



## Sirolimus in Kidney Transplant Donors and Clinical and Histologic Improvement in Recipients: Rat Model

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### ABSTRACT

**Background.** Ischemia-reperfusion (I/R) injury is one of the risk factors for delayed graft function, acute rejection episodes, and impaired long-term allograft survival after kidney transplantation. This antigen-independent inflammatory process produces tissue damage. Isogeneic transplantation in a rat model is a useful method for study of nonimmunologic risk factors for kidney damage.

**Objective.** To study the effect of sirolimus on I/R injury using only 1 dose of the drug in the donor.

**Materials and Methods.** Eighteen rats were allocated to 3 groups of 6 rats each: sham group, control group, and rapamycin group.

**Results.** Improved renal function and systemic inflammatory response were observed in the rapamycin group compared with the control group ( $\Delta$ urea,  $\Delta$ creatinine, and  $\Delta$ C3, all  $P < .01$ ). The number of apoptotic nuclei in the renal medulla in the control group was higher than in the rapamycin group ( $P < .01$ ). Tubular damage was less severe in the rapamycin group compared with the control group ( $P < .01$ ). Complement 3 and tumor necrosis factor- $\alpha$  expression in the kidney samples were significantly decreased when rapamycin was given to the donor rats ( $P > .01$ ). Bcl-2 protein was upregulated in the rapamycin group compared with the control group ( $P < .01$ ).

**Conclusion.** Administration of rapamycin in donors attenuates the I/R injury process after kidney transplantation in a rat model.

**I**SCHEMIA-REPERFUSION (I/R) injury, an important cause of early allograft dysfunction, is associated with an increased incidence of acute rejection episodes and decreased long-term allograft survival.<sup>1,2</sup> Ischemia followed by reperfusion has key implications for the pathogenesis of early graft damage. Ischemia is the sum of a possibly transient warm interval before or during organ removal from the donor, and cold ischemia is used for organ preservation and storage. Reperfusion, which is critical to organ viability, may amplify ischemic damage.<sup>3</sup>

Rapamycin is a potent immunosuppression drug that acts by inhibiting proliferation and clonal expansion of IL-2-stimulated T cells via blockade of kinase, which is necessary for cell cycle progression.<sup>4,5</sup>

Isogeneic transplantation in a rat model is the most widely used method for study of nonimmunologic risk factors for kidney damage. Complement 3 (C3) is an inflammatory mediator involved in I/R injury.<sup>6-11</sup> Apoptosis has potentially conflicting effects on transplanted kid-

neys, improving allograft survival via resolution of inflammation or leading to progression of tubular atrophy and renal fibrosis via loss of parenchymal cells.<sup>12,13</sup> This dual role of apoptosis in renal I/R injury may also provide a window of therapeutic opportunity.<sup>14</sup> Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), a proinflammatory cytokine that is upregulated in I/R injury, is involved in the extrinsic apoptotic pathway.<sup>15,16</sup> Bcl-2 and Bax are antiapoptotic and proapop-

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otic genes, respectively, that are involved in the intrinsic apoptotic pathway.<sup>17</sup>

The primary objective of the present study was to examine the effect of donor treatment with rapamycin on the I/R injury process using a model of isogeneic transplantation in rats. The secondary objective was to evaluate the effect of donor treatment with rapamycin on C3 systemic levels and apoptotic pathways.

## MATERIALS AND METHODS

Eighteen isogeneic male adult Wistar rats were randomly allocated to 1 of 3 groups of 6 rats each: a sham group; a control group, in which no immunosuppression was administered; and a rapamycin group, in which 2 mg/kg of the drug was administered via oral gavage in the donor rats 6 to 12 hours before surgery. Drug dosage and route of administration were chosen on the basis of previous reports.<sup>18</sup> Recipient rats did not receive rapamycin posttransplantation. Rats were maintained in an air-conditioned environment at 68°F to 75°F (20°C–24°C) with 12-hour light and dark cycles.

### Methods

Twenty-four hours before and after transplantation, blood urea and creatinine concentrations and C3 complement fraction were determined using UV kinetic and colorimetric-kinetic assays and radial immunodiffusion, respectively.

Donors and recipients were premedicated with atropine, 0.01 mg/kg; buprenorphine, 0.04 mg/kg; and diazepam, 10 mg/kg. Ten minutes later donors, they were anesthetized with ketamine, 100 mg/kg. In donor rats, the left kidney was removed and washed and 3 mL of Ringer lactate solution at 4°C via puncture through the aorta. The vascular pedicle was sectioned at its origin on the renal vein, with an aortic cuff on the renal artery. The ureter was sectioned near the kidney pole, and the organ was stored in the same wash solution for 3 hours. In recipient rats, after bilateral nephrectomy, the infrarenal abdominal vessels were dissected to implant the kidney using an end-to-side technique between the renal vein and the cava and the aortas of the donor and recipient. The ureter was anastomosed using an end-to-end technique. The recipient rats were sacrificed 24 hours after surgery to recover the kidneys for histologic analysis.

### Histologic Analysis

The pathologic samples were analyzed by a pathologist (P.G.) blinded to the experimental source. The kidneys were fixed in a 10% neutral buffered-formalin solution, embedded in paraffin, and cut into 4- $\mu$ m sections for staining with hematoxylin-eosin. The renal sections were scored in blinded fashion for grade of cortical tubular epithelial necrosis. Counts were performed on at least 10 square micrometer fields and scored for severity of necrosis on a scale of 1 to 5, as follows: 1, 0% to 5%; 2, 6% to 25%; 3, 25% to 50%; 4, 50% to 75%; and 5, more than 75%.<sup>19</sup>

The TUNEL (terminal deoxynucleotidyl-transferase-mediated 2'-deoxyuridine 5'-triphosphate-biotin nick-end labeling) assay was performed, in essence, according to the manufacturer's instructions (Apoptag; ONCOR, Inc, Gaithersburg, Maryland). In brief, deparaffinized 4- $\mu$ m sections of paraffin-embedded tissue were pretreated with 20  $\mu$ L/mL of proteinase K (DAKO; Glostrup, Denmark) for 30 minutes at 37°C. After washing, the sections were incubated with digoxigenin-labeled deoxyuridine in the presence of deoxynucleotidyl transferase. After the enzymatic reaction was

blocked, sections were incubated with a specific peroxidase-labeled antidigoxin antibody. Peroxidase was then reduced using 0.05 diaminobenzidine (Sigma, St. Louis, Mo) in 0.1 mL/L of phosphate-buffered saline solution (PBS), pH 7.6, containing 1% water. After washing, the sections were lightly stained with hematoxylin.

Negative control reactions were performed for each reaction step. They were obtained by omitting the terminal deoxynucleotidyl transferase, antidigoxin antibody, and peroxidase substrate. Positive controls included sections of paraffin-embedded lymphoma of human origin.

### Immunocytochemistry

Four-micrometer sections were applied to slides coated with poly-L-lysine. The sections were deparaffinized using xylene, dehydrated through graded alcohols and water, and immersed in 0.3% vol/vol water in methanol for 30 minutes to block endogenous peroxidases. Antigens were reduced by microwaving at 750 W for 15 minutes in 0.01 mol/L of trisodium citrate buffer, pH 6.0, before rinsing in PBS and blocking nonspecific binding with 10% equine serum in PBS.

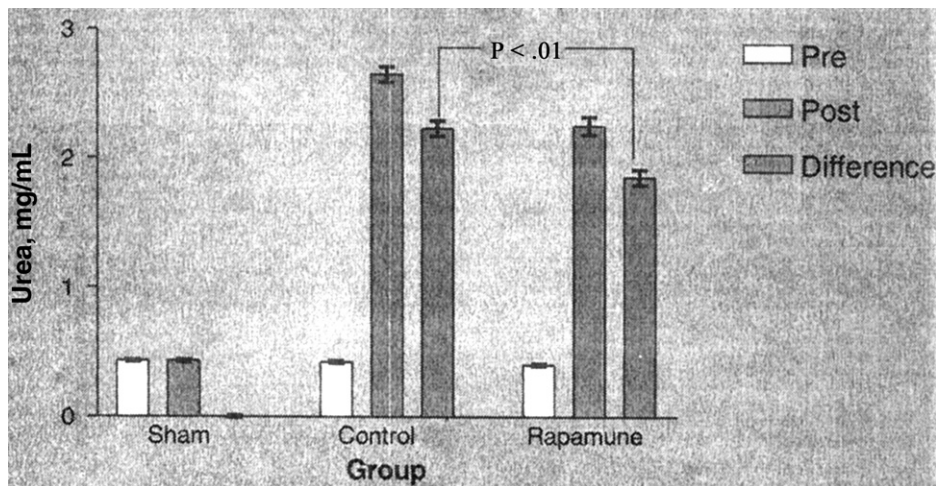
The sections were incubated with primary monoclonal antibodies against C3, polyclonal goat anti-TNF- $\alpha$ , polyclonal anti-Bcl-2 rabbit IgG, or polyclonal anti-Bax rabbit IgG (all from Santa Cruz Biotechnology, Santa Cruz, California). After the sections were rinsed with PBS, they were incubated with avidin-biotin horseradish peroxidase complex according to the manufacturer's instructions (Vectastain, Universal Quick Kits; Vector Laboratories Ltd, Peterborough, England). Peroxides were visualized by incubating the sections in 3,3'-diaminobenzidine (Sigma) with hydrogen peroxide. Negative control experiments were performed by omitting incubation with the primary antibodies. Expression of C3, TNF- $\alpha$ , Bcl-2, and Bax was calculated by assessing 10 consecutive cortical and medullary fields using a semiquantitative scale of 1 to 5, as follows: 1, 0% to 5%; 2, 6% to 25%; 3, 26% to 50%; 4, 51% to 75%; and 5, more than 75%. Each score by field reflected the extension rather than the intensity of the marking, depending on the percentage of tubulointerstitial area affected.

### Statistical Analysis

Quantitative variables were analyzed using the *t* test, or the Cochran test when heteroscedasticity was detected. For nonparametric variables, the Mann-Whitney test used. *P* < .05 (2-tailed) was considered significant.

## RESULTS

The sham group demonstrated normal C3, urea, and creatinine values. At 24 hours posttransplantation, recipient rats whose donors were treated with rapamycin exhibited lower urea and creatinine concentrations compared with the control group. Mean (SE)  $\Delta$ urea values were 2.23 (0.06) mg/dL in the control group compared with 1.85 (0.06) mg/dL in the rapamycin group (*P* < .01) (Fig 1).  $\Delta$ Creatinine values were 4.69 (0.55) mg/dL in the control group compared with 2.13 (0.04) mg/dL in the rapamycin group (*P* < .01) (Fig 2). Twenty-four hours posttransplantation, blood C3 levels were decreased in the rapamycin group:  $\Delta$ C3, 495 (38.62) in the control group compared with 166 (23.33) in the rapamycin group (*P* < .01) (Fig 3).



**Fig 1.** Mean (SE) urea values and mean  $\Delta$  values (difference 24 hours after surgery) in the 3 groups. The difference between the control and rapamycin (Rapamune) groups was statistically significant ( $P < .01$ ).

**Histologic Analysis**

Significant improvement in acute tubular necrosis and apoptosis in recipient rats was observed after administration of rapamycin in the donors. There was a marked decrease in acute tubular necrosis score (median [maximum] values) in the rapamycin group compared with the control group: 2.2 (2–3) vs 4 (3–5) ( $P < .01$ ) (Fig 4).

In the rapamycin group, apoptotic nuclei in the outer and inner medullae were significantly decreased compared with the control group at 24 hours posttransplantation. Apoptotic nuclei scores were 138 (10.12) in the control group compared with 22.3 (1.84) in the rapamycin group ( $P < .01$ ) (Fig 5).

**Immunohistochemistry**

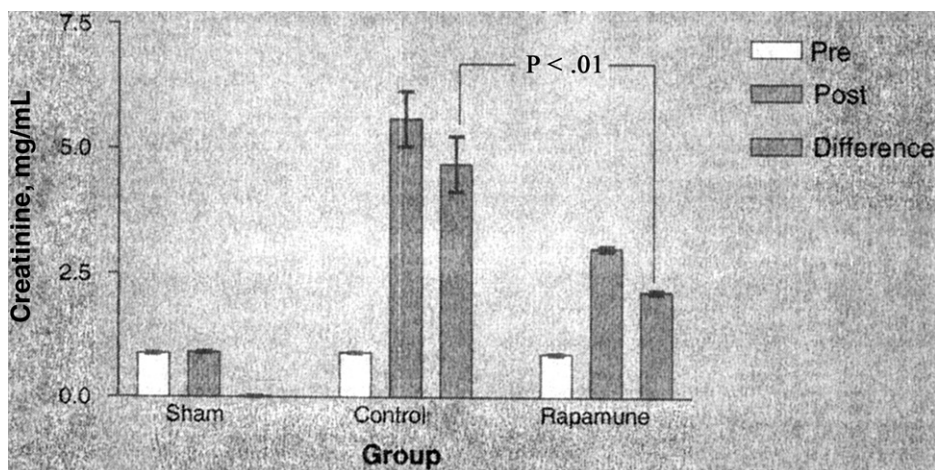
Expression of C3 and TNF- $\alpha$  in kidney samples was upregulated in the outer and inner medullae in the control group. The presence of these inflammatory mediators in situ was significantly decreased when rapamycin was given to the donors: C3, 3.3 (3–4) in the control group vs 1.0 (0–2) in the rapamycin group; and TNF- $\alpha$ , 3.67 (3–4) in the control

group vs 1.5 (1–2) in the rapamycin group ( $P < .01$ ). The control and rapamycin groups demonstrated low Bax in situ expression in the apoptotic intrinsic pathway. In both groups, Bcl-2 expression in the outer and inner medullae was increased compared with the sham group. Moreover, Bcl-2 expression in situ in the outer and inner medullae was higher in the rapamycin group compared with the control group: 4.00 (3–5) vs 1.83 (1–2) ( $P < .01$ ).

**DISCUSSION**

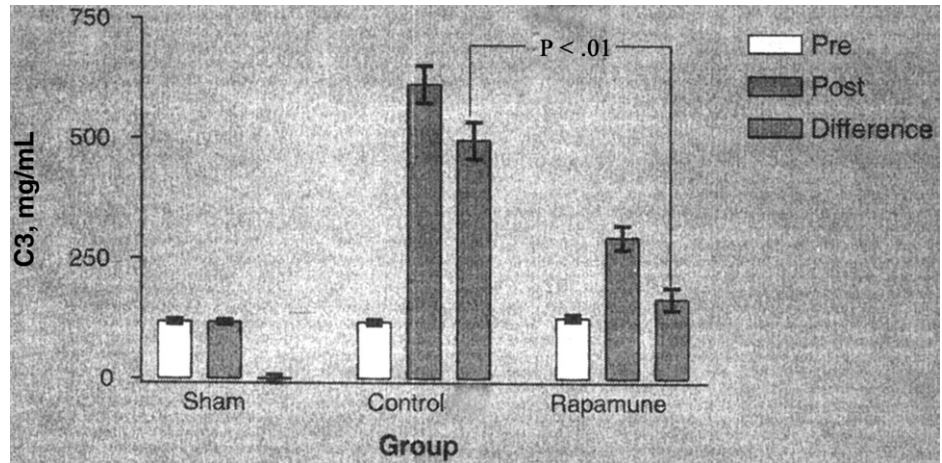
Donor preconditioning with rapamycin, 2 mg/kg, was renoprotective for subsequent function of syngeneic kidney transplants. Both necrosis and apoptosis associated with I/R injury were attenuated when only 1 dose of rapamycin was administered to donors 6 to 12 hours before kidney retrieval.

This model might be clinically relevant because it simulates transplantation-associated I/R events in human beings. We previously reported that preconditioning with rapamycin protected again I/R injury in an autotransplantation kidney model in rats.<sup>20</sup> In the present model, we



**Fig 2.** Mean (SE) creatinine values and mean  $\Delta$  values (difference 24 hours after surgery) in the 3 groups. The difference between the control and rapamycin (Rapamune) groups was statistically significant ( $P < .01$ ).

**Fig 3.** Mean (SE) C3 values and mean  $\Delta$  values (difference 24 hours after surgery) in the 3 groups. The difference between the control and rapamycin (Rapamune) groups was statistically significant ( $P < .01$ ).

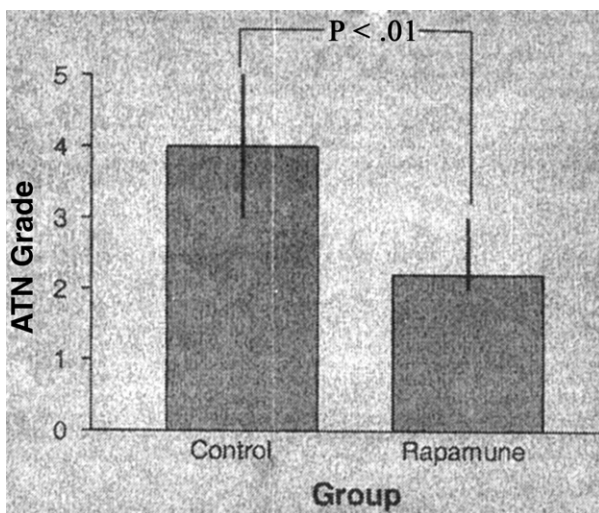


reproduced the sequence in deceased donors: removal from the donor, washing, cold storage, and reperfusion in the recipient. Cold ischemia for 3 hours was sufficient to cause more than 50% involvement with acute tubular necrosis in the control group. Therefore, the time used in this model was considered appropriate to reproduce I/R injury, which is in accord with the 2-hour cold ischemia time used by Reutzel-Selke et al.<sup>21</sup> Induction of apoptotic cell death after I/R has been observed in animal models including I/R injury to the liver, heart, brain, and kidney.<sup>22-25</sup>

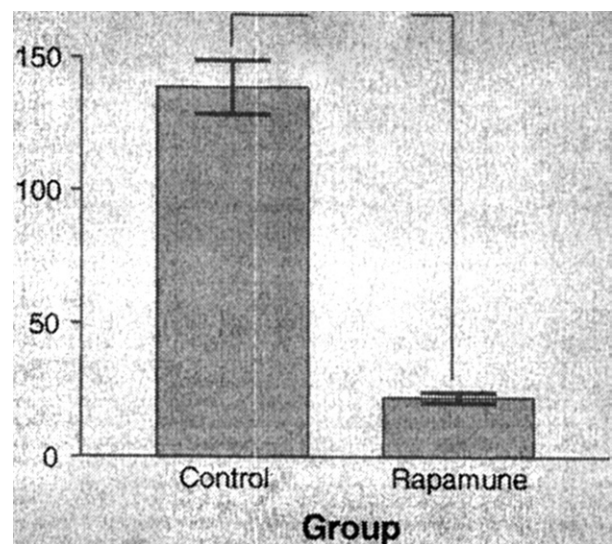
Rapamycin therapy in donors was associated with significantly decreased urea and creatinine concentrations in recipients at 24 hours posttransplantation. In addition, both necrosis and apoptosis were also decreased in treated recipients. Tubular damage was less severe in the rapamycin group than in the control group. Also, the number of

apoptotic nuclei in tubular epithelial cells in the inner and outer medullae was significantly reduced.

We evaluated both the intrinsic and extrinsic apoptotic pathways. Tumor necrosis factor- $\alpha$  is an inflammatory mediator that induces apoptosis in renal epithelial cells. The cytokine is involved in the extrinsic pathway.<sup>16</sup> Apoptosis is triggered by the interaction of TNF with the corresponding receptors (TNFR1 or Fas), which finally leads to activation of intranuclear endonucleases that cleave nuclear DNA and triggers cellular apoptosis. Tumor necrosis factor promotes neutrophil and monocyte migration toward the kidney, increasing expression of adhesion molecules such as intercellular adhesion molecule-1 and L-selectin.<sup>26</sup> In situ expression of TNF- $\alpha$  was decreased when donor rats received rapamycin before kidney retrieval. Moreover, we evaluated the intrinsic pathway with



**Fig 4.** Median (minimum-maximum; black lines) acute tubular necrosis (ATN) grade in control and rapamycin (Rapamune) groups. The difference between these groups was statistically significant ( $P < .01$ ).



**Fig 5.** Mean (SE) apoptotic nuclei values in the control and rapamycin (Rapamune) groups. The difference between these groups was statistically significant ( $P < .01$ ).

pro and antiapoptotic genes. Compared with the sham group, both the control and rapamycin groups demonstrated increased Bcl-2 expression in the inner and outer medullae. This may be a protective mechanism in medullary epithelium in the presence of cortical necrosis. Patches of Bcl-2 expression in the inner and outer medullae and cortex may represent a regenerative mechanism following ischemic cortical tubular necrosis. Furthermore, high expression of Bcl-2 in situ with lower levels of apoptosis in the rapamycin group compared with the control group may determine tubular cell protection by modulating the apoptotic intrinsic pathway. As recently demonstrated by Isaka et al,<sup>27</sup> Bcl-2 protects tubular epithelial cells from I/R injury. Yang et al<sup>28</sup> observed decreased apoptosis associated with use of tacrolimus, mycophenolate mofetil, and rapamycin in a warm ischemia model. However, these drugs were given after the surgical procedure and not before, as in the present study. In contrast, Serr et al<sup>29</sup> administered sirolimus before and after the surgical procedure and demonstrated increased apoptosis. Although rapamycin may delay kidney recovery from acute renal failure,<sup>30</sup> in our model, it attenuated the inflammatory process and improved renal function in the recipient rats. Rapamycin administered in the donor before transplantation may reduce subsequent inflammation associated with I/R injury; however, it should not be used in the presence of acute tubular necrosis after renal transplantation. Loverre et al<sup>31</sup> demonstrated that rapamycin can promote a proinflammatory state.

The complement cascade consists of sequential activation of more than 30 proteins. The damaging effects of complement depend primarily on cleavage of C3, which is the central component to which all activation pathways converge.<sup>32</sup> Activation may occur via the mannose-binding lectin pathway as well as via the alternative pathway in kidney transplantation.<sup>33–36</sup> Production of C3 by epithelial tubular cells contributes to the inflammatory process in kidney transplantation.<sup>34,35</sup> It is an essential part of the process that ends with synthesis of the membrane attack complex, which in turn leads to production of TNF- $\alpha$  and IL-6, promoting injury. In the present study, systemic C3 levels in recipients in the control group were upregulated at 24 hours after kidney transplantation, in contrast to significantly lower levels observed in the rapamycin group. The decrease in C3 plasma concentrations in the rapamycin group may have been related to lower expression of C3 expression in situ. The lower renal expression of C3 and TNF- $\alpha$  in the rapamycin group showed a close relationship to the fewer numbers of apoptotic nuclei in the inner and outer medullae.

This study has certain limitations. We did not measure drug concentrations before surgery. However, inasmuch as a positive response was observed, we believe that they were appropriate to decrease the inflammatory response. We did not perform dose-response studies of the immunosuppressant. In addition, we did not perform quantitative cytokine analysis of tissue samples. Although immunohistochemistry

demonstrated convincing results, quantitative data are necessary to specifically establish treatment effects.

In conclusion, using immunosuppression therapy in deceased donors before organ recovery may decrease damage associated with I/R injury and, consequently, improve recipient graft function. Systemic inflammatory responses triggered by brain death may also be decreased with immunosuppression drugs.<sup>37,38</sup> Thus, the 2 most important determinants of nonimmunologic injury, brain death and I/R injury, may be mitigated by administration of immunosuppression drugs in the donor. Graft immunogenicity would be decreased, with resulting positive effects on delayed graft function, acute rejection episodes, and long-term allograft survival.

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