation between MCP-1 and TGF-β1 in renal injury independent of macrophage recruitment. Thus, it is conceivable that AMT, through MCP-1 downregulation, avoid modulation on extracellular matrix compounds production by fibroblasts and activation of tubular cells to transdifferentiate into fibroblast-like cells.

In addition, we analyzed ICAM-1 expression along AMT treatment. ICAM-1 plays an important role in inflammatory processes and, in normal conditions, is constitutively expressed by capillary endothelial cells, glomerular mesangial cells, and renal tubular epithelial cells.[33] However, in response to stimuli from inflammatory mediators, expression of ICAM-1 can be markedly upregulated in mesangial and epithelial cells of the kidney.[34] AMT treatment also promoted a significant inhibition of ICAM-1 gene expression after ureteral obstruction. Although ICAM-1 by itself probably does not elicit an inflammatory response, decreased ICAM-1 expression might contribute to the benefic effect of AMT on interstitial fibrosis.

In this study, we found that administration of AMT ameliorates tubulointerstitial fibrosis developed after UUO. Macrophage infiltration, one of the key mechanisms involved in the progression of interstitial fibrosis, was suppressed by AMT. To highlight the effects of AMT in inhibiting macrophage infiltration, we compared its effects to MMF or Xi. The sublethal method of Xi employed in our study gives a reproducible transient monocytopenia and reduction in the renal macrophage number. MMF is an immuno-

suppressive agent that blocks the proliferation of activated B cells and T cells and decreases the expression of adhesion molecules.[35] It has been used widely with solid organ transplantation to prevent or reverse acute rejection.[36] Mycophenolic acid, the active metabolite of MMF, has been shown to inhibit the expression of adhesion molecules and the production of lymphocyte- and macrophage-derived cytokines and growth factors such as TGF-β1 and tumor necrosis factor-α.[37] Of interest, MMF has an anti-inflammatory effect mediated by inhibition of the production of lymphocyte and macrophage-derived growth factor TGF-β1[38] and also attenuates the progression of the fibrogenic process of UUO.[10]

Our data suggest that AMT might present better results of gene expression profile when compared to MMF administration or Xi (Figure 7). Although a weak signal is observed in animals treated with AMT, the fact that an antidepressive drug is able to avoid macrophage infiltration (when compared to nontreated obstructed kidneys) is of relevance to raise the possibility of using AMT to treat tubulointerstitial fibrosis and several other related injuries. All treatments successfully ameliorated the progression of renal injury induced by UUO. However, AMT takes advantage of MMF and Xi when cost and side effects were concerned.

Of note, AMT also elicited other effects. For instance, analgesia induced by AMT may account for some beneficial effects observed after UUO. Mice were active and did not lose appetite or weight, differently from nontreated mice. Tanda *et al.*[39] have recently demonstrated that histamine ameliorates antiglomerular basement membrane antibody-induced glomerulonephritis in rats, possibly due to immunomodulatory effects of H4 receptor agonism. Noteworthy, our findings suggest that the anti-inflammatory properties of AMT are independent of its antagonism on histaminergic receptors.

In conclusion, in this model of renal interstitial fibrosis, AMT significantly attenuated interstitial inflammation and fibrosis. These anti-inflammatory effects are greatly explained by the downregulation in osteopontin, MCP-1, and ICAM-1, and the decrease in interstitial fibrosis appears to result from less severe tubular phenotypical changes, myofibroblast differentiation, and renal TGF-β1 downregulation. We are currently engaged in define the cellular and molecular mechanisms involved in AMT blockade on interstitial fibrosis and inflammation. Due to the considerable number of patients treated with AMT in the past decades, its pharmacokinetics and adverse effect profiles are well recognized. Taken together, these elements suggest that AMT can be a potential candidate in the treatment of chronic renal disease.

MATERIALS AND METHODS

Urinary unilateral obstruction

Urinary unilateral obstruction model was induced in adult male C57BL/6 mice (3–5 months, 20–25 g) under pentobarbital-induced anesthesia. The right ureter was ligated with 4-0 silk at two locations and cut between ligatures to prevent urinary tract infection (obstructed kidney). Mice were given AMT (1 mg/Kg body weight/day; Merck Sharp Dhome Laboratories, São Paulo, Brazil) or saline solution by daily gastric gavage. Additional groups of mice with UUO ($n = 4$–6 per timepoint) received MMF (10 mg/Kg body weight/day; Sigma-Aldrich, St Louis, MO, USA) by daily gastric gavage or a solitary 6-Gy dose of whole body Xi, from a cobalt source (Discipline of Radiology, Federal University of São Paulo), with bilateral kidney shielding,[40] 7 days before ureteral ligation. Both obstructed and contralateral kidneys were harvested 1, 3, 5, or 10 days after surgery and either snap frozen in liquid nitrogen or fixed in buffered formalin. All procedures were approved by the Ethics Committee on Research from Federal University of Sao Paulo.

Immunohistochemistry

Kidneys fixed in buffered formalin were embedded in paraffin, sectioned (4 μm thick), and immunohistochemistry or Masson's trichrome stain (Sigma-Aldrich) were performed. The slides were processed for identification of macrophage infiltration (anti-CD68, clone KP1; Dako Cytomation, Carpinteria, CA, USA) and myofibroblast differentiation (anti-α-smooth muscle actin antibody, clone HHF35; Dako Cytomation), followed by diaminobenzidine detection system (Sigma-Aldrich). All samples were evaluated with an Olympus IX51 microscope (Olympus Inc., Miami, FL, USA). Images were acquired using a DP70 Digital Camera (Olympus Inc.) associated with DP Controller v1.2 software (Olympus Inc.). The semiquantitative evaluation of macrophagic population and interstitial myofibroblastic was done by and independent pathologist.

Quantitative real-time PCR analysis

RNA was extracted from crushed frozen tissue by homogenization in Trizol (Invitrogen, Carlsbad, CA, USA), and after DNAseI treatment and inhibition (Amersham Biosciences, Piscataway, NJ, USA), 1 μg-aliquots of RNA were used in a reverse transcription reaction with Improm-II reverse transcriptase (Promega, Madison, WI, USA). The resulting cDNA was used as template for qPCR analysis. Primers were obtained from Invitrogen. GAPDH: 5′-ACCACAGTCCATGCCATCAC-3′ (sense), 5′-TCCACCAC CCTGTTGCTGTA-3′ (antisense); ICAM-1: 5′-GTGATGCTC AGGTATCCATCCA-3′ (sense), 5′-CACAGTTCTCAAAGCAC AGCG-3′ (antisense); MCP-1: 5′-TTAACGCCCCACTCAC CTGCTG-3′ (sense), 5′-GCTTCTTTGGGACACCTGCTGC-3′ (antisense); osteopontin: 5′-TAGCTTGGCTTATGGACTGA GG-3′ (sense), 5′-AGACTCACCGCTCTTCATGTG-3′ (antisense); TGF-β1: 5′-ATCCTGTCCAAACTAAGGCTCG-3′ (sense), 5′-ACCTCTTTAGCATAGTAGTCCGC-3′ (antisense). Gene quantification was performed in duplicate on the Applied Biosystems 7700 Real-Time PCR System (Applied Biosystems, Warrington, UK). Quantification was obtained from a dilutional standard curve from a given sample. Data were normalized by GAPDH values, ratios between obstructed and contralateral kidneys were $\log_2$-

transformed (for linearization) and conventional paired '*t*' tests or ANOVA were performed. Results were expressed as fold changes in a log scale for convenience. Specificity of each reaction was confirmed by dissociation curve and electrophoresis.

DISCLOSURE

All the authors declared no competing interests.

REFERENCES

1. Klahr S, Schreiner G, Ichikawa I. The progression of renal disease. *N Engl J Med* 1988; **318**: 1657–1666.
2. Becker GJ, Hewitson TD. The role of tubulointerstitial injury in chronic renal failure. *Curr Opin Nephrol Hypertens* 2000; **9**: 133–138.
3. Klahr S, Morrissey J. Obstructive nephropathy and renal fibrosis. *Am J Physiol Renal Physiol* 2002; **283**: F861–F875.
4. Eddy AA. Molecular insights into renal interstitial fibrosis. *J Am Soc Nephrol* 1996; **7**: 2495–2508.
5. Klahr S, Morrissey JJ. Comparative study of ACE inhibitors and angiotensin II receptor antagonists in interstitial scarring. *Kidney Int Suppl* 1997; **63**: S111–S114.
6. Diamond JR, Kees-Folts D, Ding G *et al.* Macrophages, monocyte chemoattractant peptide-1, and TGF-beta 1 in experimental hydronephrosis. *Am J Physiol* 1994; **266**: F926–F933.
7. Kitagwa K, Wada T, Furuichi K *et al.* Blockade of CCR2 ameliorates progressive fibrosis in kidney. *Am J Pathol* 2009; **165**: 237–296.
8. Giachelli CM, Pichler R, Lombardi D *et al.* Osteopontin expression in angiotensin II-induced tubulointerstitial nephritis. *Kidney Int* 1994; **45**: 515–524.
9. Ricardo SD, Levinson ME, DeJoseph MR *et al.* Expression of adhesion molecules in rat renal cortex during experimental hydronephrosis. *Kidney Int* 1996; **50**: 2002–2010.
10. Goncalves RG, Biato MA, Colosimo RD *et al.* Effects of mycophenolate mofetil and lisinopril on collagen deposition in unilateral ureteral obstruction in rats. *Am J Nephrol* 2004; **24**: 527–536.
11. Vieira Jr JM, Mantovani E, Rodrigues LT *et al.* Simvastatin attenuates renal inflammation, tubular transdifferentiation and interstitial fibrosis in rats with unilateral ureteral obstruction. *Nephrol Dial Transplant* 2005; **20**: 1582–1591.
12. Wu MJ, Wen MC, Chiu YT *et al.* Rapamycin attenuates unilateral ureteral obstruction-induced renal fibrosis. *Kidney Int* 2006; **69**: 2029–2036.
13. Achar E, Achar RA, Paiva TB *et al.* Amitriptyline eliminates calculi through urinary tract smooth muscle relaxation. *Kidney Int* 2003; **64**: 1356–1364.
14. Max MB, Lynch SA, Muir J *et al.* Effects of desipramine, amitriptyline, and fluoxetine on pain in diabetic neuropathy. *N Engl J Med* 1992; **326**: 1250–1256.
15. Theoharides TC. The mast cell: a neuroimmunoendocrine master player. *Int J Tissue React* 1996; **18**: 1–21.
16. Mochizucki D. Serotonin and noradrenaline reuptake inhibitors in animal models of pain. *Hum Psychopharmacol* 2004; **19**(Suppl 1): S15–S19.
17. Hanno PM. Amitriptyline in the treatment of interstitial cystitis. *Urol Clin North Am* 1994; **21**: 89–91.
18. Moldwin RM, Sant GR. Interstitial cystitis: a pathophysiology and treatment update. *Clin Obstet Gynecol* 2002; **45**: 259–272.
19. Chew DJ, Buffington CA, Kendall MS *et al.* Amitriptyline treatment for severe recurrent idiopathic cystitis in cats. *J Am Vet Med Assoc* 1998; **213**: 1282–1286.
20. Abdel-Salam OM, Nofal SM, El-Shenawy SM. Evaluation of the anti-inflammatory and anti-nociceptive effects of different antidepressants in the rat. *Pharmacol Res* 2003; **48**: 157–165.
21. Obuchowicz E, Kowalski J, Labuzek K *et al.* Amitriptyline and nortriptyline inhibit interleukin-1 release by rat mixed glial and microglial cell cultures. *Int J Neuropsychopharmacol* 2006; **9**: 27–35.
22. Kubera M, Holan V, Mathison R *et al.* The effect of repeated amitriptyline and desipramine administration on cytokine release in C57BL/6 mice. *Psychoneuroendocrinology* 2000; **25**: 785–797.
23. Leduc C, Gentili ME, Estebe JP *et al.* The effect of local anesthetics and amitriptyline on peroxidation *in vivo* in an inflammatory rat model: preliminary reports. *Anesth Analg* 2002; **95**: 992–996, table of contents.
24. Xiao L, Eneroth P. Tricyclic antidepressants inhibit human natural killer cells. *Toxicol Appl Pharmacol* 1996; **137**: 157–162.
25. Yaron I, Shirazi I, Judovich R *et al.* Fluoxetine and amitriptyline inhibit nitric oxide, prostaglandin E2, and hyaluronic acid production in human synovial cells and synovial tissue cultures. *Arthritis Rheum* 1999; **42**: 2561–2568.
26. Chevalier RL. Molecular and cellular pathophysiology of obstructive nephropathy. *Pediatr Nephrol* 1999; **13**: 612–619.
27. Fan JM, Ng YY, Hill PA *et al.* Transforming growth factor-beta regulates tubular epithelial-myofibroblast transdifferentiation *in vitro*. *Kidney Int* 1999; **56**: 1455–1467.
28. Jinde K, Nikolic-Paterson DJ, Huang XR *et al.* Tubular phenotypic change in progressive tubulointerstitial fibrosis in human glomerulonephritis. *Am J Kidney Dis* 2001; **38**: 761–769.
29. Kaneto H, Morrissey J, Klahr S. Increased expression of TGF-beta 1 mRNA in the obstructed kidney of rats with unilateral ureteral ligation. *Kidney Int* 1993; **44**: 313–321.
30. El Chaar M, Chen J, Seshan SV *et al.* Effect of combination therapy with enalapril and the TGF-beta antagonist 1D11 in unilateral ureteral obstruction. *Am J Physiol Renal Physiol* 2007; **292**: F1291–F1301.
31. Persy VP, Verhulst A, Ysebaert DK *et al.* Reduced postischemic macrophage infiltration and interstitial fibrosis in osteopontin knockout mice. *Kidney Int* 2003; **63**: 543–553.
32. Wolf G, Jocks T, Zahner G *et al.* Existence of a regulatory loop between MCP-1 and TGF-beta in glomerular immune injury. *Am J Physiol Renal Physiol* 2002; **283**: F1075–F1084.
33. Brady HR. Leukocyte adhesion molecules and kidney diseases. *Kidney Int* 1994; **45**: 1285–1300.
34. Ishikura H, Takahashi C, Kanagawa K *et al.* Cytokine regulation of ICAM-1 expression on human renal tubular epithelial cells *in vitro*. *Transplantation* 1991; **51**: 1272–1275.
35. Fulton B, Markham A. Mycophenolate mofetil. A review of its pharmacodynamic and pharmacokinetic properties and clinical efficacy in renal transplantation. *Drugs* 1996; **51**: 278–298.
36. Hughes SE, Gruber SA. New immunosuppressive drugs in organ transplantation. *J Clin Pharmacol* 1996; **36**: 1081–1092.
37. Nadeau KC, Azuma H, Tilney NL. Sequential cytokine expression in renal allografts in rats immunosuppressed with maintenance cyclosporine or mycophenolate mofetil. *Transplantation* 1996; **62**: 1363–1366.
38. Bayazit AK, Bayazit Y, Noyan A *et al.* Comparison of mycophenolate mofetil and azathioprine in obstructive nephropathy. *Pediatr Nephrol* 2003; **18**: 100–104.
39. Tanda S, Mori Y, Kimura T *et al.* Histamine ameliorates anti-glomerular basement membrane antibody-induced glomerulonephritis in rats. *Kidney Int* 2007; **72**: 608–613.
40. Diamond JR, Pesek-Diamond I. Sublethal X-irradiation during acute puromycin nephrosis prevents late renal injury: role of macrophages. *Am J Physiol* 1991; **260**: F779–F786.