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Melatonin Prevents Cyclosporine-induced Nephrotoxicity in Isolated and Perfused Rat Kidney

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Cyclosporine A (CsA) is a potent and effective immunosuppressive agent, but its action is frequently accompanied by severe renal toxicity. The precise mechanism by which CsA causes renal injury is not known. Reactive oxygen species (ROS) have been shown to play a role, since CsA-induced renal lipid peroxidation is attenuated *in vivo* and *in vitro* by the concomitant administration of antioxidants such as vitamin E. We show here the effect of the antioxidant melatonin (MLT), a hormone produced by the pineal gland during the dark phase of the circadian cycle, in a model of CsA nephrotoxicity in the isolated and perfused rat kidney.

Kidneys isolated from rats were divided into seven groups. At the end of perfusion, malondialdehyde and 4-hydroxyalkenals (MDA + 4-HDA), metabolites of nitric oxide NO_2^- + NO_3^- were measured and histopathological examination was performed.

CsA treatment induced a significant increase in MDA + 4-HDA while not affecting the nitric oxide metabolite level. MLT remarkably prevented glomerular collapse and tubular damage as revealed by morphometric analysis.

Our study suggests that lipid peroxidation is an early important event in the pathogenesis of CsA nephrotoxicity and that MLT is able to protect kidneys from CsA at a relatively low concentration.

Keywords: Oxidative stress; Immunosuppressant; Antioxidant; Pineal hormone; Lipid peroxidation; Nitric oxide

INTRODUCTION

Cyclosporine A (CsA) is the standard immunotherapeutic drug in the prevention of organ allograft rejection. However, its use can lead to the development of many side effects, including nephrotoxicity.[1] Several studies have demonstrated that CsA causes vasoconstriction of the afferent and efferent glomerular arterioles[2] with a reduction in renal blood flow and glomerular filtration rate (GFR), and tubular damage.[3] There appears to be substantial impairment of endothelial cell function, leading to a reduced production of vasodilators (prostaglandins and nitric oxide) and an enhanced release of vasoconstrictors (endothelin and thromboxane).[2,4,5] However the exact mechanism of CsA-induced nephrotoxicity remains unclear. Some evidence suggests a possible role of free radicals in CsA toxicity *in vitro*[6,7] and *in vivo*.[8,9] CsA-induced functional and structural deterioration of the kidney was accompanied by renal lipid peroxidation and the administration of antioxidant agents attenuated renal injury.[10,11] The indole amine *N*-acetyl-5-methoxytryptamine or melatonin (MLT) is a good scavenger of hydroxyl radicals[12–14] and protects, in a concentration-dependent manner, against δ-aminolevulinic acid-induced lipid peroxidation in liver and in kidney, both *in vitro* and *in vivo*.[15] Intriguingly, Viljoen and co-workers[16] reported that in chronic renal failure (CRF) the circadian rhythm of MLT secretion is abnormal and is not restored by successful renal transplantation. In particular, the nocturnal rise in MLT levels was absent in all

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hemodialyzed ($n = 39/39$) and in 80% ($n = 43/54$) of the post-transplantation patients included in that study. Similar results were also obtained from studies performed in rats with CRF.[17]

MLT has been found to protect renal tissues against oxidative damage generated by a variety of toxic agents.[18,19] Furthermore, MLT has recently been found to protect against cyclosporine nephrotoxicity in rats.[20] While vitamin E, a well-known antioxidant, has been shown to protect isolated and perfused rat kidney from oxidative damage,[21] the role of melatonin as an antioxidant has not yet been investigated. The aim of this study was to evaluate the effect of melatonin administration on cyclosporine-induced acute nephrotoxicity in isolated and perfused rat kidney,[22] a model which allows to study the effect of the drug without any metabolic or neurohormonal interference.

MATERIALS AND METHODS

Experimental Design

The experiments were carried out on male Wistar rats (Morini, Italy) each weighing 300 ± 30 g. Rats were fed *ad libitum* with a standard rat chow (MIL Morini, Italy), with free access to water. Seven sets of experiments were performed with six rats each: control perfused kidneys, vehicle perfused kidneys (cremophor Sigma, MO, USA), CsA (Sandimmun-Sandoz, Switzerland) perfused kidney at a dose of 1 mg/l, MLT (Sigma, MO, USA) perfused kidney at the doses of 100 nM and 1 μM, and CsA + MLT 100 nM or 1 μM perfused kidney. After a stabilization period the perfusion was started according to the following time scheme.

Forty minutes of perfusion with Krebs Henseleit solution in control kidneys; 20 min with Krebs Henseleit solution plus 20 min with cremophor in vehicle perfused kidneys; 20 min with MLT (100 nM or 1 μM) plus 20 min with Krebs Henseleit solution in MLT perfused kidneys; 20 min Krebs Henseleit solution plus 20 min CsA (1 mg/l) in CsA perfused kidneys; 20 min with MLT (100 nM or 1 μM) plus 20 min CsA (1 mg/l) in CsA plus MLT perfused kidneys.

MLT infusion started during the stabilization period and continued for all the experiment. At the end of perfusion, samples of the eluate were collected for determination of $NO_2^- + NO_3^-$ and renal samples were analyzed for lipid peroxidation or paraffin-embedded for histological evaluation.

Operative Technique

The isolation and perfusion of the kidney were performed as previously described.[3] Briefly, kidneys were taken from the rats anesthetized by an intraperitoneal injection of thiopental (5 mg/100 g b.w.) and placed on a heated operating board to keep the body temperature near 38°C. The abdomen was opened by a midline incision and the intestine was moved to the left side and covered with moist warm saline pads. Loose ligatures were placed around the aorta below the left renal artery and above the mesenteric artery. The suprarenal artery was ligated and tied. Furthermore, loose ligature threads were placed around the left renal vessel and the mesenteric artery. The right kidney was freed from the perirenal fat, care being taken not to disrupt the renal capsule. Heparin (200 units, 0.2 ml) was injected into the right femoral vein. The mesenteric and left renal arteries were tied; the arterial catheter (PE, OD 1.6 mm, filled with 0.9% saline and closed at the distal end) was inserted into the abdominal aorta below the right kidney and tied. Perfusion was started *in situ*. The distal end of the catheter was connected with the perfusion system and opened; the aorta above the mesenteric artery was tied and then, a renal vein catheter (PE, OD 3 mm) was inserted into the right renal vein and also tied. Thereafter, all connections between the cannulated vessels and the animal were cut off and the animal was removed by lowering the operating table. The arterial catheter was inserted into the right renal artery and also tied. The kidney was transferred to a warm (37°C) water jacket chamber.

The perfusate entered the renal artery from a thermostated oxygenation reservoir. The driving force was provided by a roller pump (STA Peristaltic Pump 131900, DESAGA-Germany). Just proximal to the kidney, a side arm was connected to the perfusion circuit with a pressure transducer (Statham model P23AC, UK) and the perfusion pressure was maintained at 120 mm Hg. Renal perfusate flow and perfusion pressure were continuously monitored.

Perfusion Medium

The perfusate, continuously gassed with a mixture of 95% O_2 and 5% CO_2, consisted of a modified Krebs Henseleit bicarbonate buffer containing bovine serum albumin (fraction V) at a concentration of 7.5 g/ml, creatinine 1.5 mg/ml, urea 30 mg/ml, and an aminoacidic cocktail, as previously described.[23]

Lipid Peroxidation Evaluation

Renal cortical samples were analyzed for lipid peroxidation after homogenization with Polytron PT 10 for 20 s in ice cold 20 mM TRIS-HCl buffer, pH 7.4. Tissue homogenates were diluted in the sample buffer to approximately 10% (w/v) and then centrifuged at 3000*g* for 10 min at 4°C. Supernatants (200 μl) were used for the assay. A lipid peroxidation

assay kit purchased from Calbiochem (Italy) was used to measure malondialdehyde (MDA) and 4-hydroxyalkenal (4-HDA) end products of lipid peroxidation. This kit takes advantage of the chromogenic reagent *N*-methyl-2-phenylindole, which reacts with MDA and 4-HDA, yielding a stable chromophore that absorbs light at 586 nm. Readings were taken by using a Cary-13, Varian (Italy) spectrophotometer.

Lipid peroxidation was expressed as the normalized content of MDA and 4-HDA. The protein concentration was measured in accordance with the method of Lowry *et al*.[24]

NO_2^- + NO_3^- Determination

The stable $NO^\cdot$ end products, NO_2^-–NO_3^- in the eluate, were quantified colorimetrically as previously described.[25–27] Briefly, samples were incubated for 1 h at 37°C with *Escherichia coli* nitrate reductase, effecting complete reduction of NO_3^- in the sample to NO_2^-. NO_2^- in the sample was then quantified using the Griess reagent. Known concentrations of NO_2^- and NO_3^- were used as standards in each assay and each sample was run in duplicate. The NO_2^- measured in this way reflects the sum of NO_2^- and NO_3^- in the original sample.

Histology and Morphometry

At the end of perfusion, the kidneys were fixed in 10% phosphate buffered formalin and thereafter embedded in paraplast. Serial sections were cut by the same technician using the same microtome at thickness 4 and were stained with haematoxylin-eosine and periodic-acid Schiff (PAS). Individual volumes of 20 glomeruli were measured in these serial sections by using the Cavalieri principle.[28,29] Histological morphometry was performed using an image analysis system consisting of a personal computer equipped with a graphic tablet (Acecat II, Acecad) and scanner (Epson FilmScan 200). This system was programmed (Mocha Image Analysis Software, Jandel Scientific) to calculate the mean glomerular volume and the tuft area shape factor which is a measure of how nearly circular a given object is; a perfect circle has a shape factor of 1.000 and a line has a shape factor approaching 0.000. The histological sections were examined with a Leitz microscope (Germany), photographed, and the images were stored in the computer memory by the scanner until quantitative examinations were performed.

Statistical Analysis

Results were expressed as the mean ± standard error; the differences between groups were studied by one-way analysis of variance and by the Student–Neumann–Keuls' *t*-test. Statistical significance was defined as $p < 0.05$.

RESULTS

As shown in Fig. 1 CsA administration significantly increased the renal level of MDA + 4-HDA from 1.8 ± 0.2 mmol/mg protein in the control group to 6.3 ± 0.6 nmol/mg protein ($p < 0.05$). Pretreatment with MLT 1 μM significantly decreased CsA-induced MDA + 4-HDA production to 3.4 ± 0.3 nmol/mg protein. Lower MLT concentrations (100 nM) were not able to induce any significant change (6.0 ± 0.6 mmol/mg protein). This observation indicates that CsA-induced oxidative stress, expressed by the significant increase in the level of lipid peroxidation by-products MDA + 4-HDA, is decreased by the administration of MLT 1μM.

NO_2^- + NO_3^- levels in the eluate were higher in CsA-perfused kidneys than in controls (2.61 ± 0.05 vs 0.18 ± 0.02 μM). Pretreatment with MLT did not affect this parameter (Fig. 2).

Histological analysis of kidney specimens showed the typical pattern of nephrotoxicity with glomerular

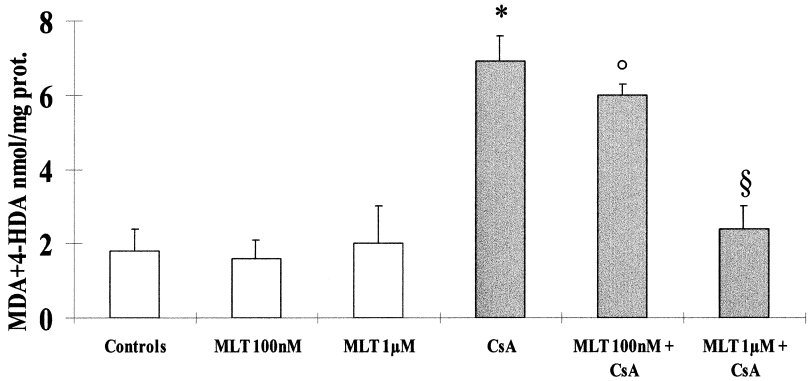

FIGURE 1 MDA + 4-HDA in isolated rat kidney perfused with CsA and different doses of MLT. MLT 1μM significantly reduces lipid peroxidation induced by CsA (*$p < 0.01$ vs control groups; °$p < 0.01$ vs control groups and n.s. vs CsA alone; §$p < 0.05$ vs CsA alone and vs control groups).

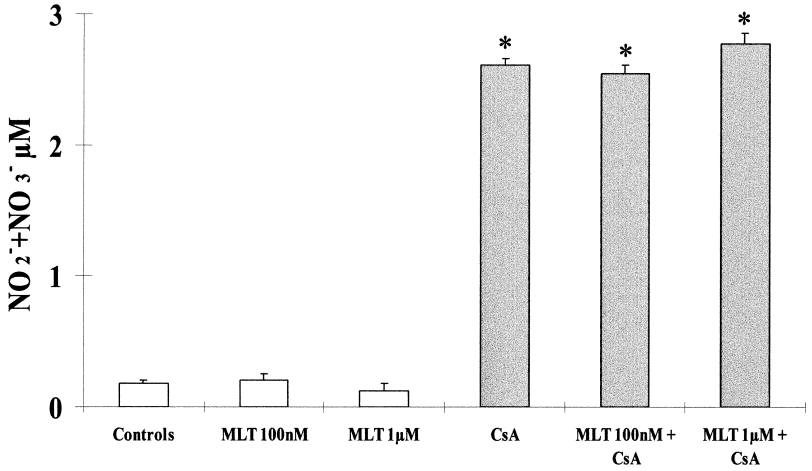

FIGURE 2 $NO_2^- + NO_3^-$ in the eluate of isolated rat kidneys perfused with CsA and different doses of MLT. CsA induces a significant increase in $NO_2^- + NO_3^-$ with respect to the controls. Pretreatment with MLT did not affect this parameter ($*p < 0.01$ vs control groups).

collapse and widespread tubular damage in the CsA-treated group (Fig. 3, panel B). Specimens from kidneys treated with 1 μM MLT alone (Fig. 3, panel C) did not show any significant difference from those of controls treated with saline (Fig. 3, panel A). Treatment with CsA + 1 μM MLT (Fig. 3, panel D) clearly prevented the tubular damage and the glomerular collapse observed in the CsA-treated group. No significant effect was observed in a specimen obtained from a kidney treated with 100 nM MLT (data not shown). These results indicate that the antioxidant action of MLT protects renal tissue from the morphological alteration induced by CsA. Mean glomerular volume was significantly lower in the CsA-treated group ($38,765 \pm 3113\ \mu m^3$) vs controls ($60,320 \pm 4338\ \mu m^3$). Figure 4 shows that pretreatment with 1 μM MLT, but not with 100 nM, preserved the normal volume ($50,321 \pm 4500$ and $40,235 \pm 3865\ \mu m^3$, respectively). The tuft area shape factor confirmed the glomerular tuft collapse in the CsA-treated group (0.49 ± 0.08 vs 0.83 ± 0.07 in the control group) and the protective effect of the higher dose of MLT (0.69 ± 0.07, $p < 0.05$) (Fig. 5).

DISCUSSION

The exact mechanism by which treatment with CsA induces nephrotoxicity is not completely understood. As extensively reviewed by Ischikawa *et al.*[30] many drugs may cause nephrotoxicity either directly or by increasing free radical generation. Experimental evidence has suggested that oxygen free radicals are also involved as

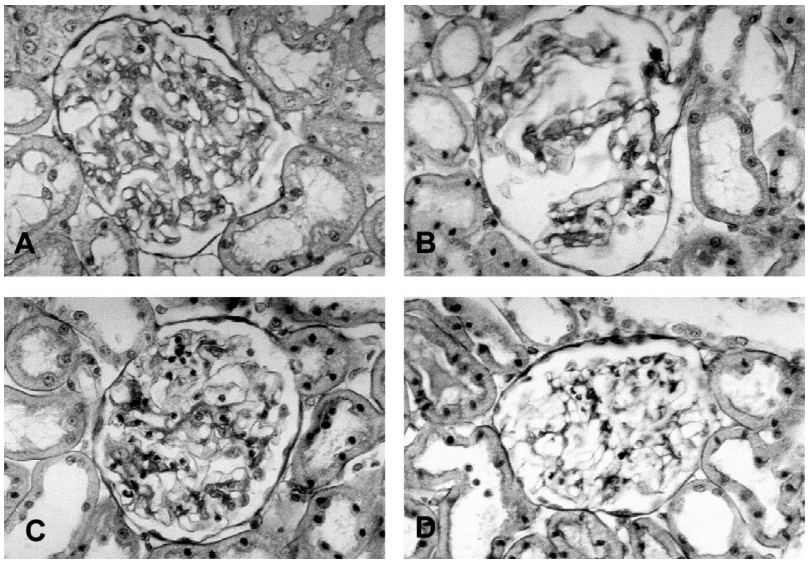

FIGURE 3 Light microscopy (PAS × 650) of isolated rat kidneys perfused with CsA and of MLT 1 μM. The typical pattern of nephrotoxicity with glomerular collapse and widespread tubular damage in the CsA-treated group is shown in panel B. The specimen from a kidney treated with 1 μM MLT alone (panel C) did not show any significant difference from that of the control treated with saline (panel A). Treatment with CsA + 1 μM MLT (panel D) clearly prevented the tubular damage and the glomerular collapse observed in the CsA-treated group. No significant effect was observed in specimens obtained from kidneys treated with 100 nM MLT (data not shown).

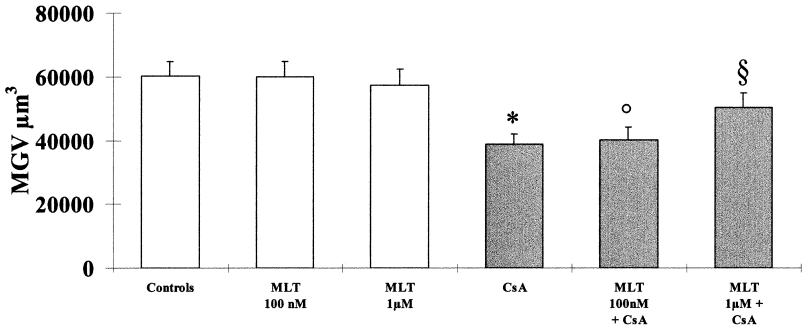

FIGURE 4 Mean glomerular volume (MGV) of isolated rat kidneys perfused with CsA and different doses of MLT. CsA induces a significant glomerular collapse with respect to the controls. Pretreatment with MLT 1 µM but not 100 nM preserved normal volume ($*p < 0.01$ vs control goups; $°p < 0.01$ vs control groups and p n.s. vs CsA alone; $§p < 0.01$ vs CsA alone, CsA + MLT 100 nM and p n.s. vs controls).

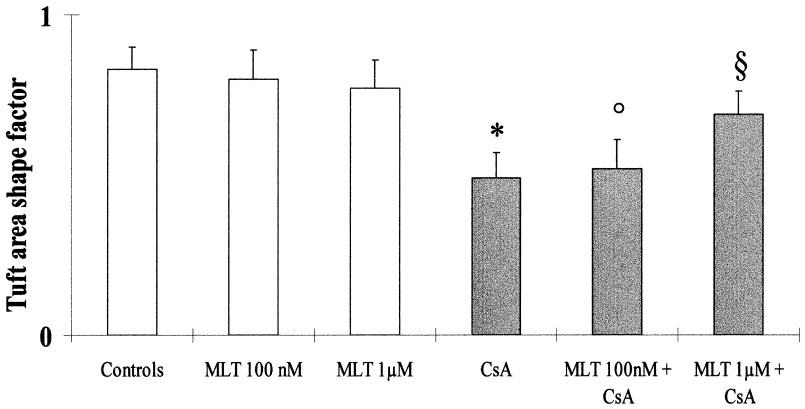

FIGURE 5 Tuft area shape factor (TSF) of isolated rat kidneys perfused with CsA and different doses of MLT. The shape factor is a measure of how nearly circular a given object is; a perfect circle has a shape factor of 1.000 and a line has a shape factor approaching 0.000. TSF confirmed the glomerular tuft collapse in the CsA-treated group) and the protective effect of the higher dose of MLT ($*p < 0.01$ vs control goups; $°p < 0.01$ vs control groups and p n.s. vs CsA alone; $§p < 0.01$ vs CsA alone, CsA + MLT 100 nM and p n.s. vs controls).

mediators of CsA nephrotoxicity. Some studies have reported a protective effect of free radical scavengers and antioxidants against CsA-induced renal lipid peroxidation. In a previous study in the IPRK we highlighted that CsA directly induces lipid peroxidation after a few minutes of perfusion.[31]

CsA-induced oxidative damage may be critically important in patients with CRF undergoing renal transplantation. Both predialytic and dialytic CRF are conditions associated with a pro-oxidative status because of impairment of endogenous antioxidant systems and increased free radical generation.[32] Studies in humans and in rats have shown alterations in the circadian rhythm of MLT secretion in CRF with a suppression of the nocturnal rise of this hormone. Neither hemodialysis nor kidney transplantation are able to restore this dysfunction.[33] Interestingly, Nava *et al.*[34] reported that *in vivo* MLT pretreatment prevented the increase in renal content of MDA and the decrease in GSH levels resulting from $HgCl_2$ toxicity. Furthermore, Kumar *et al.*[20] have recently shown that *in vivo* MLT administration to rats has a protective effect against CsA-induced nephrotoxicity.

Our present data, which are in agreement with MLT protection of renal function after *in vivo* administration to animals, clearly show that MLT action indeed occurs at the renal level. Perfusion of isolated kidney with MLT remarkably resulted in less tissue damage by oxygen free radical formation. MLT has recently been shown to protect different tissues[35] and cells[36–39] from oxidative damage due to the generation of highly toxic reactive oxygen intermediates. Furthermore, Montilla *et al.*[40] reported that MLT acts in the kidney as a very powerful scavenger of free radicals by preventing the biochemical alterations induced by adriamycin.

In the present study MLT, when administered in the isolated rat kidney, significantly protects renal injury by decreasing the lipid peroxidation produced by the administration of CsA. We conclude that the beneficial effects of MLT are due to its antioxidant properties exerted in the kidney.

Despite an action through MLT receptors, which are localized at renal level, cannot be excluded at the moment, our interpretation of an antioxidant role for MLT is substantiated by similar results obtained in previous studies performed with a different antioxidant.[31]

CsA administration was also associated with an increased production of vasoconstrictor factors such as endothelin and thromboxane,[2,4] or inhibition of relaxing factors such as $NO^{\cdot}$.[5]

In our experimental model CsA-induced histological alterations comparable with the reported acute nephrotoxicity *in vivo*, as revealed by morphometric analysis, are associated with a significant increase in tissue lipid peroxidation and in $NO^{\cdot}$ metabolite concentration in the eluate.

An impairment of $NO^{\cdot}$ release has been proposed as one of the most important factors in the pathogenesis of CsA-induced nephrotoxicity. Previous studies have reported that CsA administration induces structural damage to endothelial cells in culture[41] and impairment of endothelium-dependent vasorelaxation *in vitro*.[42] Finally, Wu *et al.*[43] have reported that CsA down-regulated the production of $NO^{\cdot}$ in cultures of rat medullary thick ascending limb cells (mTAL) expressing inducible NOS mRNA. However, these findings are in contrast with the increased activity of the $NO^{\cdot}$ system in the kidney exposed to CsA reported by other studies. CsA acute administration in freshly isolated glomeruli of rats enhances cGMP generation by a mechanism involving the endothelin B receptor; moreover it has been demonstrated that CsA up-regulates endothelial nitric oxide synthase expression both in cell cultures[44,45] and in transplant recipients.[46] Intriguingly, these authors suggested that reactive oxygen species (ROS) may participate in the regulation of eNOS gene expression in these conditions.

It is known that $NO_2^- + NO_3^-$ reflect $NO^{\cdot}$ production. However, $NO^{\cdot}$ may react with superoxide radical to give peroxynitrite, which spontaneously gives NO_3^-.[47]

MLT predominantly neutralizes hydroxyradicals, the most reactive and noxious free radical. This may explain the protective effect on CsA-induced nephrotoxicity and the lack of modifications on $NO_2^- + NO_3^-$ levels.

The direct involvement of CsA in inducing ROS generation in cultured human endothelial cells, has recently been demonstrated in our laboratories.[48] We have shown that in a single endothelial cell the generation of oxidative stress by CsA leads to modifications of the cell morphology. Although a direct change in $NO^{\cdot}$ release needs to be further investigated, the ROS-induced change in cell shape seems to be a good candidate for the endothelial dysfunction characteristic of CsA-induced nephrotoxicity.

In conclusion, our study suggests that lipid peroxidation is an important early event in the pathogenesis of CsA nephrotoxicity and that MLT is able to protect kidneys from CsA at a relatively low concentration.

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