THE UNIVERSITY OF MANITOBA

IN VITRO AND IN VIVO ASSESSMENT OF RAPAMYCIN AS AN IMMUNOSUPPRESSIVE AGENT IN THE RABBIT

BY

JONATHAN PAUL FRYER, M.D., F.R.C.S.(C)

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ABSTRACT

Although Cyclosporine A has greatly improved the success of organ transplantation, it has some serious side effects such as nephrotoxicity. Furthermore, rejection still accounts for fifty percent of graft failures. For these reasons, better and less nephrotoxic immunosuppressive agents have been sought and as a result, several promising new drugs have been identified. Rapamycin is one such drug and preliminary studies indicate that its immunosuppressive potency is one hundred times greater than Cyclosporine's, and that it may be less nephrotoxic. FK506 is another new immunosuppressive drug which is a more potent than Cyclosporine, although it appears to be equally nephrotoxic.

The first section of this thesis consists of an in vitro assessment of Rapamycin based on its effects on endothelial and mesangial cells. Comparisons were made with both Cyclosporine and FK506. Rapamycin inhibited cell growth but not DNA synthesis in endothelial cells. Rapamycin did not cause the release of prostacyclin or endothelin from mesangial cells but it caused an increase in the release of both of these vasoactive substances from endothelial cells. This balanced release of vasoconstrictors and vasodilators may protect Rapamycin-treated kidneys from the nephrotoxicity seen with Cyclosporine or FK506 use, since this appears to result from the effects of prolonged vasoconstrictor influences.

The second part of this paper involves an evaluation of the pharmacokinetics, immunosuppressive potential and toxicity

of Rapamycin in a rabbit model. To date, in vivo assessment of this drug has been limited by the fact that no effective method of measuring it in the blood has been available. However, such a method has recently been developed in our own lab using high performance liquid chromatography. Rapamycin elimination is best described by a two compartment model. Its pharmacokinetics are non-linear as both its volume of distribution and its total body clearance increase with increasing dose. Using model independent parameters, the half life of Rapamycin was 12.81 +/- 2.14 and 15.33 +/- 1.61 hours for doses of .05 mg/kg and .5 mg/kg respectively.

With the administration of various doses of Rapamycin intravenously in a rabbit heterotopic cardiac transplant model, resultant twenty-four hour trough blood levels less than 10 ug/L were associated with a high incidence of rejection while blood levels greater than 60 ug/L were not well tolerated mainly due to the development of septic complications. Based on creatinine clearance determinations, no significant nephrotoxicity was encountered in the Rapamycin treated animals while in those given Cyclosporine who had blood levels consistently greater than 150 ug/L, a statistically significant decrease in creatinine clearance was observed.

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A. INTRODUCTION

I. CYCLOSPORINE A

(a) IMMMUNOSUPPRESSION

Cyclosporine A (CSA) is a unique cyclic endecapeptide isolated from the fungus tolyplocadium inflatum Gams[1,2]. Its discovery has had a major impact on the success of solid organ transplantation by improving graft and patient survival and decreasing associated morbity[3-9]. Its effectiveness has been demonstrated in human kidney, heart, lung, liver, pancreas and small bowel transplantation, as well as in graft versus host disease associated with bone marrow transplantation[3-11].

CSA prevents allograft rejection by inhibiting the host's normal immune response to the donor antigens. This immunosuppressive effect results from CSA's influence on T lymphocytes and possibly macrophages[12]. Specifically, CSA blocks the production of interleukin 2 (IL-2) and probably its receptor as well. This inhibition results when CSA binds with its intracellular binding protein, cyclophilin, and forms a complex which then interacts with calcineurin and prevents it from carrying out key dephosphorylation reactions in specific transcriptional proteins[13]. IL-2, a T-cell lymphokine, acts as a chemical signal to promote the expansion of clones of antigen effector lymphocytes after the process has been initiated by exposure to foreign antigens[12,14]. CSA has also been

shown to inhibit interaction between macrophages and T-cells and to suppress the production of other T-cell lymphokines such as gamma-interferon, macrophage inhibitory factor and macrophage chemotactic factor[15-17].

(b) TOXICITY

Unfortunately, despite the undisputed advantages of CSA over previously employed immunosuppressants, its use has been associated with significant side effects including nephrotoxicity, hypertension, hepatotoxicity, vascular complications, seizures and tremors, gastrointestinal problems, hypertrichosis, gingival hyperplasia and lymphoma[18]. Of these, nephrotoxicity has occurred most frequently and has caused the most concern[18-22].

(c) NEPHROTOXICITY

The nephrotoxicity caused by CSA has been studied in a variety of patient populations and clinical settings. In a group of cardiac transplant patients who were studied one year after transplantation, inulin clearance and renal plasma flow were reduced compared to historical controls with similar cardiac performance who had received azathioprine immunosuppression[23]. In addition, renal morphologic changes typical of CSA nephrotoxicity were found in the CSA treated group[23]. Hemodialysis has been required in several cardiac transplant patients and in some renal function continues to deteriorate despite reductions in dosage. Liver transplant

patients have experienced similar long term renal dysfunction[24]. In the renal transplant patient population, which constitutes most of the experience with CSA, clinical investigation of nephrotoxicity is not optimal since coexistant acute or chronic rejection makes interpretation of changes in renal structure or function difficult[18-22,25-27].

II. NEW IMMMUNOSUPPRESSIVE DRUGS: RAPAMYCIN AND FK-506

(a) IN VITRO IMMUNOSUPPRESSION

Because of side effects such as nephrotoxicity plus the fact that although one-year graft survival is better than 70% with most organs rejection still accounts for 50% of graft losses, a search for new and better immunosuppressive agents has been initiated[28,29]. One of the agents being evaluated is the macrolide antibiotic, Rapamycin (RPM). Rapamycin is isolated from the fungus streptomyces hygroscopicus. Its chemical structure is quite different from CSA's but is similar to that of FK506[30], another macrolide with promising immunosuppressive potential. Early reports indicate that RPM's immunosuppressive potency is approximately one hundred times greater than CSA's[31,32]. In vitro, RPM has been shown to produce immunosuppression by a mechanism that differs from that of CSA and FK506[32] since it does not affect the expression of

interleukin-2 or its receptor and appears to inhibit T cell proliferation at a later point in the activation process. Addition of exogenous IL-2 can reverse the inhibition of cell division produced by CSA and FK506 but is ineffective with RPM[32]. While CSA and FK506 inhibit cell division by preventing the progression from the G_0 phase to the G_1 phase of the T-lymphocyte cycle, RPM appears to interfere with the cycle at a point beyond the G_1 phase[32-35]. RPM can inhibit T-cell proliferation when added 12 hours after activation whereas CSA and FK506 are effective only when added within a few hours of activation. Rapamycin inhibits both the calcium dependent and calcium independent pathways of T-cell and B-cell activation, while CSA and FK506 influence only calcium dependent mechanisms[31,32,35-37].

(b) IN VIVO IMMUNOSUPPRESSION

In vivo studies involving RPM have demonstrated its ability to prevent rejection in skin, heart, kidney, pancreas and small bowel allografts using various animal models including mouse, rat, dog, pig and monkey[38-45]. It has been shown to induce tolerance, inhibit ongoing and accelerated rejection, and prevent graft coronary disease in rodent cardiac allograft models[41-43]. In a murine model, RPM has been shown to inhibit graft versus host and host versus graft reactions in popliteal lymph nodes and to induce thymic involution[43,46].

(c) TOXICITY

Little is known about the toxicity of Rapamycin. In one study[39], gastrointestinal ulcers developed in dogs administered RPM orally in doses of 0.25 to 3.0 mg/kg and histology revealed a necrotizing vasculitis of the smaller arteries. RPM significantly inhibited weight gain in Sprague-Dawley rats treated for 14 days with an IP dose of 1.5 mg/kg[47]. The same rats were shown to have a statistically significant increase in serum creatinine and although a corresponding 20% decrease in creatinine clearance was noted, it was not significantly different than that seen in controls[47]. Glomerular filtration rate as estimated by clearance of ^{51}Cr EDTA was not affected by RPM[47]. Although no renal histologic changes were noted in RPM treated rats, some animals were found to have focal myocardial necrosis, focal hepatic necrosis or thymic involution[47]. Based on these early studies, RPM has been identified as a promising new immunosuppressive drug which is more potent and possibly less nephrotoxic toxic than CSA. Further in vitro and in vivo studies involving direct comparisons of RPM, CSA and other new immunosuppressive agents such as FK506 are essential to establish which agent is superior. The purpose of this thesis was to evaluate the toxicity and immunosuppressive capabilities of RPM in comparison to other important immunosuppressive agents. This was acheived by studying its effects on rabbit endothelial cell and mesangial cells in vitro; determining its single dose pharmacokinetics in the

rabbit; and assessing its ability to prevent rejection and its potential to produce toxicity at various blood concentrations in a rabbit heterotopic cardiac allograft model. Based on these parameters, comparisons were made with Cyclosporine A and FK506.

B. IN VITRO EXPERIMENTS

I. EVALUATION OF THE EFFECTS OF RAPAMYCIN ON RABBIT ENDOTHELIAL AND MESANGIAL CELLS AND COMPARISON WITH OTHER IMMUNOSUPPRESSIVE AGENTS

(a) INTRODUCTION

The mechanisms by which CSA and FK506 induce irreversible chronic changes to the kidneys are poorly understood. With CSA there is evidence to suggest that the interstitial fibrosis and arteriolopathy are a result of prolonged vasoconstriction of the afferent arteriole of the glomerulus [25,48,49].At one time, CSA itself was thought to directly induce vasoconstriction, but this has never been substantiated[50-53], although its vehicle for parenteral administration, cremophor-El, appears to cause some degree of vasoconstriction[54]. Similar functional and morphological changes are seen with FK506 nephrotoxicity[55,56] indicating that it may result from the same mechanism. The phenomenon may be the result of direct endothelial damage, sympathetic nerve activity, or an altered balance of local or circulating

vasodilator and vasoconstrictor agents.

The endothelial cell is recognized as an important regulatory center for local vasomotor activity and hemostasis[57]. It is capable of synthesizing many active substances such as heparan sulfate, fibronectin, interleukin-1, tissue plasminogen activator, endothelium derived relaxing factor, platelet activating factor, thromboxane, prostacyclin(PGI_2) and endothelin(ET), and modulates others such as angiotensin II, bradykinin, serotonin and adenosine[57].

Mesangial cells probably play a pivotal role in the regulation of glomerular blood flow[58]. These cells release and respond to vasoactive substances such as endothelin, a potent vasoconstrictor and to vasodilators such as prostacyclin and prostaglandin E_2 [59-61].

The interaction between some of these factors is influential in regulating renal blood flow. It has been shown previously that CSA results in altered release of vasoactive substances, including endothelin and PGI_2, from cells cultured in vitro[61-66]. These changes have also been reported in vivo in both animal and clinical studies[67-71]. CSA is toxic to endothelial cells[72,73] and thereby may alter the release of prostacyclin(PGI_2)[62,63] and endothelin(ET)[74], both of which have been implicated in the etiology of chronic cyclosporine nephrotoxicity[63,67,75]. An excessive release of ET, a potent vasoconstrictor, and/or a decreased release of PGI_2, a potent vasodilator, from renal endothelial cells in response to

chronic administration of CSA could potentially produce a state of prolonged vasoconstriction consistent with that implicated in the etiology of chronic CSA nephrotoxicity.

By evaluating and comparing the influences of RPM, CSA and FK506 on isolated endothelial and mesangial cells in vitro we attempted to identify any differences in response that in turn will help to explain the different patterns of toxicity observed in vivo. Specifically, we studied the effects of RPM, CSA and FK506 on cell growth and DNA synthesis with endothelial cells and, in addition, we determined the effects of FK506 and Rapamycin on ET and PGI_2 release in both endothelial and mesangial cells.

(c) MATERIALS AND METHODS

(i) ISOLATION AND CULTURE OF RABBIT ENDOTHELIAL CELLS

Primary cultures of rabbit endothelial cells were obtained using a modification of the technique described by Rone and Goodman[79]. Briefly, the thoracic aorta was harvested aseptically from a male New Zealand white rabbit(2.0 kg) and all excess fat was trimmed away. The aorta was turned inside out to externalize the endothelial surface which was irrigated with serum-free media to eliminate the red blood cells. The everted vessel was then placed in a shallow pool of type II collagenase (Worthington CLS II), and incubated for 15 minutes at 37°C. Next, the artery was transferred to a fresh dish containing Dulbecco's modified eagle medium (DMEM)

supplemented with 20% fetal calf serum (FCS) where the endothelial surface was gently stroked with the back of a scalpel blade. After each unidirectional stroke, the scalpel blade was dipped into additional dishes containing DMEM supplemented with Hepes (25mM), ascorbic acid (50ug/ml), glutamine (2mM), 20% FCS, penicillin (100 IU/ml) and streptomycin (100mg/ml). Cells were then incubated in this media at 37°C. When small colonies of cells began to form in a few days, those with the classic "cobblestone" appearance typical of endothelial cells[80] were isolated from the remaining cells using cloning rings.

(ii) CONFIRMATION OF ENDOTHELIAL CELLS BY IMMUNOHISTOCHEMISTRY

Immunohistochemistry was used to confirm the identity of the cell line. Factor VIII-related antigen[79,81] and angiotensin converting enzyme[79,82] are specific to endothelial cells and therefore the cells were stained to determine their presence. An avidin-biotin modification of a standard immunoperoxidase procedure was utilized[83]. Cells isolated as per above were grown on coverslips for 24 hours, rinsed one time with serum-free media and then three times with Osborn-Weber phosphate-buffered solution(OWPBS) (pH 7.30)[84]. Cells were then fixed in neutral-buffered formalin saline(10%) for one minute at room temperature followed by absolute methanol at -20°C for six minutes. The cells were then rehydrated in

OWPBS for 15 minutes and then incubated at room temperature for 15 minutes in heat-inactivated nonimmune rabbit serum diluted 100 fold in0.1% gelatin OWPBS. Individual coverslips were then incubated overnight with various dilutions of either goat anti-human factor VIII related antigen(F8RAG) (Atlantic Antibodies), goat anti-rabbit angiotensin converting enzyme(ACE) (Dr. Peter Caldwell, Columbia University) or nonimmune goat serum(NGS). All three goat sera were serially diluted to the following range of concentrations; 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280 and 1:2560, with each concentration represented on a seperate coverslip. The next morning, coverslips were rinsed three times with OWPBS and incubated with biotinyl-rabbit anti-goat IgG(Sigma) diluted 400 fold in nonimmune rabbit serum in gelatin OWPBS for 45 minutes. Another rinse with OWPBS followed this step and then the coverslips were incubated for 45 minutes in an avidin DH(5 ul/ml) + biotinyl-horseradish peroxidase (5ul/ml) complex (Sigma). Coverslips were again washed in OWPBS and incubated in a filtered diaminobenzidine tetrahydrochloride(Sigma) mixture (50 mg in 100ml OWPBS with 25ul 30% H_2O_2). Coverslips were rinsed and dehydrated through alcohols to xylene and then mounted on glass slides for viewing by a pathologist. Endothelial cells stained with both anti-F8RAG and anti-ACE antibodies at dilutions greater than 1:80 while NGS did not. Although a positive control was not used, smooth muscle cells and mesangial cells, serving as negative controls, did not

stain with either antibody thus providing strong evidence that the cells were endothelial cells.

(iii) DETERMINATION OF ENDOTHELIAL CELL GROWTH AND DNA SYNTHESIS

Endothelial cells were plated at a density of 2×10^4 cells per 9 cm^2 well in six well culture plates with a range of concentrations of RPM and FK506 (0, .05, .10, .50, 1, 5, 10, 100, 1000, 5000 ug/l), and CSA (0, 500, 1000, 2000, 5000, 10,000 ug/l). The vehicle for all drugs was ethanol and the final ethanol concentration in each well was 0.2%. The culture media used was DMEM with 20% FCS, and this combined with 0.2% ethanol therefore represented the vehicle control or "0" concentration for each drug. Tween 80 was also used to dissolve CSA and therefore this was included in its control. The controls for each drug was kept in seperate vials. All experiments were performed in duplicate and repeated a minimum of 3 times using the same endothelial cell line.

After incubating cells with drugs for 5 days, all media was removed from the wells and the cells were washed twice with normal saline and trypsinized. Recovered cells were then washed with media and counted to determine the cell growth in each well. DNA synthesis was determined by measuring the incorporation of ^{3}H-thymidine (70-85 Ci/mmol, Amersham Canada Ltd., Oakville, Ontario) into trichloroacetic acid (TCA)

insoluble macromolecules. Eighteen hours prior to trypsinization 2 uCi of ^{3}H-thymidine was added to each well. Cells were then recovered as above. After removal of an aliquot for determination of cell growth, the remaining cells were lysed by freeze-thawing and cellular material was precipitated using 10% TCA. The precipitate was resuspended in saline with a portion of the suspension being taken for protein determination as per the method described by Lowry et al[85]. The remaining amount was transferred to a sampling manifold (Millipore Corporation, Bedford, MA) fitted with Whatman GF/C glass fibre filters . The filters were thoroughly washed with 10 ml of 50% (vol/vol) ethanol. The radioactivity retained on the filters was counted in 10 ml of scintillation fluid(Beckman Redi Gel, Beckman Inc, Brea, CA) using a quench-corrected counting program on a LKB Rack Beta liquid scintillation-spectrophotometer (LKB-Praudukete AB, Bramme, Sweden).

(iv) ISOLATION AND CULTURE OF RABBIT MESANGIAL CELLS

Primary cultures of mesangial cells were obtained from outgrowths of collagenase-treated rabbit glomeruli. Intact glomeruli were isolated as previously described[65]. Briefly, kidneys were removed from New Zealand white rabbits (2.5 kg), and placed in sterile Hank's balanced salt solution (HBSS) containing 25 mM HEPES and antibiotics (penicillin 100 u/mL, streptomycin 100 u/mL, and amphotericin 0.25 ug/mL), all from Gibco Laboratories (Burlington, Ontario, Canada). The kidney

was sliced into sections of 1-3 mM and the cortex removed with forceps and minced. Differential sieving of the cortex homogenate was used to isolate the glomeruli. The homogenate was passed through a series of sieves with decreasing pore sizes of 250 microns, 150 microns and 88 microns with the glomeruli staying on top of the 88 micron mesh. Microscopic examination revealed that the glomeruli were > 95% free of tubule contamination. The glomeruli were incubated with trypsin (0.2% w/v) for 20 min at 37° C followed by incubation with 0.1% type IV collagenase (Sigma Chemical Co., St. Louis MO). The glomeruli were washed once with HBSS and resuspended in growth media (Dulbecco's minimal essential media, MEM) supplemented with 20% heat inactivated fetal calf serum (FCS), 50 u/mL penicillin, 100 ug/L streptomycin and 1% non-essential amino acids (all from Gibco). The culture medium was also supplemented with 0.66 u/mL protamine zinc insulin. Using cloning rings, the cells were treated with trypsin (0.05% w/v) and ethylenediaminetetraacetic acid (EDTA) (0.02% w/v) and replated. To inhibit the growth of any remaining fibroblasts, the cells were grown for three passages in media in which L-valine was replaced by D-valine. Fibroblasts are unable to proliferate in D-valine containing media since they lack the enzyme D-amino acid oxidase required for its utilization[76]. The cells were then replated every seven days in 6 cm^2 culture flasks in medium devoid of antibiotics since previous investigators have demonstrated a synergistic toxic effect

between CSA and streptomycin[77]. Microscopic examination revealed a homogenous population of spindle shaped cells that stained positively for myosin, actin, desmin and vimentin using specific antibodies and immunohistochemical techniques. These antigens known to be present in mesangial cells. Factor VIII which is specific for endothelial cells was absent[78].

(v) MEASUREMENT OF ENDOTHELIN RELEASE FROM ENDOTHELIAL AND MESANGIAL CELLS

Endothelin release from rabbit mesangial and endothelial cells was determined using a modification of the procedure described by Nakahama et al[64]. Briefly, media was removed from confluent endothelial cells grown for five days in the $9cm^2$ six-well tissue culture plates. Cells were washed three times with serum-free DMEM and then either RPM or FK506 at concentrations of either .01 or 10 ug/l were added. After incubation for four hours at 37°C, the media was removed and stored at 4°C until the endothelin immunoassay was performed. Because of the potential instability of endothelin, all assays were initiated within 12 hours of collecting the supernatant and were performed using a standard commercially prepared kit (Amersham Canada, Ltd.).

A second set of experiments was performed similarly except that 25 ng of transforming growth factor beta (TGF-β) was added prior to the 4 hour incubation. TGF-β was reconstituted in 4mM HCl with 1mg/ml bovine serum albumin

(BSA) and diluted in serum-free DMEM to a concentration of 12.5 ng/ml. Additional controls of TGF-β in media only and TGF-β vehicle in media were included. All experiments were performed in duplicate and repeated a minimum of three times using the same mesangial cell line. After removal of supernatant, cells were trypsinized and removed for protein determination. Results were determined as fmol endothelin per mg protein and were expressed as a percentage of the vehicle control +/- SEM.

(vi) MEASUREMENT OF PROSTACYCLIN RELEASE FROM ENDOTHELIAL AND MESANGIAL CELLS

The release of 6-keto-prostaglandin F1 alpha (6-keto-PGF1α), the stable metabolite of prostacyclin(PGI_2), from rabbit mesangial and endothelial cells was measured after incubation with RPM or FK506 using the technique described by Voss et al[56]. Briefly, the media was removed from confluent cells grown in 9cm^2 six-well culture plates and the cells washed twice with serum-free DMEM. Next, the cells were incubated for 16 hours at 37°C after combination with either RPM or FK506 at concentrations of either .01 or 10 ug/l. All drugs were prepared with a 95% ethanol vehicle in DMEM with 0.5% FBS. The final ethanol concentration in all experiments was 0.2%. After incubation, the media was removed and the cells washed three times with serum-free media and then incubated in 2ml of media for 15 minutes at 37°C.

Subsequently, the media was removed and stored at 4°C until analysed for 6-keto-PGF1-α by radioimmunoassay. All assays were initiated within 12 hours of collecting the supernatant and were performed using a standard commercially prepared kit (Amersham Canada, Ltd.). In addition to this, a second set of experiments was performed in a similar manner except that 10 umol of arachidonic acid was added prior to the 15 minute incubation. Arachidonic acid was initially made up in serum-free media to acheive a concentration of 5 umol/ml. All experiments were performed in duplicate and repeated a minimum of three times using the same cell line. After removal of supernatant, cells were trypsinized and their protein content determined by the Lowry method. Results were determined as pmol of 6-keto-PGF1-α released/ 15 minutes/ mg protein and expressed as a percent of vehicle control +/- SEM.

(c) RESULTS

(i) ENDOTHELIAL CELL GROWTH AND DNA SYNTHESIS

Data from Table 1, representing results obtained with the drug vehicles only, were used as controls (ie. 100%) in determining the results described below. CSA did not significantly affect cell growth at concentrations less than 10,000 ug/L but at this concentration it completely inhibited cell growth (Figure 1). Although CSA appeared to increase DNA synthesis at lower concentrations, this was not statistically significant. However, at a concentration of 10,000 ug/L, DNA

synthesis was completely inhibited (Figure 1).

RPM significantly inhibited cell growth at concentrations greater than 0.1 ug/L and this effect appeared to be concentration dependent (Figure 2). Paradoxically, DNA synthesis did not decrease with RPM (Figure 2).

With FK506, both cell growth and DNA synthesis were unaffected at the concentrations studied (Figure 3).

(ii) PROSTACYCLIN AND ENDOTHELIN RELEASE

The release of PGI_2, measured as its stable metabolite 6-keto $PGF_1\alpha$ by endothelial and mesangial cells in the presence and absence of arachidonic acid is shown in Table 2. Arachidonic acid did not have any significant effect on the release of this analyte from the two cell lines tested. The release of endothelin by these two cell lines in the presence and absence of TGF-β is also shown in Table 2. The latter results in a greater than two-fold increase in endothelin release from endothelial cells, but did not result in any significant change in release from mesangial cells. The data in Table 2 were used as the controls (ie. 100%) in determining the results described below.

RPM at 0.01 ug/L resulted in significant increase in endothelin release in TGF-β treated endothelial cells. The drug at 10 ug/L resulted in a significant increase in PGI_2 in non-treated endothelial cells (Figure 5a).No other significant changes were seen with the drug.

RPM at a concentration 10.0 ug/L resulted in a significant decrease in PGI_2 in non-treated endothelial cells (Figure 5b). In addition, at both concentrations tested, FK506 resulted in a significant increase in endothelin in TGF-β treated endothelial cells (Figure 4b).

Neither FK-506 nor RPM had any significant effect on endothelin or PGI_2 release in mesangial cells.

(d) DISCUSSION

The antiproliferative effect which CSA and FK506 impart on activated T-lymphocytes, thereby suppressing the immune response, does not appear to manifest with endothelial cells as cell growth was not inhibited at most concentrations of these agents. This finding is not unexpected since interleukin-2 has not been implicated as a stimulator of endothelial proliferation. The absolute inhibition of cell growth and DNA synthesis seen with the highest CSA concentration likely reflects a severe toxic insult with disruption of cellular integrity as only a few severely deformed cells were found to survive the incubation period. In a previous in vitro study involving incubation of mesangial cells with various concentrations of CSA, both cell growth and DNA synthesis were similarly inhibited at a concentration of 10,000 ug/L although lesser concentrations also appeared to inhibit cell growth[65].

RPM, which prevents T-cell proliferation by mechanisms unrelated to interleukin-2[31] production, appears to influence

endothelial cell growth in a concentration dependent manner. Whether or not this indicates that RPM has antiproliferative effects not specific to lymphocytes needs to be clarified by further studies using other cell lines. The maintenance of DNA synthesis seen with concentrations which significantly inhibited cell growth suggests that RPM may influence the cell cycle at a step occurring after the S phase, and this concept has been supported in previous studies[31-34].

It has been speculated that the renal side-effects of CSA may be mediated by an alteration in the balance of vasoactive substances leading to the vasoconstriction of the afferent arteriole of the glomerulus[25,48,49]. Several studies have clearly shown that CSA can alter the synthesis and release of vasoconstrictor substances such as endothelin and vasodilatory substances such as prostacyclin both in vitro and in vivo[61-71]. The in vitro work presented here investigates the effects of RPM and another new immunosuppressive agent FK506 on the release of these analytes from primary rabbit mesangial and endothelial cell lines. These two cell lines were chosen since they are known to release both endothelin and PGI_2 and are both involved in the maintenance of renal hemodynamics in vivo[57].

Experiments were performed both in the presence and absence of arachidonic acid, a precursor in the synthesis of prostaglandin which has been shown to stimulate the release of prostacyclin[62,63] as well as TGF-β, a known stimulator of

endothelin synthesis[86,87]. Evaluation of the effects of these immunosuppressive drugs on both the stimulated and basal release of vasoactive substances was thought to represent a more comprehensive and perhaps more accurate assessment of the in vivo situation.

We found that arachidonic acid had no effect on the release of PGI_2 with both mesangial and endothelial cells. This lack of effect was not concentration dependent since alteration of the arachidonic acid concentration above and below that used did not alter the response. Other investigators have shown arachidonic acid to stimulate the release of PGI_2[62,63]. These studies were performed on endothelial cells obtained from other species, which may account for the differences seen with the results reported here. In contrast, TGF-β resulted in a significant increase in release of endothelin from endothelial, but not mesangial cells. The latter cell line could be less sensitive to the effect of TGF-β [86,87]. The effect of both RPM and FK506 varied between cell lines. Both drugs had little effect on the release of PGI_2 or endothelin from mesangial cells. It is possible that these drugs do not effect this cell in vivo. Alternatively, the conditions of incubation and drug concentrations tested may not have been optimized for PGI_2 release. It is unlikely that the latter is true since similar results were obtained with drug concentrations both above and below the concentrations tested. In contrast, both RPM and FK506 influenced the release

of PGI_2 and endothelin from endothelial cells. The effect was concentration dependent. At the highest concentration tested (10 ug/L), RPM resulted in a significant increase in release of PGI_2 in non-stimulated endothelial cells. The drug also resulted in a significant release of endothelin at 0.01 ug/L in TGF-β stimulated cells. FK506 at 10 ug/L resulted in a decrease in PGI_2 in non-stimulated endothelial cells with a stimulation in endothelin release in TGF-β stimulated cells at both concentrations of drug tested. These latter results are consistent with those recently reported by Moutabarrik et al[88], who showed that FK506 stimulated the release of endothelin from cultured renal cell lines.

The concentration range tested for these drugs spanned that over which they produce an immunosuppressive effect[31,89]. The variation in the release of prostacyclin and endothelin from cell line to cell line and over the concentration range of drug tested has been observed by other investigators[88]. Moutabarik et al have shown that FK506 at 1.0 ug/L resulted in a greater release of endothelin than at 10 ug/L. Although this was not observed for FK506 in the present study, a similar situation was observed with RPM where the drug at 0.01 ug/L resulted in a significant increase in the release of endothelin while at 10 ug/L this increase was not seen. It is possible that the higher concentrations of drug may impair the release of vasodilatory substances due to other effects the drug may have on cellular metabolism which may indirectly

affect the ability of the cell to release vasoactive substances. Furthermore, as Figure 2 demonstrates, RPM at a concentration of 10 ug/L significantly inhibits cell growth and therefore the decreased endothelin release may merely reflect cell death, although this seems unlikely since drug and cells were incubated together for only 4 hours.

CSA has been shown to result in a decreased release of prostacyclin in primary rabbit mesangial cells and in human endothelial cells[62,63,65]. The drug has also been shown to inhibit prostaglandin E_2 formation by rat mesangial cells[61]. The in vitro data is corroborated by in vivo studies in both animals and patients which indicate that CSA may alter the balance of vasodilatory and vasoconstrictor substances , which may be the etiologic factor in its role in nephrotoxicity[68-71]. FK506 has been shown to be as nephrotoxic as CSA[56,90,91]. The findings from these experiments suggest that this may be mediated by an imbalance of vasodilatory and vasoconstrictor substances. In contrast, in animal studies RPM has been shown to result in fewer renal side-effects than CSA and FK506[47,92-94]. Although RPM resulted in an increased release of endothelin, it also stimulated release of prostacyclin, which may counterbalance the release of the former, thus resulting in maintenance of normal vascular tone in vivo. Although in our in vivo studies, blood RPM levels of 10 ug/L provided inadequate immunosuppression and no evidence of cellular toxicity, it is not known what the corresponding tissue levels were.

In summary, both RPM and FK506 appear to alter the release of vasoactive substances from cell lines in vitro. However, it should be noted that in vitro findings may not be representative of the situation in vivo. Further studies are required in which the concentration of both vasodilatory and vasoconstrictor substances are measured in blood subsequent to the administration of the drugs in vivo.

IN VIVO STUDIES

I. INTRODUCTION

Both CSA and RPM have been studied in vivo using a variety of animals models. CSA nephrotoxicity has been studied most extensively in the rat[95,96], but in this animal it has been difficult to reproduce the renal histologic changes commonly seen in man, even using pharmacological doses[25-27,95-98]. The rabbit has also been used to study CSA, and it appears to be a good model for studying nephrotoxicity[99-102]. Recent studies in our own lab have demonstrated that CSA administered intravenously to rabbits in doses as low as 2.5 mg/kg/day over 30 days will produce renal histologic changes consistent with those seen in man[101]. Others have also reported on the usefulness of the rabbit model for studying CSA nephrotoxicity[103].

Reports of cardiotoxicity are rare with most immunosuppressive agents, while hepatoxicity and nephrotoxicity are more commonly reported. Use of a cardiac allograft, therefore, prevents much of the confusion encountered in the assessment of a failing renal or hepatic allograft which could be due to either rejection or to drug toxicity. The use of a cardiac allograft also simplifies daily monitoring of graft function since palpation is all that is required. Palpation is easier if the heart allograft is placed heterotopically in the neck[104], since it is quite superficial in this location. Heterotopic cervical transplantation is also

safer in the rabbit because it is less likely to result in major hemmorrhage or paraplegia, both of may occur with abdominal placement[105,106].

II. SINGLE DOSE PHARMACOKINETICS OF RAPAMYCIN IN THE RABBIT

(a) INTRODUCTION

In all in vivo work with RPM to this point, it has been administered on a dose per weight basis with no knowledge of its pharmacokinetics or even its peak and trough blood concentrations. Previously, the lack of a suitable assay has prevented the elucidation of the pharmacokinetics of RPM. Our laboratory has recently developed a method for measuring RPM in whole blood using high performance liquid chromatography[107]. The object of this study was to study the disposition of intravenously administered RPM in the rabbit as such information would be helpful in planning the appropriate doses and dosing intervals required to acheive therapeutic blood levels in further experimental work as well as in a clinical setting.

(b) MATERIALS AND METHODS

(i) ANIMAL PROCEDURES

Five New Zealand white rabbits weighing between 3.1 and 4.4 kg were used for the study. The rabbits were housed in individual cages with food being restricted during the two study days. A heparin lock was placed in the left central ear artery on the day of the study for the purpose of blood

sampling. Patency was maintained with a heparin solution (1-2 ml of 100/ml) which was discarded prior to each blood sampling and replaced after the sample was obtained.

(ii) RAPAMYCIN PREPARATION

Rapamycin, (Wyeth Ayerst Inc., Princeton, New Jersey), was initially solubilized in 1 ml of dimethylacetamide(Sigma Chemical Co., St. Louis, MO.). A sufficient volume of a 1:9 solution of polysorbate 80(Sigma) and polyethylene glycol 400(Sigma) was added to provide a drug concentration that would allow a final volume of approximately 2 ml to be injected.

(iii) RAPAMYCIN ADMINISTRATION AND BLOOD SAMPLING

Five rabbits were randomized in a crossover fashion, to receive RPM in doses of 0.05 and 0.5 mg/kg. The two doses were separated by a minimum of 7 days. RPM was administered via slow push intravenously over 2 minutes into the right marginal ear vein. Blood samples were obtained at 0, 2, 4, 6, 10, 20, 30 and 60 minutes, and at 2, 4, 6, 8, 12, 20 and 26 hours. All samples were collected in tubes containing EDTA and were stored at -40°C until analyzed.

(iv) ANALYTICAL METHODS

RPM was analyzed in whole blood by reverse-phase high performance liquid chromatography[107](HPLC). Briefly, the procedure utilizes desmethyl-rapamycin as the internal standard with subsequent extraction using potassium carbonate, and diethyl ether. The diethyl ether layer is evaporated to

dryness and the remaining extract is reconstituted in a mobile phase containing methanol/water (70/30 vol/vol) and further addition of hexane. The top hexane layer is removed following centrifugation and the remaining mobile phase is utilized for analysis. Chromatographic separation is performed isocratically on two spherisorb C-8 columns (5 um, 25 x 0.21 cm)(Chromatography Sciences Company, Montreal, Quebec) joined in tandem, preceeded by a pellicular and silica precolumn (30-40 um)(Upchurch Scientific, Oak Harbor, WA) using the previously described mobile phase. Precolumn and column temperatures are maintained at 45°C. The eluted peaks are monitored at 278 nm. The within day coefficient of variation (CV) of the assay at 10 and 50 ug/l is 8.1% and 1.9% respectively. The between day CV of these concentrations is 14.4% and 9.8% respectively. The method was shown to have a sensitivity of 0.5 ug/l(signal to noise ratio of 3:1).

(v) PHARMACOKINETIC AND STATISTICAL ANALYSIS

Blood concentration-time curves following administration of RPM were fitted using PCNONLIN V 3.0 (Statistical Consultants Inc, Lexington, Kentucky), with initial parameter estimates derived from ESTRIP using the computer program PKCALC[108]. The data were fit to a two-compartment open model. From the fitted parameters, other pharmacokinetic parameters were calculated, including the alpha (α) and beta (β) half-lives, the volume of distribution of the central compartment (V_c), the volume of distribution at

steady state (Vdss), and the total body clearance (CL). The following equations were used:

$$CL = Dose / AUC \qquad V_{\beta} = Dose / AUC_{\beta}$$

$$V_c = Dose / A+B \qquad Vdss = Dose[(A/\alpha^2)+(B/\beta^2)] / AUC^2$$

$$t\ 1/2\ terminal = 0.693 / \beta$$

where α and β are the exponents of the biexponential equation describing the data and A and B are the corresponding zero-time intercepts. The terminal disposition constant (β) was obtained from the terminal slope. The model independent parameters were calculated using PKCALC[108]. Parameters included the total body clearance (CL), the steady state volume of distribution (Vdss), the mean residence time (MRT), and the terminal elimination half-life (t 1/2 terminal). The following equations were used:

$$CL = Dose / AUC \qquad Vdss = Dose \times AUMC / (AUC)^2$$

$$MRT = AUMC / AUC \qquad t\ 1/2\ terminal = 0.693 / \lambda_z$$

where the area under the curve (AUC) was calculated using the trapezoidal rule and the extrapolated AUC from the last data point to time infinity was calculated by dividing the last experimental blood concentration value by the terminal slope

(λ_z). The first moment of the concentration-time curve (AUMC) was calculated from AUMC = [(AUC) x time]. Differences between pharmacokinetic parameters of the two dosages were analyzed using the paired T-test. A p value less than 0.05 was considered significant.

(c) RESULTS

The mean (+/- SEM) RPM blood concentrations of the two dosages are illustrated in Figure 6. The blood concentration time curves were best described by a two compartment model following IV administration. Table 3 summarizes the fitted pharmacokinetic parameters obtained from NONLIN analysis of the two dosages of RPM. The t 1/2β, Vc, Vb, Vdss, and CL increased significantly as the dose increased. No difference in the t 1/2α was observed.

Table 4 summarizes the model-independent parameters obtained with the two IV doses of RPM. The Vdss, CL and MRT all increased significantly with an increase in dose. No difference in the t 1/2 terminal was found with the higher dose.

(d) DISCUSSION

This is the first study to describe the pharmacokinetics of RPM in a animal model. RPM pharmacokinetics appear to be dose dependent in the rabbit following IV administration with the doses studied. An apparent nonlinearity of RPM pharmacokinetics is evident with the increases in Vdss and CL. A similar dose dependent effect

on Vdss and CL has been observed in the rabbit with CSA[109]. The observed increases in Vdss and Cl may be attributable to saturable binding of RPM to a component(s) in rabbit blood. Whether or not RPM binding to rabbit blood components actually occurs has yet to be demonstrated.

The doses used in this study have been shown to be efficacious in the prolongation of allograft survival in animal models[92,110]. The observed blood concentrations 24 hours post dose were well within the range of the analytical procedure.

The preliminary assessment of RPM pharmacokinetics presented will allow further refinement of dosing when additional information is available regarding the concentration effect relationship of RPM. Future dose ranging studies with RPM should consider its apparent nonlinear pharmacokinetics. Further studies should also determine the extent of RPM binding to blood components and investigate other factors which may account for the dose dependent pharmacokinetics.

III. RELATIONSHIP OF BLOOD CONCENTRATIONS OF RAPAMYCIN AND CYCLOSPORINE TO SUPPRESSION OF ALLOGRAFT REJECTION IN A RABBIT HETEROTOPIC HEART TRANSPLANT MODEL

(a) INTRODUCTION

The monitoring of blood concentrations of CSA and other

immunosuppressive agents such as FK506 have been utilized to adjust dosages in order to bring the drugs' concentrations within a therapeutic range in order to minimize toxicity while maximizing immunosuppression[95,111,112]. To date little is known about the blood concentrations of RPM that are associated with the immunosuppressive doses used in the allograft survival studies in animals. This is partly due to the lack of a suitable method for measurement of the drug. We have recently reported a HPLC method which has a sensitivity of 1.0 ug/l for measurement of the drug in whole blood[107]. As previously mentioned, we have used this method to study the pharmacokinetics of RPM in rabbits[113]. It has not yet been shown whether a relationship exists between blood concentrations and either the immunosuppressive or toxic effects of the drug. The ability of RPM and CSA to prevent allograft rejection was compared using a rabbit heterotopic cardiac transplant model. This animal was chosen because it was previously demonstrated to be a good model for assessing CSA-induced nephrotoxicity[101] and to facilitate surgical, biochemical and pharmacological studies.

(b) MATERIALS AND METHODS

(i) TECHNIQUE OF CERVICAL CARDIAC TRANSPLANTATION

Fifty heterotopic cardiac transplants were performed on male New Zealand white rabbits using the method described by Heron[104]. Small donors (.5 - 1.5 kg) and large recipients (3.0 - 4.0 kg) were utilized to minimize the size discrepancy

between anastomosing vessels. Donor and recipient animals were anaesthetized with a "cocktail" containing proportions of Ketamine, Xylazine and Acepromazine 65/22/13 v/v/v. Anaesthesia was maintained in the recipient using halothane inhalation via a medishield anaesthetic machine. The recipient's right neck was shaved and prepped with Betadine. A longitudinal incision was made over the sternocleidomastoid muscle and the external jugular vein and common carotid artery were isolated. The donor's thoraco-abdominal surface was then shaved and prepped and both animals were heparinized (100 U/kg). The chest of the donor was then opened. The inferior vena cava (IVC) was clamped just above the diaphragm and cold Stanford solution (mannitol 12.5 g, $NaHCO^3$ 25 mg and KCL 20 mg in 1000 ml of 5% dextrose) infused proximal to the clamp. The IVC was then transected, the aortic arch clamped and cold Stanford solution infused into the proximal ascending aorta to perfuse the coronary microcirculation. The ascending aorta and main pulmonary artery were then transected and the pulmonary veins, superior and inferior vena cava were "gang-tied" with a 2-0 mersilene suture and subsequently transected below this ligature. The donor heart now free of its vascular connections was placed immediately in cold Stanford solution. The recipient's external jugular vein and common carotid artery were then ligated cephalad, clamped caudad and transected just below the ligation point. Using a non-sutured silastic cuff technique[104](Figure 7), continuity was re-established between

the recipient common carotid artery and donor ascending aorta as well as between the recipient external jugular vein and the donor pulmonary artery. During the performance of the non-sutured anastomosis (10-25 minutes) the donor heart was continuously irrigated with cold isotonic saline. Upon completion of the anastomoses both vessels were unclamped, and with the re-establishment of myocardial blood flow, the heart started beating immediately. The skin was then closed using 3-0 Vicryl. The overall technical failure rate for the procedure was 8%. The surgeon was blinded as to the treatment group designation and after completion of the surgery the animal was transferred to its cage where the initial intravenous injection of drug , vehicle or saline was administered by a separate team.

Animals were examined daily and the cervical allografts were palpated to determine if they were still beating. This assessment was performed by individuals unaware of each animals treatment group assignment.If the heart graft ceased beating within 72 hours of surgery it was considered a technical failure and was eliminated from the study. If the allograft stopped beating after 72 hours, it was removed under anaesthesia, and the animal continued to receive its daily intravenous injection until the study was completed 60 days from the time of surgery. Animals were sacrificed prior to this time if the following manifestions of treatment intolerance occurred:

1. emaciation with loss of all subcutaneous fat, muscle wasting and a loss of greater than 20% of original body weight.
2. lethargy, obtundation and anorexia with no food intake for five days.
3. severe gangrene of both ears precluding further intravenous drug administration.

The reasons for destroying these animals were threefold:

1. manifestations of treatment intolerance constitute logical study end points.
2. post mortem changes occurring after the unwitnessed death of a sick animal would confound the final biochemical and histopathologic analysis.
3. to prevent unnecessary suffering.

The animals were sacrificed with a fatal intravenous injection of Euthanyl and post mortem examinations were performed.

(ii) DRUG ADMINISTRATION

RPM was obtained as a gift from Wyeth-Ayerst (Princeton NJ). The drug was administered using a vehicle consisting of the following: polyethylene glycol, 400 polysorbate, dimethyl-acetamide, isotonic saline 58/20/20/2 (v/v/v/v). Sandimmune IV (CSA) and its vehicle (cremophor-El) were obtained as a gift from Sandoz Inc. (East Hanover, NJ). The fifty animals were randomly divided into ten groups of 5

animals each. The groups received drug or vehicle as a single daily bolus IV (total volume 2.5 ml) via the marginal ear vein for 60 days at the doses listed below: RPM at doses of 0.05, 0.1, 0.5 and 1.0 mg/kg/day; CSA at 5, 10 and 15 mg/kg/day; RPM and CSA vehicle and normal saline.

(iii) LABORATORY MONITORING

The animals were placed into metabolic cages preoperatively, monthly and just prior to sacrifice for 24 hour urine collections for measurement of creatinine, sodium and potassium. Blood samples were obtained preoperatively from a marginal ear vein and then weekly until the end of the study. The resulting sera were analyzed for the following analytes using routine procedures in the Clinical Chemistry Laboratory at the Health Sciences Centre: creatinine, sodium, potassium, urea, bilirubin, aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALK). Blood was also drawn on a weekly basis into tubes containing EDTA as anticoagulant for measurement of drug levels. CSA and RPM in whole blood were measured by reverse phase HPLC as previously described[101,107]. The interassay coefficient of variation of both methods is <10%. Blood, urine and lung cultures were obtained in those animals that became severely ill or died suddenly.

(iv) STATISTICAL EVALUATION

Statistical assessment of the data obtained was performed by using analysis of variance and Duncan's multiple-range test and the paired t-test, where appropriate. A p value

of less than 0.05 was considered statistically significant.

(c) RESULTS

In the animals administered RPM (Figure 8) no grafts were lost to rejection prior to study end points except for in the lowest dose group (0.05 mg/kg/d), which attained a 60% survival rate. The two animals that rejected their grafts in this group on days 9 and 13, had trough RPM concentrations consistently below 10 ug/l. It must be pointed out that in the two highest RPM dose groups, the majority of animals were destroyed prior to 60 days because they developed severe septic complications and therefore technically it cannot be assumed that they would not have rejected if they had survived. In the animals treated with CSA (Figure 9) no animals rejected prior to study end points. In contrast to the above, all control animals rejected their grafts at a mean survival time of less than 15 days.

Trough blood concentrations in the animals administered RPM were relatively stable in contrast to CSA where much more variation was noted (Table 5). The trough concentrations of RAPA in individual animals at the four doses tested are shown in Figure 10. A slight decline in blood levels near the end of the study likely reflects the increased difficulty in performing daily intravenous injections due to the diminishing availability of suitable ear veins. A relationship was shown to exist between dose, trough blood concentrations and tolerance of animals to RAPA treatment. As the dose and trough

blood concentrations were increased the proportion of animals tolerating the treatment decreased (Figure 8). One animal in each of the 0.05 and 0.1 mg/kg/d groups was destroyed while 3 and 4 were destroyed in the 0.5 and 1.0 mg/kg/d group respectively. One of the 4 animals destroyed in the 1.0 mg/kg/d group was found to have a large wound abscess which grew Staphylococcus aureus. This infection likely contributed to its deterioration. Three of the 4 animals destroyed in the 1.0 mg/kg/d group were terminated because of severe bilateral gangrene of the ears, precluding intravenous injections. Despite moderate weight loss, these animals appeared to be otherwise well. Several other animals treated with RPM were found to have moderately heavy growth of Bordetella bronchiseptica in their lungs. As this was present to some degree in all specimens in animals administered either drug, the significance of this finding in relation to the deterioration of the animals health was unclear.

In the CSA treated animals morbidity was not as directly related to dose and blood concentration. Two of 5 animals in the 5 mg/kg/d group were lost prior to sixty days. One was destroyed because of severe emaciation, and the second died suddenly. In the 10 mg/kg/d group none of the animals were destroyed but one died suddenly, while in the 15 mg/kg/d group two animals were destroyed. Both animals that died suddenly were subsequently found to have positive blood and lung cultures growing Pasteurella multocida. One animal in the 15

mg/kg/d group was found to have a perforated jejunum which had sealed resulting in a bowel obstruction.

Only animals surviving at least 30 days were included in the determinations of weight change.There was a tendency to lose or to fail to gain weight in most of the drug-treated animals (Figure 11,12). In the RPM treated animals, (Figure 11) the magnitude of the weight loss tended to increase with increased dosage and trough level concentration of the drug. In the CSA treated groups (Figure 12), weight loss was minimal. In contrast, all of the vehicle and saline control groups gained weight.

Biochemical studies were performed on the blood and urine of all animals at the beginning and end points of the study (Tables 6 and 7). None of the animals in any of the control groups exhibited significant changes in the biochemical parameters throughout the study. Only animals surviving longer than 30 days were included in the determinations of creatinine clearance. Of the animals treated with RPM, none exhibited a significant change in creatinine clearance at the endpoints of the study although a trend to decreased values was seen in the group given the highest dose (1.0 mg/kg/d) (Figure 11). A similar situation existed for the animals receiving CSA, with the exception of the group who received the highest dose (15 mg/kg/d), which exhibited a significant ($p<0.05$) decrease in creatinine clearance (Figure 12). A significant increase ($p<0.05$) in serum bilirubin occurred in the group of animals

receiving RPM at a dose of 1.0 mg/kg/d. No other significant increases were detected for either RPM or CSA, although some decreases in biochemical parameters were noted (Table 6 and 7).

(d) DISCUSSION

The present study examined the relationship between blood concentrations of RPM and the survival of heart allografts and tolerance of the drug in rabbits. To allow investigation of the side effects of the drug, animals who rejected their grafts were not sacrificed until 60 days had elapsed post-transplant unless other endpoints were reached as defined in the protocol. In rabbits administered an RPM dose in the range of 0.1 - 1.0 mg/kg/day, no grafts were lost to rejection although, as mentioned earlier, most animals in the higher dose groups did not survive the full 60 days of the study . In contrast, 2 of 5 animals receiving RPM at the lowest dosage rejected their grafts. The results suggest that a minimum dose for optimal graft survival was 0.1 mg/kg/day. These results are similar to those of Stepkowski et al[114] who showed a graded dose-response immunosuppression in a rat heart allograft transplant model with RPM at doses ranging from 0.16 to 0.8 mg/kg/day. In the animals receiving CSA, graft survival parallelled animal survival over all the three doses tested as no animals rejected and no differences in animal survival between the groups was observed.

The animals' overall tolerance of their treatment with

RPM and CSA differed somewhat as did the specific side-effects they encountered with each drug. While a relationship existed between dose and tolerance to RPM treatment, such was not the case for CSA at the doses used. For the latter drug intolerance was consistent at all the doses tested. The most conspicuous examples of drug intolerance with both drugs were abscesses at the surgical site and severe gangrene of the ears due to routine IV drug administration (primarily seen in animals given RPM). Animals with these lesions were destroyed in accordance with the established protocol to prevent unnecessary suffering. Several animals in both treatment groups developed pneumonia (primarily in those given CSA), a finding that is not surprising in light of the immunosuppressive therapy. The intolerance for either drug was minimal if the animals destroyed because of severe septic complications are excluded.

There was no statistically significant weight loss in animals treated with either drug. Neither hepatic nor renal functional abnormalities, as determined by measurement of liver function tests and creatinine clearance respectively, were observed in animals treated with RPM although most animals in the 2 highest RPM dose groups were not exposed to the drug for a full 60 days since they were destroyed prior to this when they developed septic complications. In animals receiving CSA, a significant decrease ($p<.05$) in creatinine clearance in the highest (15.0 mg/kg/day) dose treatment group

was seen. This observation is consistent with that of a previous study from our laboratory in which non-transplanted rabbits were given CSA intravenously for 30 days. In this study histopathological changes, namely interstitial fibrosis, tubular atrophy and arteriopathy were noted in the CSA treated animals[101]. Histological assessment of the kidneys harvested from RPM and CSA treated animals in this study is beyond the scope of the present investigation but will be presented in a subsequent report.

This is the first study in which trough blood concentrations of RPM were monitored. For this reason a broad range of doses was used. The trough concentrations of this drug exhibited a proportionality with dose. In animals whose grafts survived to the endpoint of the study, trough concentrations were in the range of 10-60 ug/L. The two animals who rejected their allografts in the lowest dose group had trough concentrations of the drug < 10 ug/L prior to rejection. Most animals with concentrations >60 ug/L did not survive the full sixty days of the study as most developed septic complications. This indicates that such levels result in excessive immunosuppression in this species. A tentative therapeutic range of 10-60 ug/L for RPM is therefore proposed. This is supported by the fact that animals administered RPM at a dose of 0.1 mg/kg/day who consistently had concentrations within this range had the highest incidence of graft survival with the least drug-related side-effects. In contrast, for

CSA, alterations in trough concentrations over a range of 50-300 ug/L did not significantly affect graft survival, nor the incidence of drug induced side-effects although, a decrease in renal function was noted at the higher concentrations.

The concentration of RPM in blood was higher than expected in light of the doses administered assuming that its pharmacokinetic properties are similar to that of CSA. This could be due to a number of factors including a long half-life of the drug in circulation. In assessing the pharmacokinetics of Rapamycin we demonstrated that RPM's half-life was, in fact, longer than CSA's in this species[109,113]. Alternatively, the drug could be sequestered in erythrocytes, and therefore the whole blood/plasma ratio for the drug would be expected to be high. This appears to be the case as we have found the ratio to be approximately 9.5 for RPM (unpublished observation) while CSA is sequestered within erythrocytes with a WB/plasma ratio of 1.5[115]. What is not known is whether monitoring of the drug in plasma would provide a better guide for dosage adjustment than the measurement of the drug in whole blood although based on its whole blood/plasma ratio whole blood monitoring would appear to be the preferred approach. This latter issue has not been resolved for CSA, even though the drug has been routinely monitored for approximately 10 years[48].

In summary, these data indicate that RPM is a potent immunosuppressive agent which significantly prolongs heart allograft survival in the rabbit with minimal side-effects if

appropriate blood levels are maintained. Further studies with other animal transplant models must be performed to confirm these findings.

D. CONCLUSIONS

Rapamycin is a promising new immunosuppressive agent. Its half-life is slightly longer than Cyclosporine A's and like Cyclosporine A its pharmacokinetic properties vary with the dose.

Twenty-four hour trough blood levels of Rapamycin exceeding 10 ug/L prolong cardiac allograft survival beyond sixty days in a rabbit model. Rapamycin levels exceeding 60 ug/L are poorly tolerated in this species and result in a high incidence of septic complications. A tentative therapeutic range of 10 ug/L to 60 ug/L is proposed as a guide for other studies with Rapamycin. Although the results of this study were not conclusive, Rapamycin does not appear to be nephrotoxic in the rabbit when blood levels are kept within the proposed therapeutic range. Additional studies using more precise indicators of renal function such as inulin clearance and renal blood flow are required to confirm these findings.

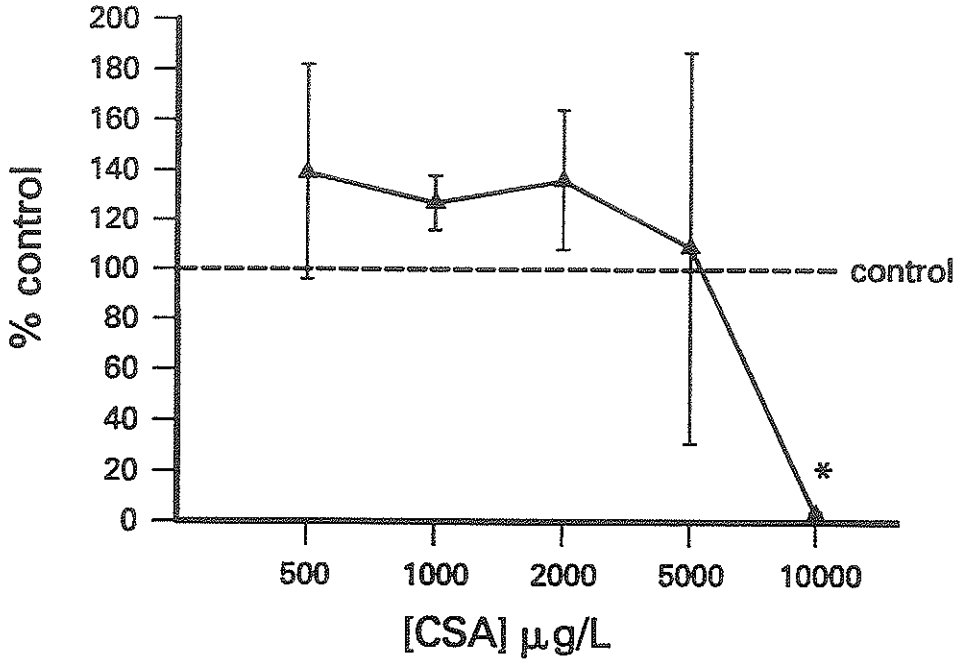
Rapamycin effects a balanced release of endothelin, a vasoconstrictor, and prostacyclin, a vasodilator, from endothelial cells in vitro, unlike Cyclosporine A and FK506 which effect the release of endothelin and/or inhibit the release of prostacyclin thus creating a chemical milieu which predisposes to vasoconstriction. If these same findings can be reproduced in vivo, they may provide an explanation as to why Rapamycin may be less nephrotoxic than Cyclosporine A and

FK506 which appear to produce nephrotoxicity as a result of prolonged vasoconstriction.

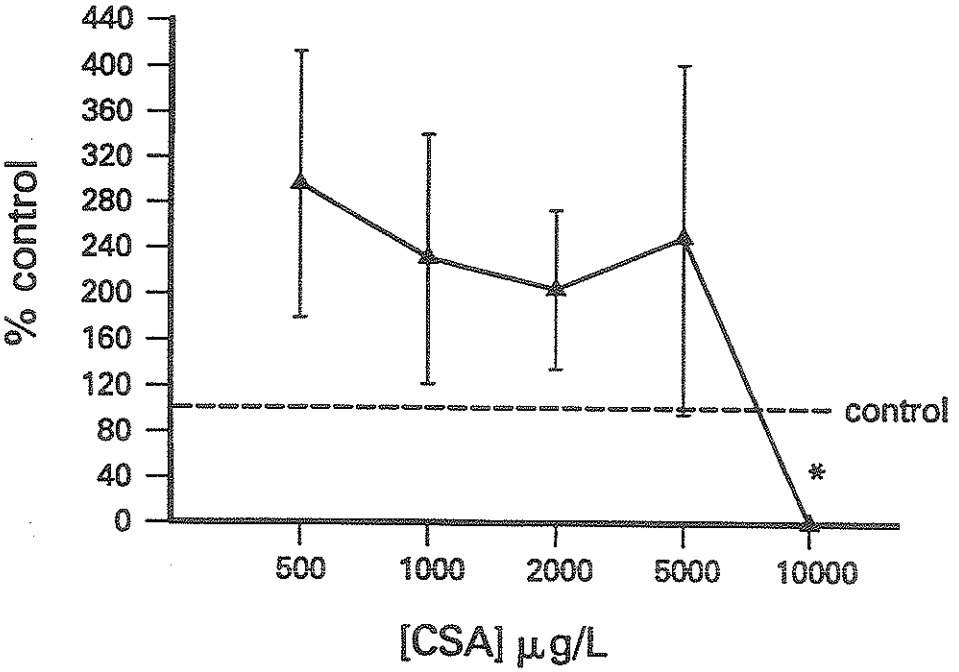
Further in vitro and in vivo animal studies followed by carefully designed prospective clinical trials are necessary to establish Rapamycin's role in transplantation.

Figure 1 **The effects of various concentrations of Cyclosporine A**

a) Effect on endothelial cell growth



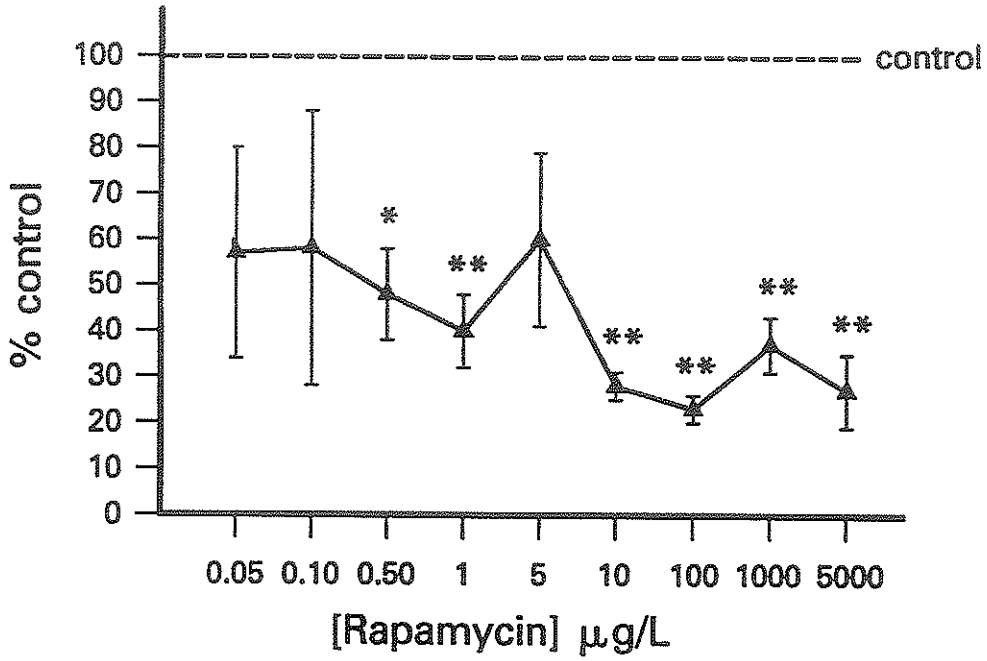
b) Effect on endothelial cell DNA synthesis



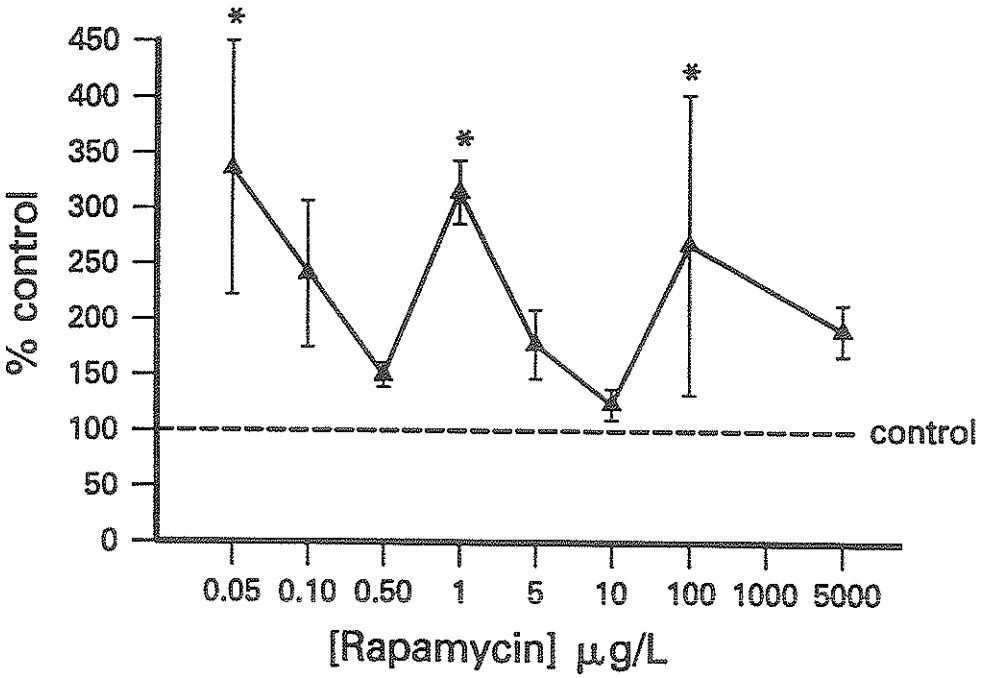
* $p < .05$

Figure 2 **The effects of various concentrations of Rapamycin**

a) Effect on endothelial cell growth



b) Effect on endothelial cell DNA synthesis

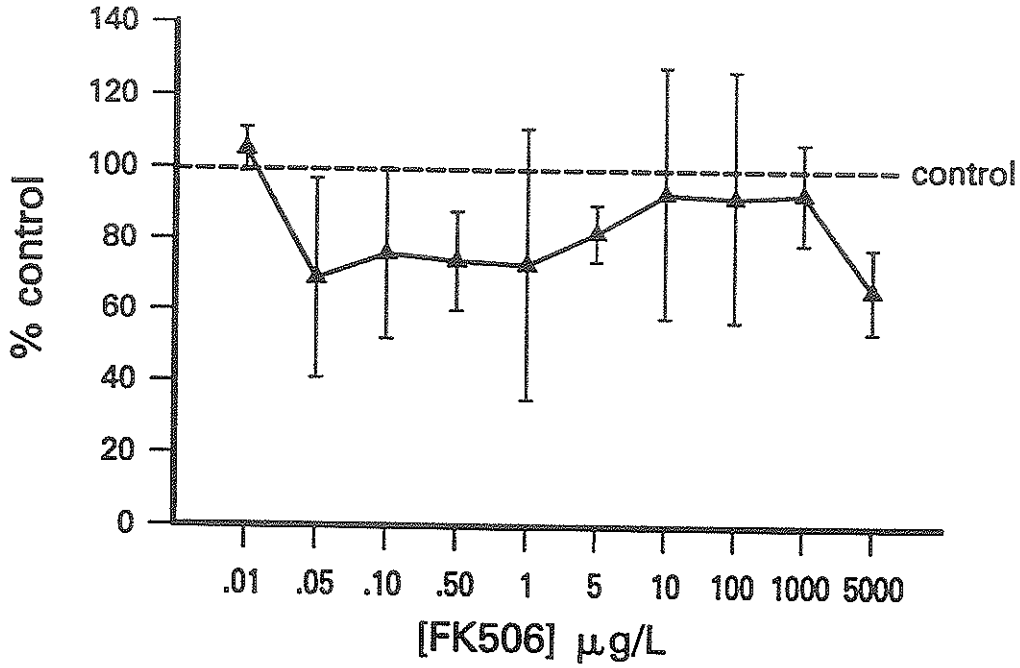


* $p < .05$

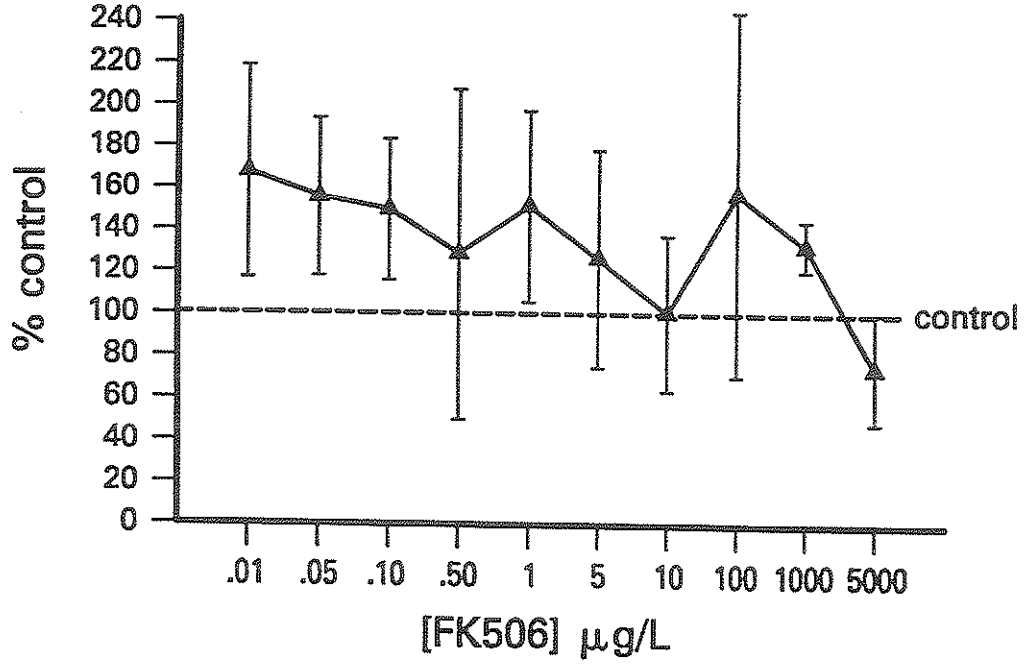
** $p < .01$

Figure 3 **The effects of various concentrations of FK-506**

a) Effect on endothelial cell growth



b) Effect on endothelial cell DNA synthesis



TGF-β. Results expressed as mean ± SEM (N = 8).

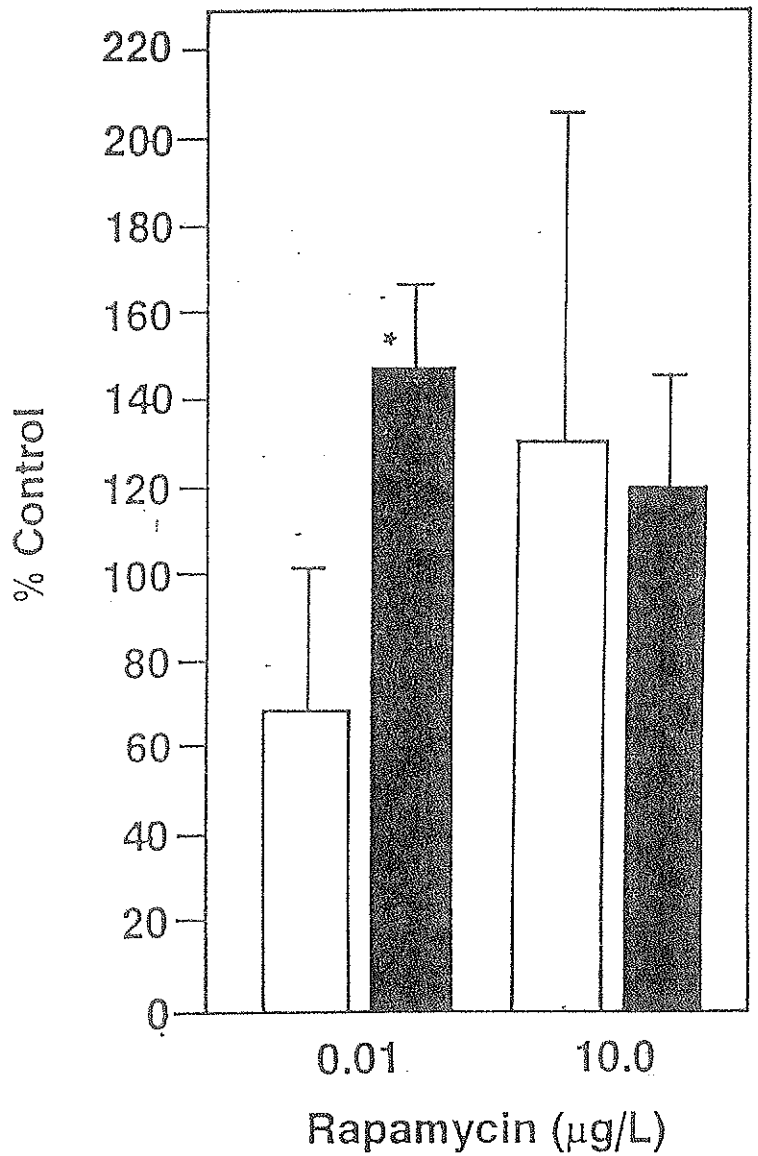


Figure 4 (b) Effect of FK-506 on release of endothelin from endothelial cells in the presence and absence of TGF-β. Results expressed as mean ± SEM (N = 8).

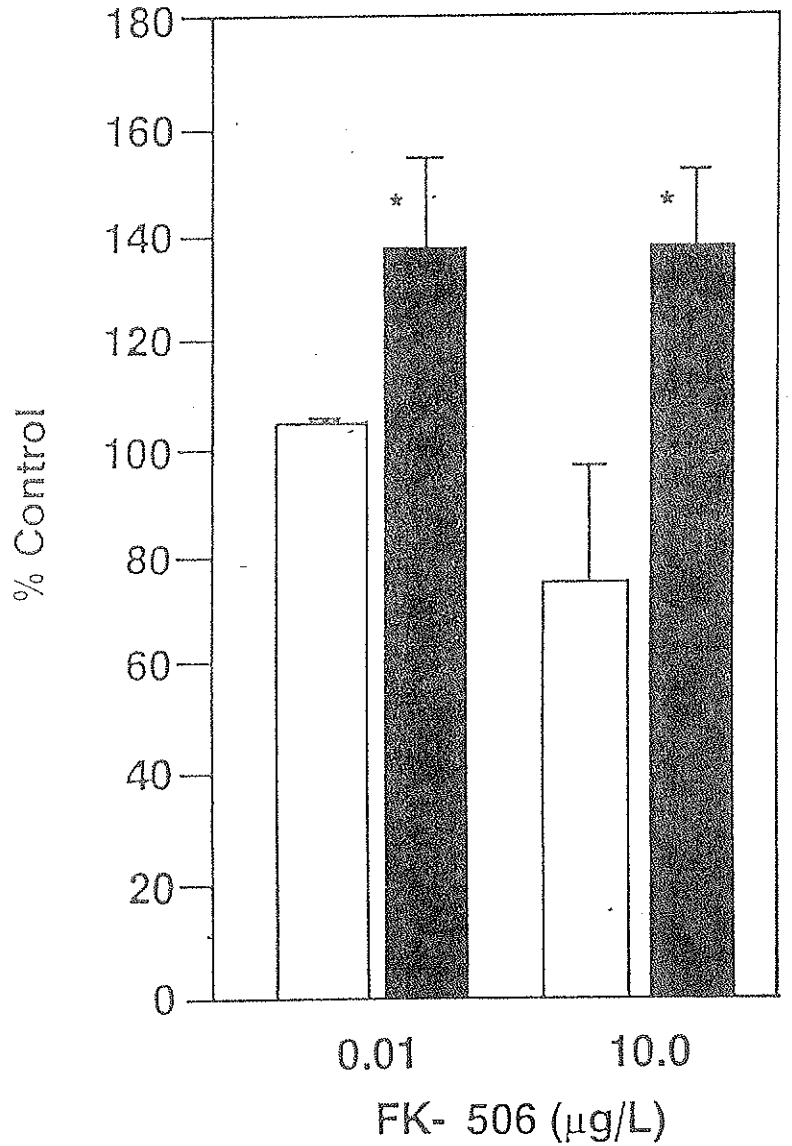


Figure 5 (a) Effects of Rapamycin on release of PGI_2 as measured by its stable metabolite 6-keto-$PGF_{1\alpha}$, from endothelial cells in the presence and absence of arachidonic acid. Results expressed as mean ± SEM (N = 8).

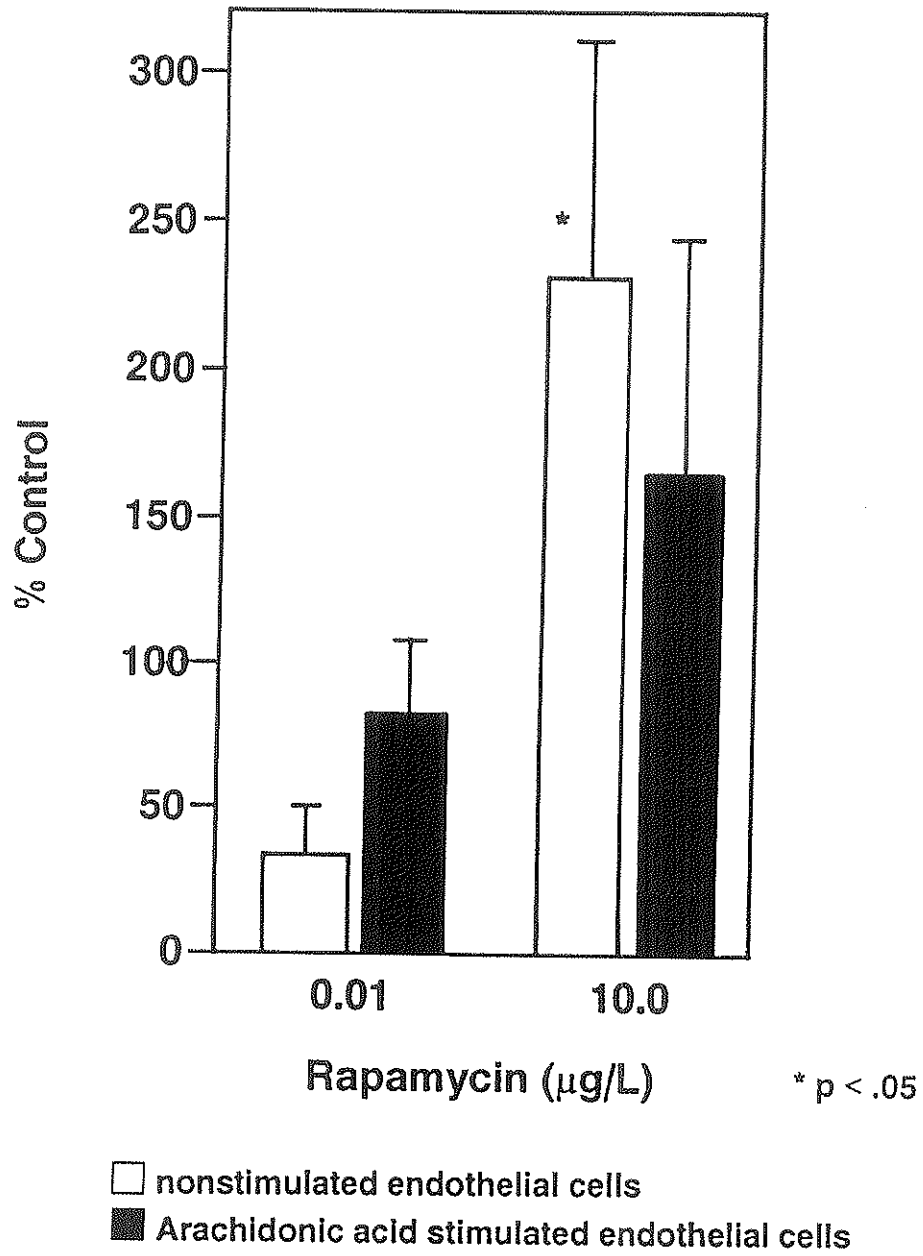


Figure 5 (b) Effect of FK-506 on release PGI_2 as measured by its stable metabolite 6-keto-$PGF_{1\alpha}$, from endothelial cells in the presence and absence of arachidonic acid. Results expressed as mean ± SEM (N = 8).

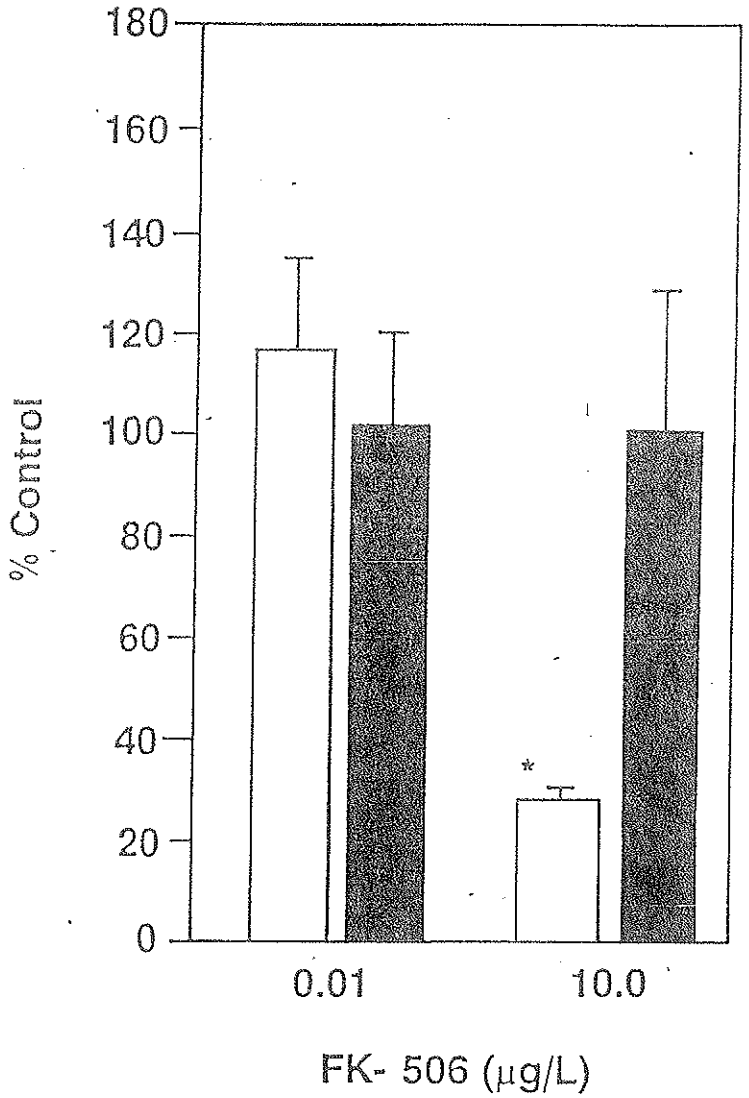


Figure 6 **Blood concentration after IV administration of Rapamycin to five rabbits. Results expressed as mean ± SEM.**

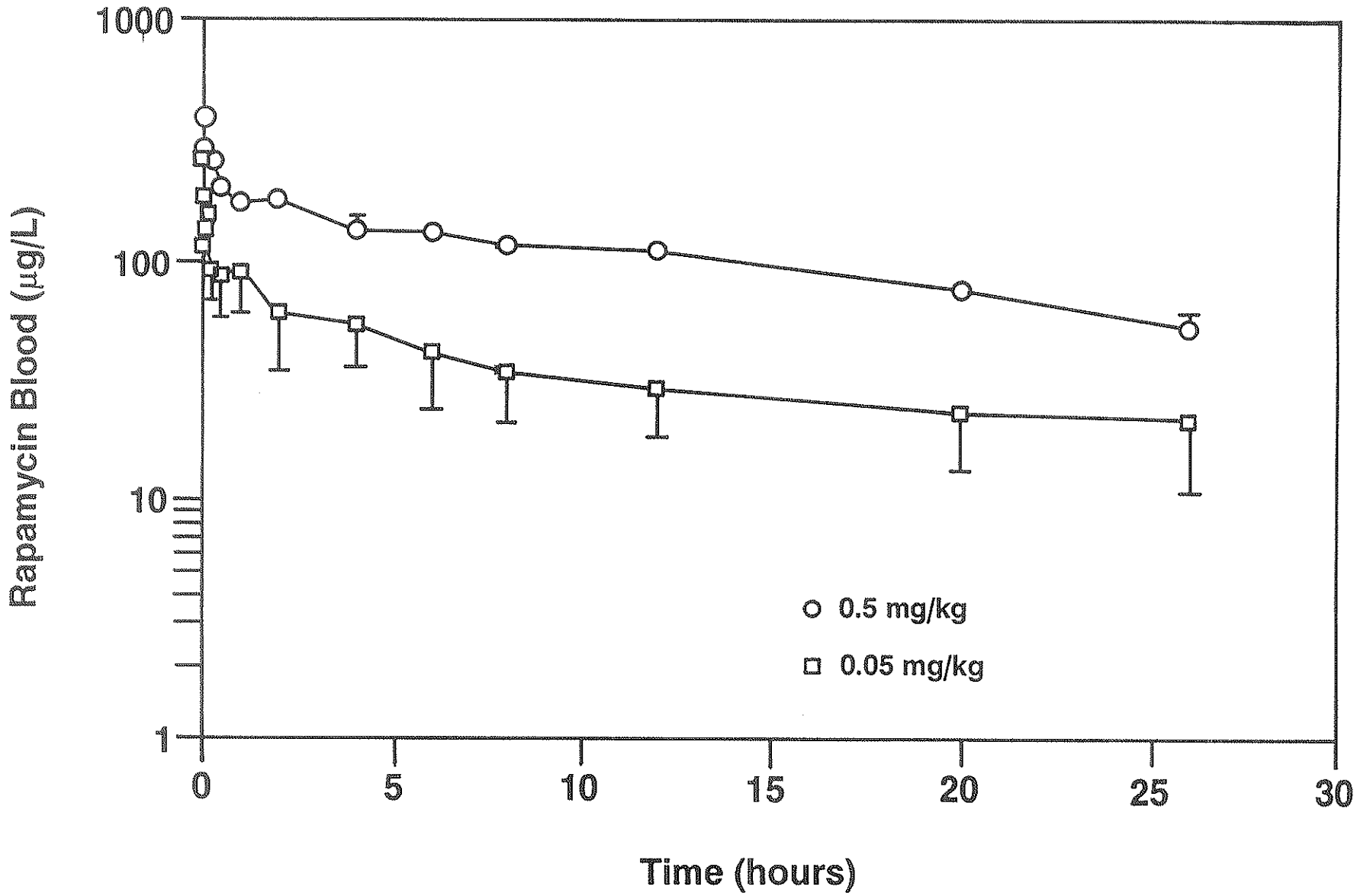


Figure 7.
Technique of non-sutured, silastic cuff vascular anastomosis

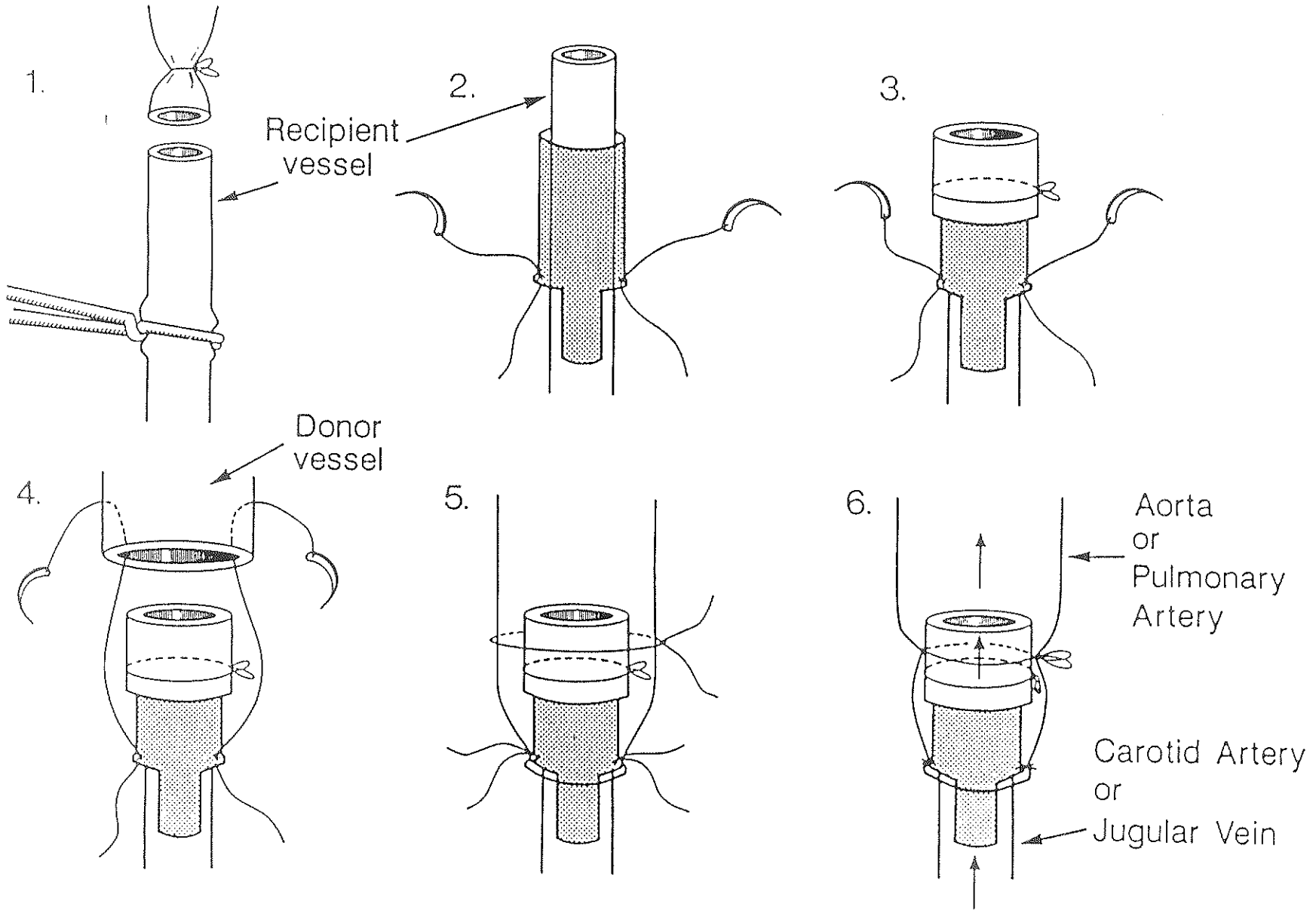


Figure 8
Animal and graft survival of rabbits which had undergone heterotopic hearts transplantation and were administered Rapamycin IV. daily at the dosages indicated.

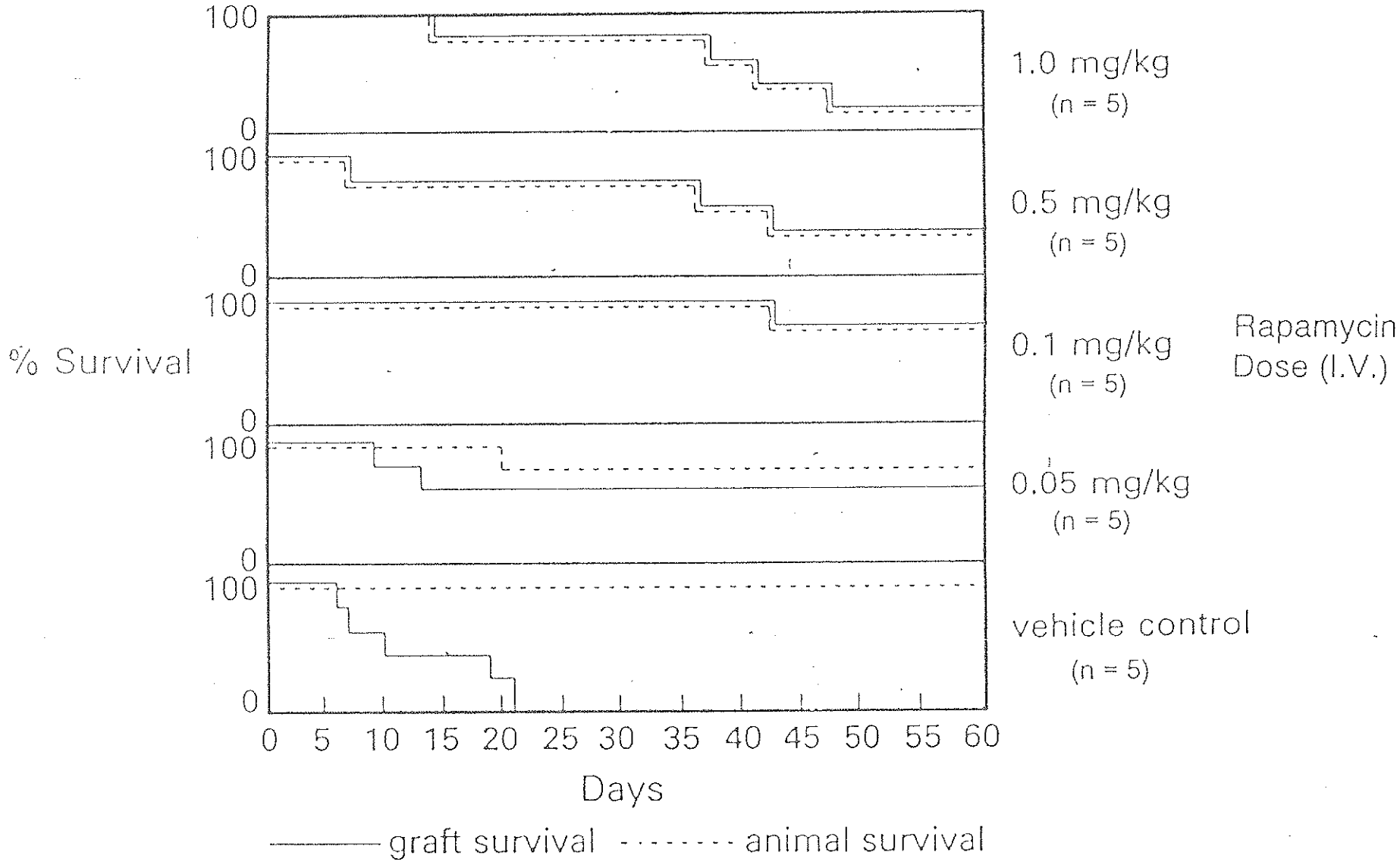


Figure 9

Animal and graft survival of rabbits which had undergone heterotopic heart transplantation and were administered Cyclosporine A IV daily at the doses indicated

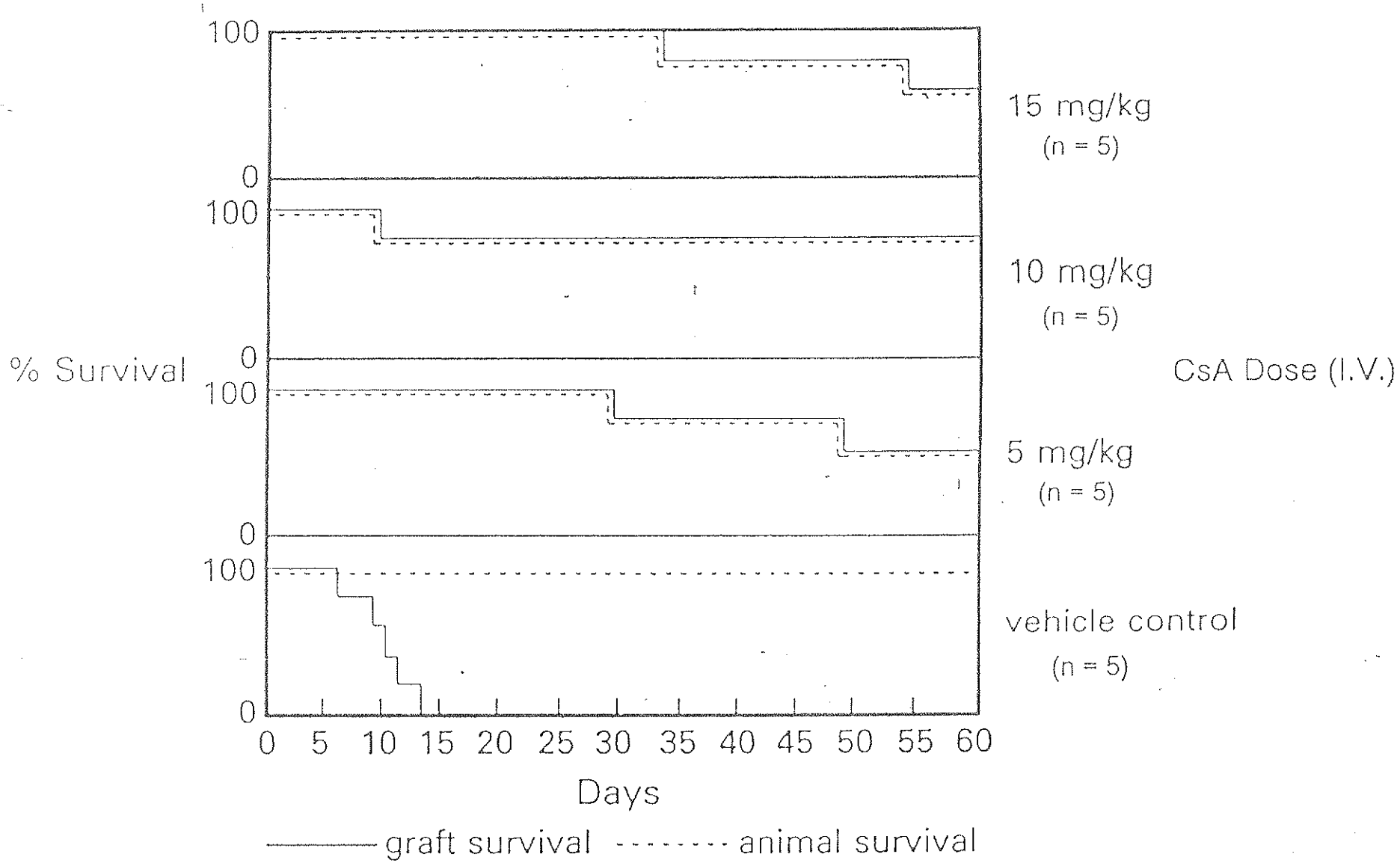


Figure 10
Trough blood concentrations of Rapamycin over the course of the study in individual animals given the four doses tested.

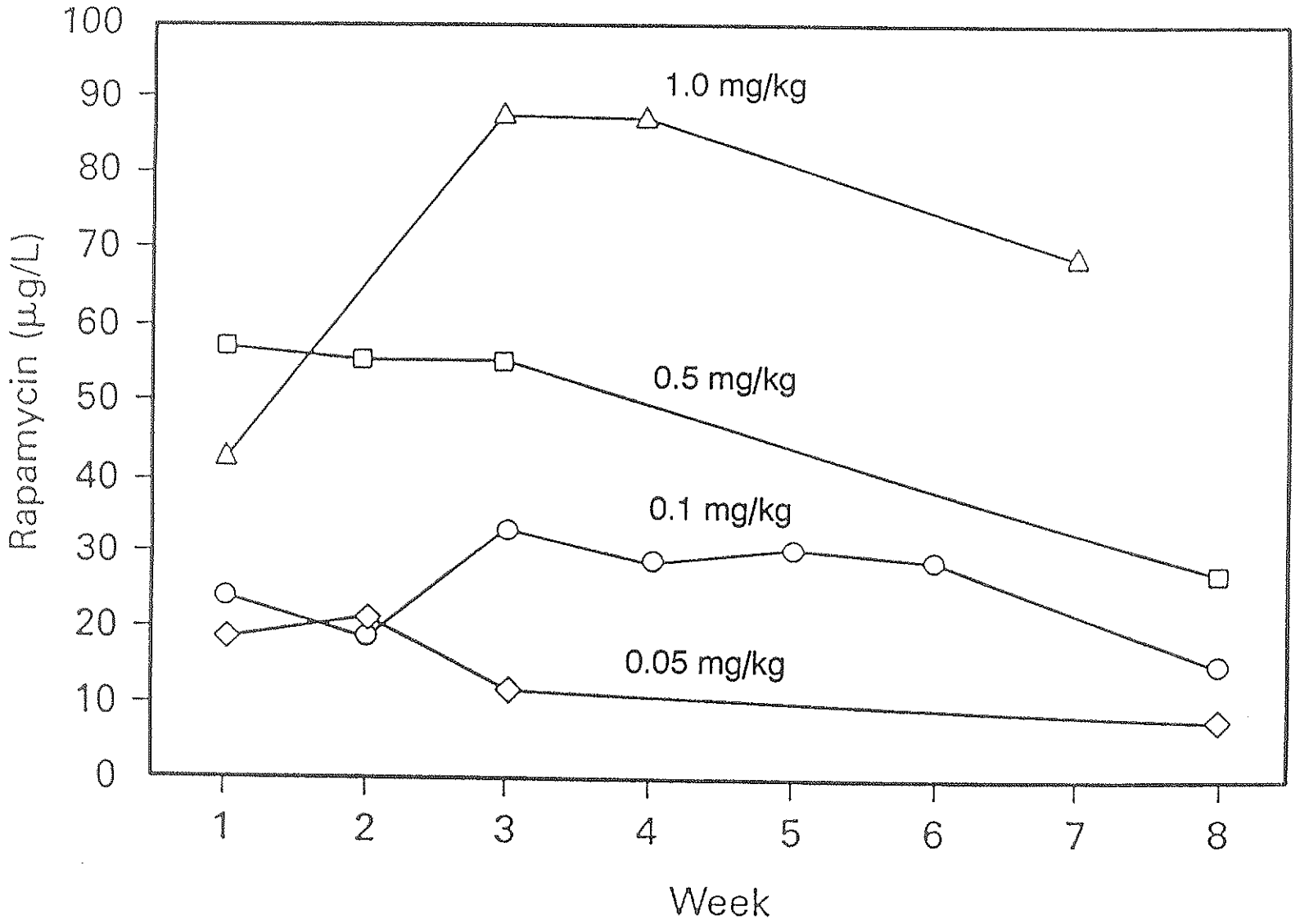


Figure 11

Changes in creatinine clearance and weight from preoperative assessment to in animals surviving more than 30 days of administration of Rapamycin at doses indicated. Results expressed as mean ± SD percentage change as compared to controls.

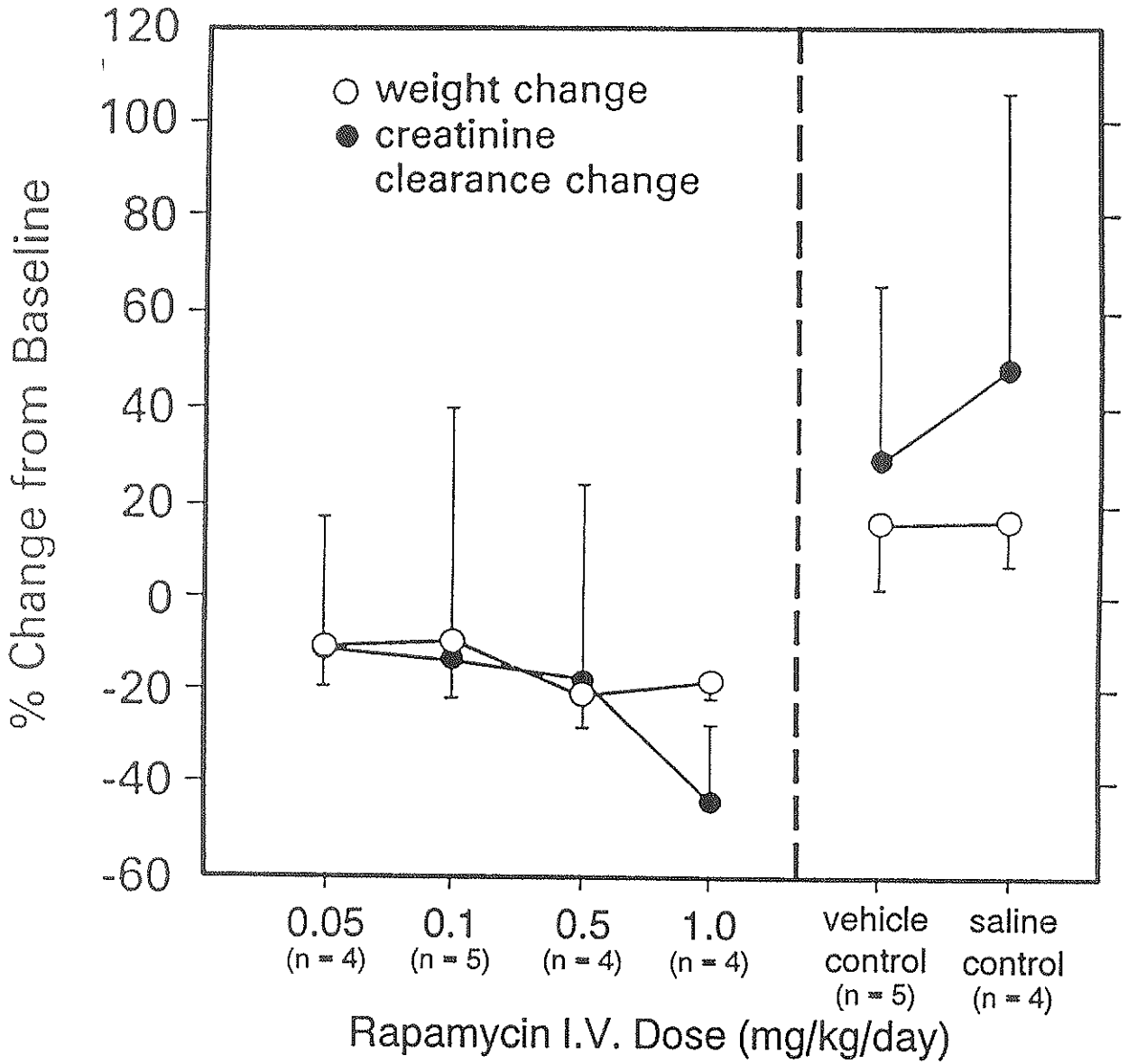
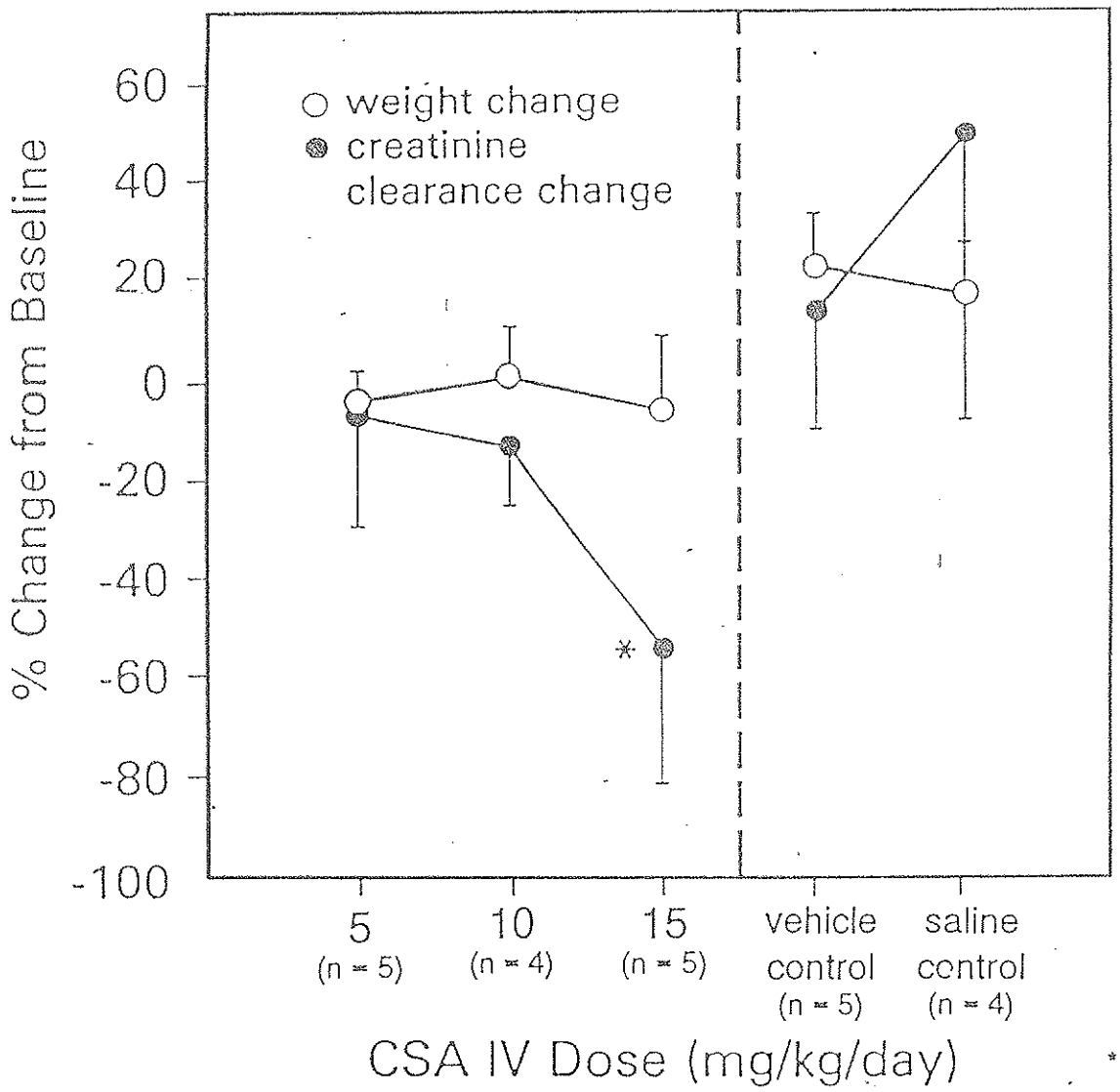


Figure 12

Changes in creatinine clearance and weight from preoperative assessment in animals surviving more than 30 days of administration of Cyclosporine A at doses indicated. Results expressed as mean ± SD percentage change as compared to controls.



* p < .05

TABLE 1

Endothelial cell growth and DNA synthesis in the absence of drug.

Vehicle Control	Average Cell Growth ($x10^4$ cells per ml)
CSA (N = 6)	32.50 ± 6.06
FK506 (N = 6)	34.00 ± 7.32
RPM (N = 6)	35.25 ± 11.52

Vehicle Control	Average DNA synthesis (CPM)
CSA (N = 6)	5307.07 ± 1486.47
FK506 (N = 6)	6005.71 ± 2996.66
RPM (N = 6)	5709.79 ± 1526.90

TABLE 2

Release of prostacyclin (6-keto-PGF$_{1\alpha}$) and endothelin in the absence of drug.

	6-Keto PGF$_{1\alpha}$ (pg/μg PROTEIN)	
	Basal	Arachidonic Acid Treated
Endothelial Cells	2018 ± 434	2048 ± 197
Mesangial Cells	311 ± 52	198 ± 67

	ENDOTHELIN (fmol/mg PROTEIN)	
	Basal	TGF-β Treated
Endothelial Cells	27.0 ± 11.4	65.4 ± 10.4*
Mesangial Cells	20.7 ± 7.3	16.7 ± 2.4

Results expressed as mean ± SEM (N = 8)

* $p < 0.05$

Table 3

Two compartment pharmacokinetic parameters obtained after IV administration of 0.05 and 0.5 mg/kg of rapamycin.

Dose (mg/kg)	Rabbit	t1/2 α (Hrs)	t1/2 β (Hrs)	Vc (L/kg)	Vβ (L/kg)	Vdss (L/kg)	Cl (ml/min/kg)
0.05	1	0.048	6.09	0.268	1.344	1.302	2.549
	2	0.077	5.07	0.322	1.036	0.999	2.362
	3	0.025	11.50	0.068	0.412	0.406	0.414.
	4	0.336	13.74	0.312	0.695	0.855	0.752
	5	0.040	5.03	0.130	0.511	0.498	1.173
Mean ± SEM		0.105 ± 0.058	8.29 ± 1.78	0.220 ± 0.051	0.840 ± 0.171	0.812 ± 0.164	1.450 ± 0.429
0.5	1	0.035	14.90	0.485	3.043	3.002	2.359
	2	0.449	11.91	1.833	1.707	2.465	1.656
	3	0.051	17.20	0.251	2.667	2.591	1.791
	4	0.270	16.09	1.380	3.775	3.669	2.712
	5	0.064	14.97	0.785	2.490	2.467	1.921
Mean ± SEM		0.174 ± 0.081	15.01 ± 0.86†	0.947 ± 0.241†	2.736 ± 0.339†	2.839 ± 0.229‡	2.088 ± 0.195

* $p < 0.05$
† $p < 0.01$
‡ $p < 0.001$

Table 4

Model-independent pharmacokinetic parameters obtained after IV administration of 0.05 and 0.5 mg/kg of rapamycin.

Dose (mg/kg)	Rabbit	t1/2 terminal (hrs)	Vd_{ss} (L/kg)	Cl (ml\min\kg)	MRT (hrs))
0.05	1	8.17	2.468	1.609	10.87
	2	9.47	1.239	1.629	12.67
	3	18.74	0.517	0.3288	26.22
	4	17.07	0.665	0.414	26.76
	5	10.60	0.614	0.904	11.82
Mean ± SEM		12.81 ± 2.14	1.101 ± 0.364	0.977 ± 0.279	17.67 ± 3.61
0.5	1	18.45	3.428	2.242	25.48
	2	11.22	1.958	2.268	14.39
	3	17.71	2.648	1.798	24.55
	4	11.57	2.618	2.608	16.74
	5	17.71	2.578	1.742	24.66
Mean ± SEM		15.33 ± 1.61	2.646 ± 0.233‡	2.132 ± 0.161*	21.16 ± 2.32

* $p < 0.05$

‡ $p < 0.001$

Table 5

Trough Blood Concentrations of Rabbits
Administered Rapamycin and CsA Doses Indicated

RPM		CsA	
Dose mg/kg/d	Concentration µg/L	Dose mg/kg/d	Concentration µg/L
0.05	18 ± 10	5.0	88 ± 35
0.1	23 ± 9	10.0	140 ± 45
0.5	59 ± 17	15.0	255 ± 125
1.0	67 ± 16		

Results expressed as mean ± sd of trough blood concentrations from all animals included in the study from start until endpoints were reached. Each value represents the concentration from a minimum of 20 measurements.

Table 6
Comparison of Biochemical Parameters at The Beginning and End of Study for Animals Administered Rapamycin at Doses Indicated [a]

URINE	0.5 mg/kg/d (n = 5)	0.1 mg/kg/d (n = 5)	0.5 mg/kg/d (n = 5)	1.0 mg/kg/day (n = 5)
Sodium (mmol/L)	42[b] ± 39.5 17[c] ± 9.5	42 ± 41.9 11 ± 1.7	55 ± 37.2 18[d] ± 15.2	234 ± 19.8 24 ± 19.8
Potassium (mmol/L)	107 ± 68.9 61 ± 34.7	118 ± 162.9 60 ± 51.4	127 ± 12.2 55[d] ± 23.9	83 ± 34.6 47[d] ± 35.7
Creatinine (mmol/L)	4.8 ± 2.9 7.8 ± 6	9.7 ± 7.1 5.5 ± 4.1	6.9 ± 2.3 7.8 ± 3.9	5.1 ± 1.8 5.0 ± 2.8
Creatinine Clearance	4.57 ± 1.88 4.06 ± 2.29	5.03 ± 2.14 4.37 ± 3.00	5.06 ± 0.72 4.21 ± 3.21	4.75 ± 1.81 2.65 ± .39
SERUM				
Sodium (mmol/L	138 ± 3.5 132 ± 11.3	138 ± 3.1 145 ± 13.2	142 ± 2.8 138 ± 5.1	140 ± 1.8 147 ± 15.9
Potassium (mmol/L)	5.5 ± 0.4 4.9 ± 0.3	6.0 ± 2.2 3.6 ± 0.4	5.3 ± 0.8 3.9 ± 1.4	5.2 ± 0.6 4.2 ± 0.5
Glucose (mmol/L)	9.3 ± 2.7 14.1 ± 7.5	8.0 ± 2.2 13.7 ± 5.7	7.8 ± 1.0 9.2 ± 2.4	8.0 ± 1.0 11.0[d] ± 2.0
Urea (mmol/L)	8.3 ± 3.6 15.8 ± 14.4	6.7 ± 1.0 14.8 ± 16.6	6.7 ± 1.0 7.9 ± 5.6	7.1 ± 0.8 9.0 ± 4.5
Creatinine (mmol/L)	113 ± 36.8 176 ± 60.5	98.0 ± 11.7 252.0 ± 294.0	105 ± 9.6 148 ± 72.2	108 ± 19.9 142 ± 44.1
Calcium (mmol/L)	3.2 ± 0.4 2.9 ± 0.4	3.4 ± 0.1 3.4 ± 0.4	3.3 ± 0.2 2.5 ± 0.9	3.4 ± 0.1 3.2 ± 0.4
Bilirubin (µmol/L)	1 ± 0.0 3 ± 1.5	2 ± 1.0 5 ± 2.5	1 ± 0.4 2 ± 1.1	1 ± 0.0 3[d] ± 1.1
Uric Acid (µmol/L)	32 ± 11.2 78 ± 148.0	73 ± 57.7 8 ± 2.2	31 ± 17.3 46 ± 83.8	28 ± 12.0 6[d] ± 3.1
ALT U/L	66 ± 134.1 67 ± 33.2	40 ± 18.8 64 ± 40.5	37 ± 10.5 47 ± 14.9	44 ± 5.8 52 ± 36.6
ALK U/L Phos	214 ± 80.0 67 ± 37.6	147 ± 32.3 76 ± 15	190 ± 80.5 58 ± 42.2	148 ± 55.9 54[d] ± 14.8
AST U/L	34 ± 19.1 88 ± 132.8	30 ± 12.2 41 ± 22.7	20 ± 8.7 31 ± 11.3	21 ± 8 28 ± 19.2

[a] Results expressed as Mean ± SD of Groups (N=5)
[b] Biochemical Parameters Pre-OP
[c] Biochemical Parameters at end point
[d] Statistiacally significant ($p < 0.05$) from values at beginning of study

Table 7

Comparison of Biochemical Parameters at the Beginning and End of Study for Animals Administered Cyclosporine at Doses Indicated [a]

URINE	5.0 mg/kg/d (n = 5)	10.0 mg/kg/d (n = 5)	15.0 mg/kg/d (n = 5)
Sodium (mmol/L)	19[b] ± 9.4 16[c] ± 8.6	22 ± 11 10 ± 0	29 ± 17.7 10 ± 0.9
Potassium (mmol/L)	106 ± 57.4 66 ± 25.5	88 ± 44.1 26 ± 13.5	83 ± 59.4 30 ± 19.6
Creatinine (mmol/L)	6.3 ± 4.2 12.8 ± 7.1	4.7 ± 2.1 2.1 ± 1	4.6 ± 2.5 2.7 ± 1.3
Creatinine Clearance (mL/min)	4.78 ± 1.13 4.44 ± 1.62	4.00 ± 1.04 3.53 ± .26	5.67 ± 1.71 2.60[d] ± 1.70
SERUM			
Sodium (mmol/L)	134 ± 4.3 139 ± 3.8	140 ± 3.0 138 ± 2.7	138 ± 3.0 141 ± 7.2
Potassium (mmol/L)	5.7 ± 0.9 4.3 ± 0.7	4.9 ± 3.8 4.2 ± 1.6	5.2 ± 0.8 4.7 ± 1.8
Glucose (mmol/L)	13.9 ± 12.9 11.9 ± 2.4	7.5 ± 2.0 13.0 ± 4.9	7.9 ± 1.8 14.8 ± 6.5
Urea (mmol/L)	7.0 ± 3.6 8.3 ± 5.3	7.7 ± 1.8 5.6 ± 1.0	6.6 ± 0.7 27.8 ± 22.0
Creatinine (µmol/L)	105 ± 19.8 139 ± 44.8	112 ± 14.2 105 ± 28.2	99 ± 9.1 226 ± 178.3
Calcium (mmol/L)	3.3 ± 0.1 2.9[d] ± 0.2	3.3 ± 0.5 3.1 ± 0.2	3.4 ± 0.1 2.6[d] ± 0.4
Billirubin (µmol/L)	2 ± 0.9 1 ± 1.5	1 ± 0.4 2 ± 0.8	1 ± 0.9 8 ± 10.0
Uriac Acid (µmol/L)	33 ± 29.2 14 ± 7.9	30 ± 14.6 15[d] ± 14.3	27 ± 13.2 21 ± 20.0
ALT U/L	41 ± 7.4 36 ± 12.3	41 ± 27.0 33 ± 8.0	39 ± 8.2 115 ± 182.6
ALK U/L Phos	153 ± 63.2 66[d] ± 33.4	210 ± 79.9 86[d] ± 42.7	188 ± 132.4 246 ± 373.0
AST U/L	19 ± 7.2 28 ± 11.4	23 ± 10.3 20 ± 5.2	15 ± 2.2 46 ± 73.8

a Results expressed as mean ± SD of Groups (N=5)
b Biochicical Parameters Pre-OP
c Biochemical Parameters at end points
d Statistically significant ($P < 0.05$) from values at beginning of study

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