

# ***In-vitro* Release of Rapamycin from a Thermosensitive Polymer for the Inhibition of Vascular Smooth Muscle Cell Proliferation**

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Received March 31, 2009; Accepted May 05, 2009; Published May 06, 2009

**Citation:** Zhu W, Masaki T, Cheung AK, Kern SE (2009) *In-vitro* Release of Rapamycin from a Thermosensitive Polymer for the Inhibition of Vascular Smooth Muscle Cell Proliferation. J Bioequiv Availab 1: 003-012. doi:10.4172/jbb.1000002

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## **Abstract**

Hemodialysis arteriovenous grafts are often plagued by stenosis at the anastomosis, which is due to the proliferation of vascular smooth muscle cells (SMCs). To prevent the stenosis, we have been developing a strategy for the sustained perivascular delivery of an antiproliferative agent, rapamycin, using an injectable biodegradable polymer, ReGel®. In this study we examined the *in-vitro* kinetics of rapamycin released from ReGel and its efficacy for inhibiting the proliferation of human and porcine venous and arterial SMCs. To study the release from ReGel, rapamycin was mixed with ReGel and incubated at 37°C in a release medium. The release medium was periodically sampled and assayed for rapamycin concentration by UV. Cellular uptake and release of rapamycin were examined by incubating SMCs with rapamycin for various durations. Intracellular drug was extracted and measured by HPLC. Antiproliferative effects and cytotoxicity of stock rapamycin and that released from ReGel were examined using cell counting and lactate dehydrogenase (LDH)-release assay, respectively. Rapamycin exhibited a sustained-release pattern from ReGel for 52 days. The kinetics of rapamycin transport through the cell membrane was compatible with a passive diffusion mechanism. Rapamycin released from ReGel exhibited antiproliferative activity similar to the free drug. Our results support the concept of sustained delivery of rapamycin using ReGel as a promising strategy to inhibit SMC proliferation for the prevention of hemodialysis arteriovenous graft stenosis.

## **Introduction**

The arteriovenous polytetrafluoroethylene (PTFE) graft has served as a predominant form of vascular access for chronic hemodialysis in the United States. Unfortunately, graft failure occurs frequently, with most cases due to stenosis at the anastomosis followed by thrombosis. The pathology of the stenosis is usually neointimal hyperplasia, which primarily results from the proliferation and migration of vascular smooth muscle cells (SMCs) (Swedberg et al., 1989). In recent years, the porcine arteriovenous graft model has been increasingly used for the investigation of the pathogenesis of the stenosis (Johnson et al., 2001; Kelly et al., 2002; Rotmans et al., 2003). This model has demonstrated

that the neointima forms early, within 2-3 weeks following graft placement. In dialysis patients, hemodynamically significant stenosis appears to develop progressively, partly attributed to the chronic turbulent blood flow and compliance mismatch between the graft and native vessels. Thus the continuous inhibition of SMC proliferation using antiproliferative agents can be a logical strategy to prevent the stenosis.

Rapamycin is a drug with potent inhibitory activities on the proliferation and migration of human and rat aortic SMCs *in-vitro* (Marx et al., 1995; Poon et al., 1996). Its

antiproliferative activity is believed to be mediated by its binding to a 12-kDa intracellular molecule, the FK506 binding protein (FKBP12) (Bierer et al., 1990). The rapamycin-FKBP complex inhibits the activity of a kinase known as mammalian target of rapamycin (mTOR), resulting in an increased level of cyclin-dependent kinase inhibitors (CDKI) with the subsequent cell cycle arrest in G<sub>1</sub>/S transition (Gallo et al., 1999).

Our group has been investigating perivascular drug delivery using a biodegradable polymer, ReGel<sup>®</sup>, which provides a platform for sustained release of the drug from the adventitial side of the graft-vessel anastomosis (Kuji et al., 2006; Masaki et al., 2004; Zentner et al., 2001; Zhu et al., 2006). ReGel is thermosensitive which allows for easy mixing with rapamycin and also easy injection into the physiologic site of interest. This enables the drug depot to be replenished if necessary which may be crucial to this application since the graft is continually subjected to stimuli which would encourage SMC proliferation.

As an initial step in this strategy, we examined the *in-vitro* release kinetics of rapamycin from ReGel, the transport properties of the drug in SMCs, and the pharmacodynamics of the drug after release from ReGel. In preparation for testing in the porcine model and clinical trials, these experiments were performed in both human and porcine SMCs. In addition, since stenosis develops in both venous and arterial anastomoses of the hemodialysis graft with increasing evidence that venous and arterial SMCs may respond differently to various antiproliferative drugs, both venous and arterial SMCs were examined (Kim et al., 2004; White et al., 2000; Yang et al., 1998). The ultimate objective is to investigate the potential of this combination of delivery system and drug for the prevention of vascular smooth muscle cell proliferation, a primary cause of hemodialysis arteriovenous graft stenosis.

## Materials and Methods

All animal experiments were conducted with approval from the IACUC of the University of Utah and the Salt Lake Veterans Administration Research Center.

### Materials

Rapamycin was purchased from LC Laboratories (Woburn, MA). ReGel<sup>®</sup> was obtained from MacroMed Inc (Sandy, UT). Two individual cell lines of human saphenous venous and aortic SMCs, and smooth muscle growth medium-2 bullet kit were purchased from Clonetics (Walkersville, MD). The smooth muscle growth medium was supplemented with 10% fetal calf serum, 5 µg/ml insulin, 0.5 ng/ml human recombinant epidermal growth factor

(EGF), 2 ng/ml human recombinant fibroblast growth factor (FGF), 50 µg/ml gentamicin and 50 ng/ml amphotericin-B. Fluorescein isothiocyanate (FITC)-conjugated anti-human smooth muscle actin and lactate dehydrogenase (LDH) assay kits (substrate, cofactor, dye and lysis solution) were purchased from Sigma-Aldrich (St Louis, MO).

### In-vitro Release of Rapamycin from ReGel

Rapamycin was mixed with 0.4 ml of ReGel at 4°C to form a 10 mg/ml liquid mixture in a 20-ml vial. It was then maintained at 37°C to form a gel in the bottom of the vial. Release medium (15 mL) made from PBS-ethanol (90:10 v/v, pH 7.6) (Alexis et al., 2004) was added to the vial. The vial was incubated in a 37°C water bath with agitation at 50 rpm. Medium was sampled daily from the vial and replaced with fresh medium. Rapamycin concentration was measured in the sampled medium by UV spectrometry (Ultrospec III, Pharmacia LKB, McAllen, TX) at 277 nm, using standards of 0-20 µg/ml.

To assess whether rapamycin released from ReGel altered its anti-proliferative activity, rapamycin (10 mg/ml in 0.4 ml of ReGel) or ReGel alone (0.4 ml) was incubated in 15 ml of culture medium at 37°C for 24 hrs. The medium was assayed for rapamycin concentration and pH, and then diluted to 0.1, 10 and 100 ng/ml. These solutions of rapamycin were then used in cell culture experiments as described below.

### Cell Culture

Porcine venous and arterial SMCs were isolated from the normal femoral veins and arteries of two Yorkshire cross domestic pigs and cultured. Briefly, the femoral veins or arteries (5-cm length) were minced into pieces of approximately 1 mm<sup>3</sup> in size. Endothelial cells were detached from the segments by incubating with collagenase A (5 mg/ml) at 37°C for 10 min. The remaining SMCs were cultured in the culture medium at 37°C in a humidified 5% CO<sub>2</sub> incubator. SMCs were identified by their typical elongated swirling and overlapping morphology under the light microscope and positive staining with FITC-conjugated anti-smooth muscle actin. Human venous and arterial SMCs were also grown in the culture medium at 37°C in a humidified 5% CO<sub>2</sub> incubator. Cells from passage 3-5 were used in experiments as described below.

### Cellular Uptake and Release of Rapamycin

Human venous SMCs were seeded at a density of 1×10<sup>5</sup> cells per well in a 12-well plate and cultured for 24 hrs. Rapamycin was dissolved in ethanol and diluted in culture medium to a final ethanol concentration of 0.5% (v/v). For uptake measurement, cells were incubated with 5, 15 or 25

$\mu\text{g/ml}$  of rapamycin at  $37^\circ\text{C}$  for incremental durations. For release measurement, cells were incubated with 5, 15 or 25  $\mu\text{g/ml}$  of rapamycin at  $37^\circ\text{C}$  for 2 hrs and further incubated in a fresh drug-free medium for incremental durations. At the end of incubation, the cells were washed with PBS, and the intracellular rapamycin was extracted with a Triton X-100 solution (the lysis buffer from a LDH assay kit). After centrifugation (TJ-6, Beckman Coulter, Fullerton, CA) at 3000 rpm for 5 min, 50  $\mu\text{l}$  of the supernatant was subjected to HPLC analysis.

High pressure liquid chromatography (HPLC) was performed using a Waters HPLC system (Waters Corporation, Milford, MA) consisting of dual pumps, an autosampler and a photodiode array detector. A  $C_{18}$  analytic column (150 mm  $\times$  4.6 mm, 3  $\mu\text{m}$ , 100  $\mu\text{m}$ , Ascentis<sup>®</sup> column, Sigma-Aldrich, St Louis, MO) was used preceded by a  $C_{18}$  guard column (2 cm  $\times$  4.0 mm, 5  $\mu\text{m}$ , 100  $\mu\text{m}$ , Ascentis<sup>®</sup> Supelguard<sup>®</sup> cartridge, Sigma-Aldrich, St Louis, MO). The mobile phase containing a mixture of 42% acetonitrile, 30% methanol and 28% water, was filtered and degassed. HPLC was carried out at the flow rate of 1 ml/min, with the analytic column temperature set at  $45^\circ\text{C}$ , the UV detection at 270-290 nm, and the running time per sample of 30 min. Rapamycin concentration was calculated by the peak height. Rapamycin concentrations of 10 - 10,000 ng/ml were used as standards. With the injection volume of 50  $\mu\text{l}$ , the minimum detectable concentration of rapamycin was 10 ng/ml. In addition to the drug extracted from the cells, the drug concentration in the remaining medium was also measured to determine the total amount in each experiment. Using the mass balance, the recovery rate of rapamycin from the cells was determined to be  $96.9 \pm 3.8\%$ .

### Cell Proliferation Assay

To examine if the incorporation of rapamycin into ReGel affects the pharmacological activity of the drug, rapamycin released from ReGel was tested for cell proliferation. Human and porcine venous and arterial SMCs were seeded at a density of  $2.5 \times 10^4$  cells per well in a 12-well plate and cultured for 24 hrs. The culture medium was replaced with a fresh medium containing various concentrations of rapamycin that had been released from ReGel as described above (0.1, 10 and 100 ng/ml) or from stock rapamycin (0.001 ng/ml - 1 mg/ml). A vehicle of 0.5% (v/v) ethanol in culture medium served as a control. The cells were cultured for 72 hrs and the proliferation was assessed by cell counting using a hemocytometer after trypsin digestion.

### Cytotoxicity Assay

Human and porcine venous and arterial SMCs were seeded

at a density of  $2.5 \times 10^3$  cells per well in a 96-well plate and cultured for 24 hrs. After incubation with various concentrations of free rapamycin (0.001 ng/ml - 1 mg/ml) or rapamycin released from ReGel (0.1, 10 or 100 ng/ml) for 72 hrs, the plate was centrifuged at 1000 rpm for 4 min. The supernatant containing the released lactate dehydrogenase (LDH) from the damaged cells was set aside. The cells remaining in the plate were lysed to release the intracellular LDH. The supernatant and the cell lysate were separately subjected to the LDH assay. In brief, 50  $\mu\text{l}$  of the mixture of LDH-assay substrate, cofactor and dye solution (1:1:1) was added to each well, and the plate was incubated at room temperature for 30 min, followed by the addition of 15  $\mu\text{l}$  of 1N HCl to each well. The absorbance at 492 nm ( $A_{492}$ ) was measured with the reference wavelength of 690 nm. The percentage of LDH released from damaged cells was calculated as  $A_{492}$  of released LDH / ( $A_{492}$  of released LDH +  $A_{492}$  of LDH from lysed cells)  $\times$  100.

### Data Analysis

Each experiment was performed 3-6 times on separate days. All values were expressed as mean  $\pm$  SD. Mean values were compared using one-way or two-way ANOVA with the Tukey test for multiple comparisons. P value less than 0.05 was considered significant.

The data on *in-vitro* drug release from ReGel were further analyzed. A mathematical model for drug release from a biodegradable matrix has been proposed with the following assumptions. (1) Drug release follows the mechanism of drug diffusion and polymer degradation that occur simultaneously. (2) The initial drug loading is well above the solubility of the drug within the matrix that mimics a pseudo-steady state of drug diffusion. (3) Polymer matrix undergoes a homogeneous erosion process and the chain cleavage follows first-order kinetics. The following equation for the cumulative absolute amount of drug released,  $Q$ , versus time  $t$  has been derived (Charlier, Leclerc, and Couarraze 2000):

$$Q = S \sqrt{\frac{2C_0 C_s D_0 (e^{kt} - 1)}{k}} \quad (1)$$

Where  $S$  is surface area of the matrix exposed to the release medium;  $C_0$  and  $C_s$  are initial drug concentration and drug solubility in the matrix, respectively;  $D_0$  is drug diffusion coefficient at time 0; and  $k$  is polymer degradation rate constant. At early time points ( $t$  near 0),  $e^{kt} \approx 1 + kt$ , therefore

$$Q = S \sqrt{2C_0 C_s D_0 t} \quad (2)$$

Thus, the product of  $C_s$  and  $D_0$ , could be an indicator of the released drug amount at early time points, given the constant values of  $S$  and  $C_0$ . Rapamycin release data were fitted to Equation 1 using the nonlinear regression function in SigmaPlot (Systat Software, Point Richmond, CA) to determine  $D_0$  and  $k$ . We have previously examined the *in-vitro* release kinetics of a relatively hydrophilic drug, dipyridamole, from ReGel or polymeric microspheres mixed with ReGel (Zhu et al., 2006). Dipyridamole release data were also analyzed for comparison with rapamycin.

The data on cellular uptake of rapamycin were further analyzed by calculating the uptake rate constant  $k_u$  and release rate constant  $k_r$ . Assuming a two-compartment model for drug permeation between the extracellular medium (compartment 1) and the intracellular space (compartment 2), and the first-order kinetics of drug permeation, intracellular rapamycin concentration  $C_2$  at time  $t$  can be expressed as:

$$V_2 \frac{dC_2}{dt} = V_1 C_1 k_u - V_2 C_2 k_r \quad (3)$$

Where  $V_1$  and  $V_2$  are volume of the extracellular medium and the intracellular space, respectively; and  $C_1$  is rapamycin concentration in the extracellular medium, respectively. Let  $C_0$  represent the drug concentration in the medium at the beginning of incubation. Substitution of  $V_1 C_1$  by  $(V_1 C_0 - V_2 C_2)$  gives:

$$V_2 \frac{dC_2}{dt} = (V_1 C_0 - V_2 C_2) k_u - V_2 C_2 k_r \quad (4)$$

By integration of Equation 4 when  $C_2 = 0$  at time 0,  $C_2$  at time  $t$  can be expressed as:

$$C_2 = \frac{V_1 C_0 k_u}{V_2 (k_u + k_r)} (1 - e^{-(k_u + k_r)t}) \quad (5)$$

At 2 hours when cellular uptake of rapamycin reached equilibrium, intracellular drug concentration  $C_{2,ss}$  can be expressed as:

$$C_{2,ss} = \frac{V_1 C_0 k_u}{V_2 (k_u + k_r)} \quad (6)$$

Therefore,

$$\frac{C_2}{C_{2,ss}} = 1 - e^{-(k_u + k_r)t} \quad (7)$$

Rapamycin cellular uptake data were fitted to Equation 7 for the calculation of  $k_u$  and  $k_r$  using the nonlinear regres-

sion function in SigmaPlot.

Temperature-dependent cellular uptake data were fitted to the following bi-exponential equation using the nonlinear regression function in SigmaPlot.

$$M = ae^{bT} \quad (8)$$

Where  $M$  is the percentage of intracellular rapamycin amount at the steady state at temperature  $T$  relative to that at 37°C;  $a$  and  $b$  are factors related to the membrane transport properties of rapamycin. The mechanism of drug transport through the lipid bilayer of the cell membrane can be reflected by the temperature coefficient  $Q_{10}$ , defined as the factor by which the intracellular drug concentration at the steady state ( $C_{ss}$ ) increases as the temperature is raised by 10°C. A larger  $Q_{10}$  suggests a larger activation energy involved (Hille 1984; Stein 1967), which is related to the diffusion mechanism. Low temperature dependence with a low  $Q_{10}$  value ( $< 2$ ) is suggestive of a passive diffusion mechanism. High temperature dependence with a high  $Q_{10}$  value ( $> 6$ ) suggests a facilitated diffusion or active transport process. Moderate  $Q_{10}$  between 2 to 6 cannot directly indicate the diffusion mechanism (Lane et al., 1987).  $Q_{10}$  can be calculated as:

$$Q_{10} = e^{10b} \quad (9)$$

The concentrations of rapamycin which produce 50% of the maximal antiproliferative effect ( $EC_{50}$ ) and induce 50% of the maximal lethal (toxic) effect ( $LC_{50}$ ) were obtained by fitting the respective concentration-effect data to the following sigmoid  $E_{max}$  equation (or Hill equation) using the nonlinear regression function in SigmaPlot.

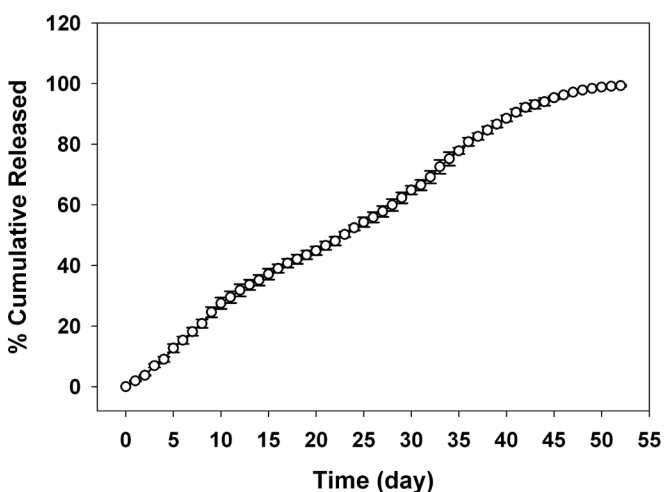
$$E = \frac{E_{max} \cdot C^n}{EC_{50}^n + C^n} \quad (10)$$

Where  $E$  is the percentage of antiproliferative or toxic effect at concentration  $C$  relative to the maximal antiproliferative or toxic effect  $E_{max}$  (100);  $EC_{50}$  is the concentration of drug that produces 50% of the maximal antiproliferative or toxic effect;  $n$  is the shape factor. Therapeutic index is calculated as the ratio of  $LC_{50}$  to  $EC_{50}$ .

## Results

In the present study, we first examined the *in-vitro* release of rapamycin from ReGel. The release was gradual and sustained over 52 days (Fig. 1). We have previously examined the *in-vitro* release of dipyridamole from ReGel (Zhu et al., 2006). Approximately 40% of dipyridamole was released as an initial burst in the first 3 days, followed by a gradual release in the subsequent 2 weeks.





**Figure 1:** *In-vitro* release of rapamycin from ReGel. The release rate is relatively constant over 45 days with no significant initial burst release. Each data point represents the mean  $\pm$  SD of 3 experiments.

	$D_0$ (cm <sup>2</sup> /s)	$D_0C_s$ (g/cm·s)	$k$ (day <sup>-1</sup> )	$R^2$
Rapamycin from ReGel	$3.89 \pm 0.24 \times 10^{-8}$	$2.60 \pm 0.16 \times 10^{-12}$	$3.95 \pm 0.24 \times 10^{-2}$	0.979
Dipyridamole from ReGel	$8.45 \pm 0.30 \times 10^{-8}$	$1.64 \pm 0.06 \times 10^{-11}$	$3.70 \pm 0.47 \times 10^{-2}$	0.963
Dipyridamole from microspheres mixed with ReGel	$1.96 \pm 0.32 \times 10^{-8}$	$3.80 \pm 0.62 \times 10^{-12}$	$1.06 \pm 0.12 \times 10^{-1}$	0.949

$D_0$ : drug diffusion coefficient at time 0;  $C_s$ : drug solubility in the polymer matrix;  $k$ : polymer degradation rate constant. Each value represents the mean  $\pm$  SD of 3 experiments.

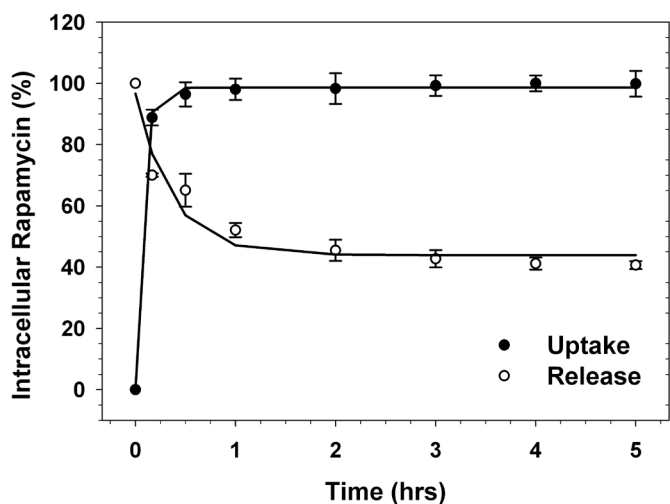
**Table 1:** The in vitro drug release parameters of Dipyridamole and Rapamycin from ReGel and ReGel with microspheres.

The drug diffusion coefficient at  $t = 0$ ,  $D_0$ , and polymer degradation rate constant,  $k$  (Table 1) was smaller for rapamycin ( $3.89 \pm 0.24 \times 10^{-8}$  cm<sup>2</sup>/s) than dipyridamole ( $8.45 \pm 0.30 \times 10^{-8}$  cm<sup>2</sup>/s). This may partly be attributed to the larger size of rapamycin (MW 914.2 vs. 504.6). It may also be a result of the difference in their hydrophobicity, since the higher hydrophobicity of rapamycin may retard the drug release from the hydrophilic domain of the polymer. The  $C_sD_0$  of rapamycin ( $2.60 \pm 0.16 \times 10^{-12}$  g/cm·s) is smaller than that of dipyridamole ( $1.64 \pm 0.06 \times 10^{-11}$  g/cm·s), indicating the slower release rate of rapamycin at early time. There was no significant difference in  $k$  of ReGel when mixed with either drug. Incorporation of dipyridamole into microspheres decreased the  $D_0$  ( $1.96 \pm 0.32 \times 10^{-8}$  cm<sup>2</sup>/s) and  $C_sD_0$  ( $3.80 \pm 0.62 \times 10^{-12}$  g/cm·s), and increased the  $k$ .

The uptake and release of rapamycin in human venous SMCs were quantified by measuring cell-associated rapamycin at various time points (Fig. 2). Cellular uptake of rapamycin was rapid and reached a plateau in 30 min. The concentrations chosen for the uptake experiments were below the  $LC_{50}$  but high enough to assure that they could be accurately assayed during the washout experiments. Cellular uptake and release rate constants ( $k_u$  and  $k_r$ ) of

rapamycin showed no significant differences among the three drug concentrations tested (Table 2), which is compatible with a passive diffusion mechanism for rapamycin transport through the cell membrane. The uptake of rapamycin reached a steady state in 30 min at 37°C and 60 min at 4°C, which persisted for several hours thereafter when the extracellular drug was maintained in the medium. Therefore, the temperature-dependence study was performed using a constant incubation time of 2 hrs to ensure the equilibrium of drug uptake.  $Q_{10}$ , the temperature coefficient, was calculated to be below 2 over the range of concentration evaluated (Table 2) which is also consistent with a passive diffusion model of drug transport.

The proliferation of human and porcine venous SMCs was attenuated in a dose-dependent manner by treatment with rapamycin (Fig. 3A and 3B). Rapamycin  $EC_{50}$  in human and porcine arterial SMCs were significantly (1.8 and 2.0 times, respectively;  $P < 0.05$ ) higher than those in the corresponding venous SMCs (Table 3). The  $EC_{50}$  in porcine venous and arterial SMCs were significantly (1.3 and 1.5 times, respectively;  $p < 0.05$ ) higher than those in the corresponding human SMCs.



**Figure 2:** Uptake and release of rapamycin in human venous SMCs. The rate of uptake is faster than the rate of release. In addition, release is extended because rapamycin likely binds within the cell. Each data point represents the mean  $\pm$  SD of 3 experiments.

$C_{initial}$ ( $\mu\text{g/ml}$ )	$k_u$ ( $\text{min}^{-1}$ )	$k_r$ ( $\text{min}^{-1}$ )	$Q_{10}$
5	$0.00073 \pm 0.00006$	$0.0356 \pm 0.0046$	$1.13 \pm 0.16$
15	$0.00072 \pm 0.0001$	$0.0355 \pm 0.0024$	$1.22 \pm 0.22$
25	$0.00070 \pm 0.00007$	$0.0326 \pm 0.0049$	$1.35 \pm 0.27$

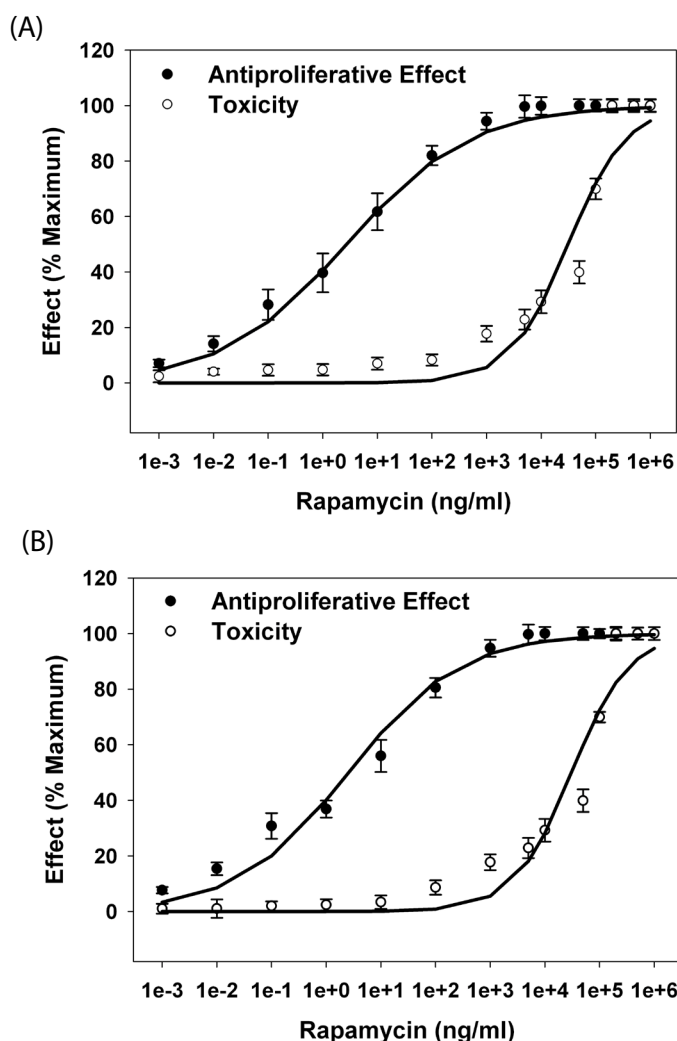
$C_{initial}$ : initial rapamycin concentration in the medium;  $k_u$ : uptake rate constant;  $k_r$ : release rate constant;  $Q_{10}$ : the factor by which the cellular drug concentration at steady state increases as the temperature is raised by  $10^\circ\text{C}$ . Each value represents the mean  $\pm$  SD of 3 experiments

**Table 2:** Pharmacokinetic parameters for uptake and release of rapamycin in human venous SMCs.

In assessing the cytotoxicity of rapamycin (Figure 3A and 3B), LDH release did not increase at rapamycin concentrations lower than 100 ng/ml, indicating the antiproliferative effect of rapamycin was a pharmacologic and not a toxic effect. At concentrations higher than 1000 ng/ml, however, LDH release was increased and significantly higher than the control ( $P < 0.05$ ), indicating that the anti-proliferative effect at these concentrations was partially due to toxicity. The rapamycin concentration which produced 50% of the maximal LDH release,  $LC_{50}$ , was significantly (1.4 and 1.5 times, respectively) higher in human and porcine venous than in the corresponding arterial cells ( $P < 0.05$ ), while it was not significantly different between porcine SMCs (venous or arterial) and the corresponding human cells (Table 3). Consequently, the therapeutic index ( $LC_{50}/EC_{50}$ ) was significantly (2.4 and 3.0 times, respectively;  $P < 0.05$ ) higher in human and porcine venous SMCs than in the corresponding arterial SMCs, and significantly (1.3 and 1.6

times, respectively;  $P < 0.05$ ) higher in human venous and arterial SMCs than in the corresponding porcine SMCs.

Comparing the effect of rapamycin released from ReGel versus rapamycin applied directly to the SMCs, there was no difference seen in between the two experimental conditions as shown in Figure 4. While this is an anticipated result – there is not an expectation that rapamycin released from the gel is altered in any significant way - this experiment does confirm that ReGel is not interacting with rapamycin in any way to alter the inhibition of cellular proliferation. Given that ReGel is composed of polymers that have been used for drug delivery applications in the past with no significant toxicity, this confirmation is for completeness.

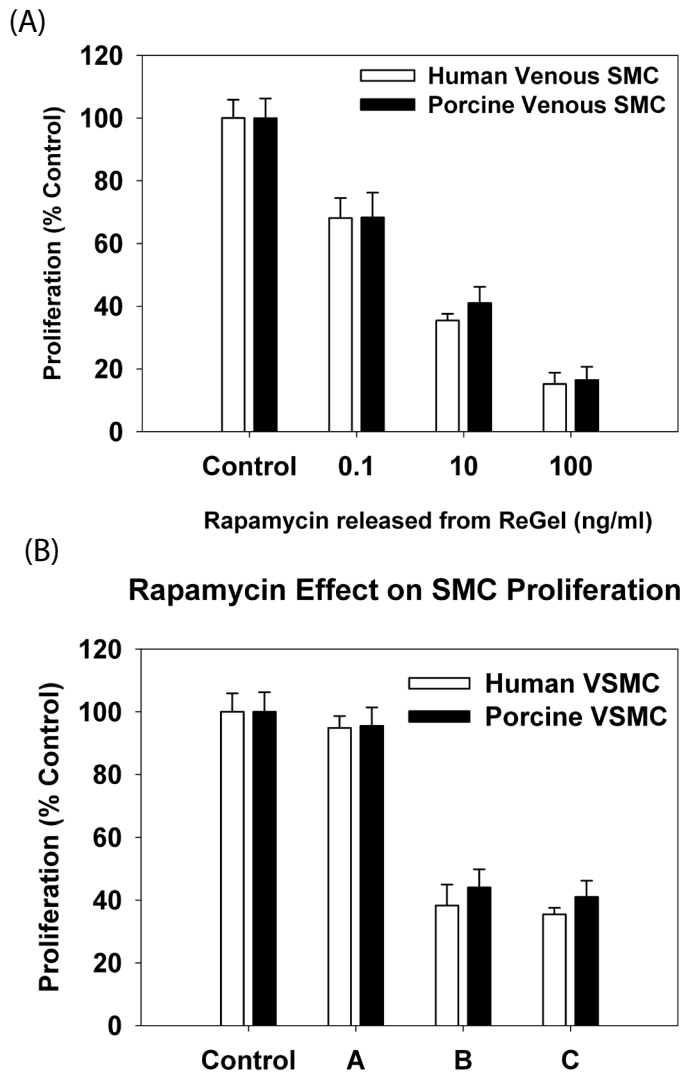


**Figure 3:** Antiproliferative effect and toxicity of rapamycin in (A) human venous SMCs and (B) porcine venous SMCs. The results show that the *in-vitro* efficacy and therapeutic index is not different between these two cell models. Each data point represents the mean  $\pm$  SD of 6 experiments.

Parameters	Human venous SMC	Human arterial SMC	Porcine venous SMC	Porcine arterial SMC
$EC_{50}$ (ng/ml)	$2.52 \pm 0.40$	$4.45 \pm 0.59$ *	$3.39 \pm 0.65$	$6.70 \pm 0.85$ *
$LC_{50}$ (ng/ml)	$(3.86 \pm 0.65) \times 10^4$	$(2.80 \pm 0.37) \times 10^4$ *	$(3.85 \pm 0.63) \times 10^4$	$(2.57 \pm 0.34) \times 10^4$ *
Therapeutic index	$(1.53 \pm 0.21) \times 10^4$	$(6.29 \pm 0.83) \times 10^3$ *	$(1.14 \pm 0.10) \times 10^4$	$(3.85 \pm 0.51) \times 10^3$ *

$EC_{50}$ , concentration of rapamycin that produces 50% of the maximal antiproliferative Effect;  $LC_{50}$ : concentration of rapamycin that induces 50% of the maximal toxicity; therapeutic index, ratio of  $LC_{50}$  to  $EC_{50}$ . Each value represents the mean  $\pm$  SD of 6 experiments. \*  $P < 0.05$  vs. corresponding value in venous SMCs of the same species.

**Table 3:** Pharmacodynamic parameters for antiproliferative effect and toxicity of rapamycin in human and porcine venous and arterial SMCs.



**Figure 4:** The impact of rapamycin on venous smooth muscle cells after it was released from ReGel *in-vitro*. (A) Rapamycin shows a dose-dependent inhibitory activity on the proliferation of human and porcine venous SMCs. (B) The inhibitory activity of rapamycin (10 ng/mL) released from ReGel compared the drug directly from the vial diluted to the same concentration. ReGel does not alter rapamycin’s ability to inhibit SMCs. Samples of the plain culture medium alone were used as a control. Each data point represents the mean  $\pm$  SD of 6 experiments. The smooth muscle growth medium was supplemented with 10% fetal calf serum, 5  $\mu$ g/ml insulin, 0.5 ng/ml human recombinant epidermal growth factor (EGF), 2 ng/ml human recombinant fibroblast growth factor (FGF), 50  $\mu$ g/ml gentamicin and 50 ng/ml amphotericin-B.

## Discussion

We found that the release of rapamycin from ReGel was sustained for up to 50 days without an initial burst effect. This compares favorably to our previous results with dipyridamole. (Zhu et al., 2006) The difference in release profile might be attributed to the difference in hydrophobicity of the drug. Rapamycin is more hydrophobic than dipyridamole and is therefore likely to partition more into the hydrophobic core and less into the hydrophilic shell, assuming a domain structure of the polymer (Jeong, Bae, and Kim 2000). It is suggested that the drug release from the hydrophilic shell in the early time period results in the greater initial burst and a faster release of dipyridamole. The drug release from the hydrophobic core, which is dependent on polymer degradation, would give rapamycin a longer release period without the initial burst. Since neointimal formation in hemodialysis arteriovenous grafts usually occurs progressively over months, sustained drug release without an initial burst is likely to be more desirable to achieve the long-term antiproliferative effect.

Stenosis in the PTFE graft develops more often at the vein-graft anastomosis than the artery-graft anastomosis (Kanterman et al., 1995). Therefore, an understanding of the biology and responses of venous SMCs to antiproliferative agents is important to design optimal strategies to prevent graft stenosis. Despite the well-established effects of many antiproliferative agents in human and animal arterial SMCs, there is a paucity of data on their effects in venous SMCs. Our group has shown that of venous SMCs were more sensitive than arterial SMCs to the inhibitory effect of drug (Kim et al., 2004), but the reverse seemed to be true for the effect of gamma radiation (Kim et al., 2005). We therefore examined the cellular pharmacodynamics of rapamycin in human venous and arterial SMCs separately. Porcine venous and arterial SMCs were also examined to facilitate the understanding of any potential inter-species difference that might occur in drug response. As shown in Table 3, venous SMCs were more susceptible to the antiproliferative effect, and less susceptible to the toxicity, of rapamycin than arterial SMCs. In addition, venous SMCs exhibited the higher therapeutic index than arterial SMCs. These differences between venous and arterial SMCs were preserved between the species tested.

We also examined the antiproliferative effect and toxicity of rapamycin after it was released from ReGel. Released rapamycin inhibited the proliferation of human and porcine venous SMCs in a dose-dependent manner (Fig. 4A), with the potency similar to the stock drug (Fig. 4B). Released rapamycin at 0.1, 10 and 100 ng/ml did not induce cytotoxicity (data not shown).

ReGel is a triblock copolymer of polylactide-co-glycolide (PLGA)-polyethylene glycol (PEG)-PLGA, which is formulated as a 23% (w/w) aqueous solution of polymers in PBS (Zentner et al., 2001). Its degradation products, monomeric or oligomeric lactic acid and glycolic acid may cause a decrease in local pH and affect SMC proliferation. An *in-vitro* experiment was therefore performed in which ReGel was incubated in culture medium and the medium was sampled daily for pH determination. The lowest pH ( $7.10 \pm 0.03$ ;  $n = 6$ ) was observed on day 1. We further examined the effect of pH in the culture medium on SMC proliferation. It was found that a pH lower than 7.0 could significantly inhibit cell proliferation, compared to the pH of 7.4; whereas a pH between 7.0 and 7.4 did not affect cell proliferation. Here we demonstrated that ReGel neither affected SMC proliferation nor caused cytotoxicity. The presence of perivascular tissues *in vivo* in which ReGel is deposited should provide potent pH buffering. Thus, it is unlikely that the degradation products of ReGel would have significant impact on SMC proliferation during clinical administration.

In this study, we have demonstrated that (a) ReGel achieved a sustained release of rapamycin *in-vitro*, which should provide a continuous inhibitory effect in SMCs to prevent the graft stenosis *in vivo*; (b) the transport of rapamycin through the cell membrane followed a mechanism that is compatible with passive diffusion, and a substantial portion of rapamycin was retained in the cells; and (c) the incorporation of rapamycin in ReGel did not alter its potency in inhibiting SMC proliferation in a dose-dependent manner. The effectiveness of rapamycin delivered by ReGel *in vivo* would, however, further depend on the pharmacokinetics and pharmacodynamics of the drug in vascular tissues. Extension of the studies in cell culture into an animal model (such as a porcine PTFE graft model) will be a critical next step in the development of antiproliferative strategies.

ReGel<sup>®</sup> achieved a sustained release of rapamycin *in-vitro*. Rapamycin inhibited the proliferation of human and porcine venous and arterial SMCs *in-vitro*. Rapamycin released from ReGel retained its antiproliferative activity. ReGel can therefore be a potential sustained delivery system for rapamycin to inhibit SMC proliferation for the prevention of hemodialysis arteriovenous graft stenosis.

## Acknowledgements

This work was supported by the National Heart, Lung and Blood Institute (RO1HL67646), the Merit Review Program of the Department of Veterans Affairs, the Dialysis Research Foundation and the National Kidney Foundation of Utah & Idaho. ReGel<sup>®</sup> was kindly provided by MacroMed



Inc. We thank Mr. Ilya Zhuplatov for assistance in cell culture studies. Drs. Donald Blumenthal, John K. Leyboldt, Li Li, Syed F. Mohammad and Christi Terry provided helpful comments.

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