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The poloxamer 407 induced hyperlipidemic rat model and its effect on renal toxicity of calcineurin inhibitors

By

Hetal Revabhai Chaudhary

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Dedicated to my loving grandparents

Abstract:

The present study characterized poloxamer 407 (P407) induced hyperlipidemia in rats and investigated its effect of on renal toxicity of the immunosuppressants tacrolimus and cyclosporine A. P407 (0.5 or 1 g/kg) was injected in rats and blood samples were collected at different time-points. Serum levels of total cholesterol, triglyceride, high-density lipoprotein (HDL), adiponectin, leptin and TNF-α were measured. *In vitro* renal toxicity studies were performed using LLC-PK1 and NRK-52E cells for tacrolimus and cyclosporine A, respectively. P407 increased serum cholesterol, triglyceride and HDL with maximum increase at 36h. Moreover, P407 significantly increased leptin and decreased adiponectin but did not affect TNF-α. Hyperlipidemic serum treated cells did not show any significant difference in toxicity compared to normo-lipidemic serum treated cells for both the drugs. Together, P407 induces hyperlipidemia and alters lipid and adipokine levels. Furthermore, hyperlipidemic serum treatment had no apparent effect on *in vitro* kidney cell toxicity by immunosuppressants.

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AUC	Area under the concentration versus time curve
β	Beta
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
CyA	Cyclosporine
Cmax	Peak plasma drug concentration
CV	Coefficient of variation
CYP	Cytochrome P450
DMEM	Dulbecco's modified eagle's media
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
GFR	Glomerular filtration rate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
h	Hour
HCl	Hydrochloric acid
HDL	High density lipoproteins
HL	Hyperlipidemia/ hyperlipidemic
HPLC	High performance liquid chromatography

IL-6	Interleukin-6
IS	Internal standard
i.p.	Intraperitonial
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
Kg	Kilogram
L	Liter
LC/MS	Liquid chromatography-mass spectrometry
LC-MS/MS	liquid chromatography mass spectrometry - tandem mass spectrometry
LDH	Lactate dehydrogenase
LDL	Low density lipoproteins
µg	Microgram
µL	Microliter
mL	Millilitre
mg	Miligram
µM	Micromolar
min	Minute
mM	milimolar
mRNA	Messenger RNA
MS	Mass spectrometer
MDR	Multi drug resistant
MTT	3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl

	tetrazolium bromide
ND	Not detectable
N	Normality
n	Number of samples
NaOH	Sodium hydroxide
NH_4OH	Ammonium hydroxide
ng	Nanogram
NL	Normal lipidemia/ Normolipidemic
O_2	Oxygen
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PD	Pharmacodynamics
PK	Pharmacokinetics
P407	Poloxamer 407
Pgp	P-glycoprotein
r^2	Correlation coefficient
RF	Rifampin
rpm	Rotations per minute
SD	Standard deviation
TNFα	Tumour necrosis factor α
$T_{1/2}$	Terminal elimination phase half-life
TC	Total cholesterol
TG	Triglyceride

Tmax	Time of maximum concentration
USA	United states of America
UV	Ultraviolet
Vd	Volume of distribution
VLDL	Very low density lipoprotein
Vs.	Versus
^{o}C	Degree Celsius
%	Percent
~	Approximately

CHAPTER: 1 Introduction

1.1 Hyperlipidemia:

Heart disease, which is one of the leading cause of death worldwide[1], is related to factors such as overweight, poor diet, alcohol, diabetes, family history or drugs. A common factor in some of these etiologies is hyperlipidemia (HL), which is a condition of elevated lipoproteins and dietary lipids such as cholesterol and triglycerides. When it is chronically present, it can lead to atherosclerosis and life threatening conditions such as heart attack and stroke. Although HL does not have any specific noticeable symptoms, monitoring of serum lipids provides an invaluable tool for the monitoring of risk for these more immediate serious cardiovascular events.

The primary cause of HL is family history , also known as familial HL, and secondary causes are diet, sedentary lifestyle, obesity, diseases or drugs. Examples of possible disease states are involved malaria, HIV/AIDS, diabetes, nephrotic syndrome, hypothyroidism, pregnancy and metabolic syndromes.[2-4] Other factors that may lead to HL are drugs such as diuretics, beta blockers, steroids, cyclosporine, sirolimus and estrogens.[3, 5]

1.2 Animal models used to study hyperlipidemia:

Various animal models have been used to study the effect of HL. HL can be induced by either high fat diet[6-10], genetic manipulation[11, 12] or by treating animals with compounds such as Triton[13, 14] and P407[15].

Chemicals like P407 are known to induce experimental HL in many species including rat[15], mouse[16] and rabbit[17].

Various literature reports that feeding animals with high fat diet is also a popular model to induce HL. In previous studies HL was induced by high fat diet consisting of normal pulverized food (92.8%), lard (5%), cholesterol (2%) and sodium cholate (0.2%) and fed for 4 weeks or containing 33.0% (cal) fat and given for 8 weeks.[6, 7] A disadvantage of this approach is that animals need to be fed with the high fat diet for long time, from 4 to 16 weeks, to get higher lipid levels.[10, 14] Another way to induce HL is to inject surfactants such as Triton, which is known to inhibit lipoprotein lipase enzyme which is responsible for metabolism of triglyceride.[13, 14] The increase in lipids was about 10-fold with a single intravenous injection of Triton WR 1339. However, Levine et al reported that Triton WR 1339 (tyloxapol) must be injected more than twice in a week to have high and sustained levels of serum cholesterol and triglycerides for an entire week or longer.[14]

P407-induced HL is also widely used model to study the effect of HL on many drugs including fluvoxamine[18], CyA[19], amiodarone[20], nifedipine[21], halofantrine[22], docetaxel[23], clomipramine[24] and carbamazepine[25]; these drugs were studied using the P407-induced hyperlipidemic rat model. In most of these studies, pharmacokinetic assessments were conducted within 24-36 hours from the time of P407 i.p. injection. Very large increases present themselves in serum at this

time. This provides for a convenient screening tool for the influence of lipoproteins on drug behavior. However, the downside is that the changes occur over a short period of time, and as such longer term changes associated with HL might not be detectable. Another issue is that lipoprotein concentrations at this time are many fold higher than are seen in humans, and as such it is possible that the pharmacokinetic changes are exaggerated compared to what would be seen in humans with HL.

1.3 Poloxamer 407-Induced hyperlipidemia:

It is known that the P407 model of HL is reversible in nature, and in conducting studies even if lipoprotein levels are not assayed, within 2-3 d from the time of injection it is visually evident that hyperlipidemia subsides, as the plasma/serum gradually changes from a milky white to a normal clear appearance. The plasma concentrations of lipids have been followed after dosing of rats with a single dose of P407 of 0.4 g/kg, and concentrations of total cholesterol (TC), triglyceride (TG) and high density lipoprotein cholesterol (HDL-C) were observed to fall 12-24 hours after dose administration. However, low density lipoprotein was still rising and the other lipid levels were still substantially higher than baseline levels after up to 48 hours after dosing with the agent.[25]

P407 increases serum lipoproteins via its actions at various levels in lipid metabolism, largely by inhibiting lipoprotein lipase (LPL), which facilitates the hydrolysis of TG [15]. Johnston et al investigated the effect of P407 on

LPL activity and found that after 3 h of P407 i.p. injection in rats the enzyme activity decreased by 95% compared to values in normal saline treated rats[15]. P407 also causes indirect stimulation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-C0A) reductase which is involved in cholesterol biosynthesis[17, 26]. A study on rat with P407 i.p. injection of 1g/kg dose reported the effect of the P407 on activity of many enzymes involved in lipid metabolism[27]. The study found that P407 elevated the activity of lecithin cholesterol acyl transferase (LCAT) and cholesteryl ester transfer protein (CETP). Furthermore, activities of hepatic lipase (HL) and LPL, which together are involved in the metabolism of TG, were significantly inhibited in P407 treated rats. [27]

1.4 Hyperlipidemia and pharmacokinetics:

HL is known to influence the pharmacokinetic, pharmacodynamic and toxic properties of various drugs. Most commonly, lipophilic drugs are affected in such a manner, as they are most likely to bind to lipoproteins. Known examples include amiodarone, cyclosporine A, amphotericin B, nystatin, nifedipine and halofantrine[2].

The reason for change in the pharmacokinetics of lipophilic drugs by HL is an increase in binding to lipoproteins. This causes increased blood levels due to the drug being restricted to the plasma. However, HL can also cause a change in pharmacokinetics by altering drug metabolizing enzyme function and expression (e.g. cytochrome P-450, CYP) and efflux

transporters such as P-glycoprotein (P-gp). Previous studies found that HL alters the QT intervals compared to control animals treated with halofantrine and halofantrine binding to lipoprotein might be the controlling factor accounting for the difference. QT prolongation might increase the risk of ventricular arrhythmias in patients with cardiac disease.[28, 29]

CyA is reported to have more toxicity in hyperlipidemia.[2] Aliabadi et al reported higher levels of CyA in HL rats compared to NL rats in blood, plasma, kidney and liver; an increase in microscopic lesions was also noted in the kidneys of HL rats.[30] The reason for increase in blood and plasma was high lipoprotein binding of CyA (lipophilic drug) and decrease in clearance as HL has effect on CYP enzymes activity.[31] CyA uptake in tissues such as liver and kidney might be result of increased bound concentration and it may lead to increase uptake as receptors for lipoprotein will be present on some tissues. Amphotericin B is another drug showing elevated kidney concentrations in obese Zucker rats.[2]

Fluvoxamine is an example of drug that shows altered plasma protein binding in HL rats compared to normal rats, with increased AUC (plasma concentration vs time curve) and decreased brain concentrations by 20 % in HL rats compared to NL.[32] Docetaxel also shows increased plasma protein binding in HL rats with increased AUC due to a decrease in hepatic metabolism in HL rats. Clomipramine, an antidepressant drug, has similar effects in HL rats as docetaxel. Kobuchi et al reported significantly higher

steady state concentrations of clomipramine and decreased brain concentration in HL rats compared to normal rats.

In vivo and in vitro studies have shown that Pgp and CYP activity may be altered in the case of increased lipid levels.[31, 33-36] The down-regulation in CYP enzymes in rat after dose of P407 was attributed to lower expression of nuclear hormone transcription factors such as constitutive androstane receptor (CAR) and retinoid X receptor (RXR).[31] The possible reason for altered Pgp activities are either cholesterol directly interact with substrate binding site of Pgp as cholesterol is being trasported by Pgp[34] or cholesterol may alter the physicochemical property of the lipid bilayer, which may affect the activity of Pgp.[36] Studies in HL rats with amiodarone showed substantial increases in plasma concentrations of drug compared to normal rats, and certain tissue concentrations also changed.[31] Lee et al reported a study in rats showing that the AUC of plasma concentration vs time curve was increased significantly in HL rats due to decreased hepatic clearance of carbamazepine after intravenous administration of carbamazepine. Moreover, reduction in hepatic expression of CYP3A1/2 altered metabolism of carbamazepine to the 10,11-epoxide metabolite. Interestingly, the study reported that HL reduced liver microsomal epoxide hydrolase expression and also resulted in a decrease in further metabolism of the 10,11-epoxide metabolite to an inactive metabolite of

carbamazepine (trans-10,11-dihydoxyl-10,11-dihydro-carbamazepine). [25]

1.5 Immunosuppressant drugs: Tacrolimus and Cyclosporine A

Immunosuppressants, the agents which suppress or prevent body immune response, are used to prevent rejection of organ transplant and for the treatment of autoimmune disease. In organ transplant patients, the immune system triggers a response against the newly transplanted organ and can damage it. The number of patients requiring solid organ transplant are increasing every year and newer immunosuppressant drugs have resulted in a significant reduction of rejection of transplanted organs.[37] Immunosuppressant drugs greatly decrease the risks of rejection by protecting the new organ and preserving its function. Immunosuppressant drugs play a critical role in decreasing the probability of allograft rejection. Tacrolimus and CyA are two drugs belonging to the calcineurin inhibitor class of immunosuppressant drugs. These agents cause several acute and chronic side effects such as nephrotoxicity, neurotoxicity, diabetes mellitus, hyperlipidemia, hypertension, cardiovascular disease, anemia and so on.[38, 39] Moreover, patients need to continue life-long immunosuppressant therapy to prevent organ rejection.

Tacrolimus, a highly lipophilic drug, is produced by fermentation of *Streptomyces tsukubaensis*.[40] Currently tacrolimus is gaining popularity

as the first choice of calcineurin antagonist immunosuppressant agents in patients receiving solid organ transplants. Although tacrolimus is often used post-transplantation, it can lead to nephrotoxicity which is a major problem with this drug. The reasons for nephrotoxicity of calcineurin inhibitors are increased vasoconstrictor factor such as endothelin and thromboxane and decrease in vasodilator factor such as nitric oxide. Reduction in ranal blood flow and glomerular filtration rate also result in kidney toxicity during the treatment of tacrolimus and CyA. Drug accumulation in kidney is known to cause morphological changes in kidney cells.[39] Moreover, tacrolimus is a narrow therapeutic index drug (5-20 ng/mL) with high inter- and intraindividual variability in pharmacokinetics which complicates its safe and effective use.[39] The possible reason reported for variability in pharmacokinetic was polymorphisms in ABCB1 and CYP3A gene, which can affect absorption and metabolism of tacrolimus.[41]

CyA, is another highly lipophilic cyclic compound isolated from *Tolypocladium inflatumgams* fungus. CyA was discovered by Sandoz in 1972 and it has since revolutionized transplant medicine in kidney, heart, liver and bone marrow recipients.[42, 43] However, like tacrolimus CyA is also associated with certain serious complications including nephrotoxicity, hypertension and increased risk of cardiovascular events. Moreover, there are significant differences in bioavailability of CyA due to interindividual

variability in intestinal absorption which is also affected by food ingestion, diabetes, gastric motility problems, and diarrhea among other things.[42]

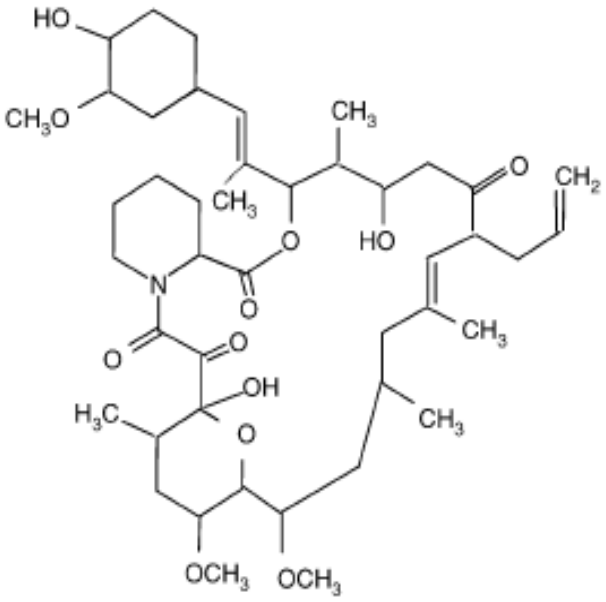

Figure 1: Chemical structure of tacrolimus

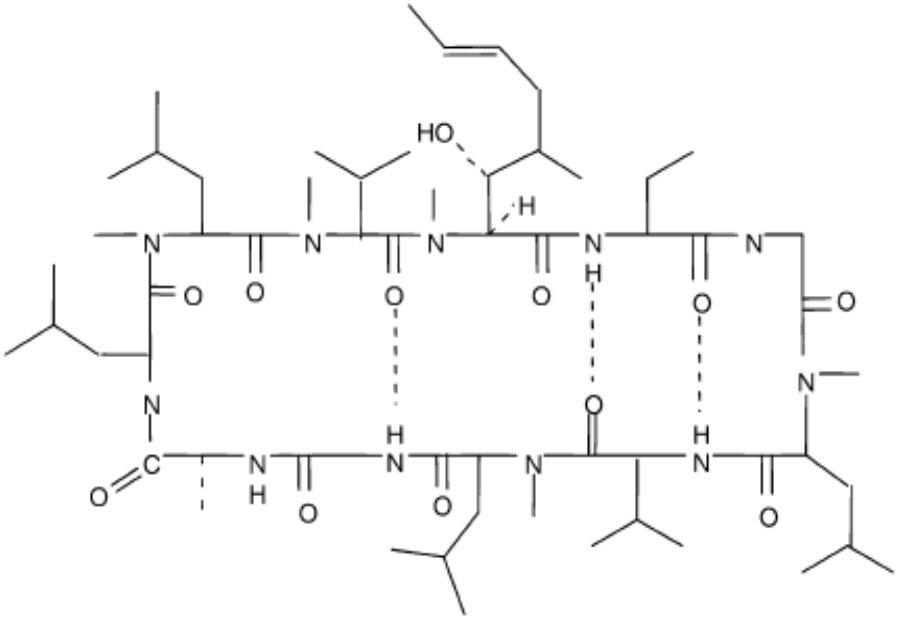

Figure 2 : Chemical structure of cyclosporine A

1.6 Mechanism of action of calcineurin inhibitors:

Tacrolimus and CyA comprise the calcineurin inhibitor class of immunosuppressants. These agents act on cytosolic protein immunophilin of T-lymphocytes. For CyA, immunophilin is known as cyclophilin, and for tacrolimus it is known as FKBP1A (FK binding protein1). The complex of drug and immunophilin inhibit calcineurin phosphatase (a serine threonine phosphatase) which is required for transcription of interleukin-2.

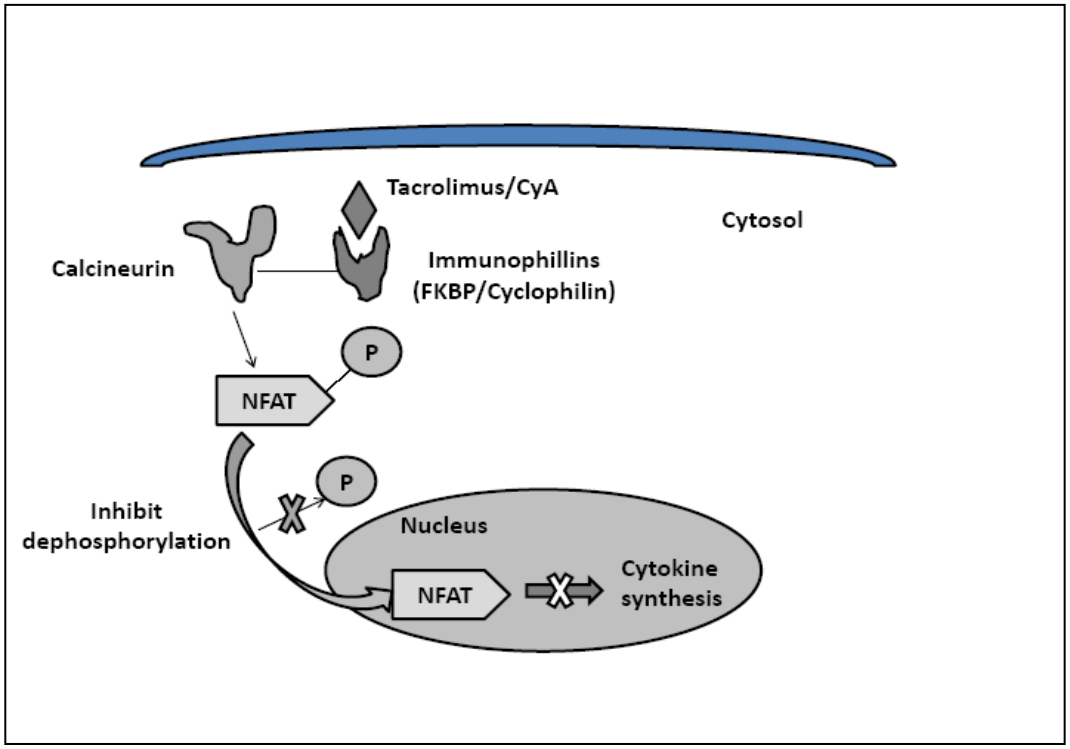

Figure 3 : Mechanism of action of immunosuppressant drugs. [43] T cell activation occurs by activation of the calcineurin pathway. Calcineurin inhibitors inhibit T cell activation by inhibition of translocation of nuclear factor of activated T cell (NAFT) in the nucleus results in inhibition of interleukin 2 and other cytokines.

Inhibition of interleukin-2 transcription then leads to inhibition of T-cell activation. Moreover, calcineurin inhibitors also inactivate a transcription

factor known as nuclear factor of activated T-cell (NF-AT) and thereby prevent cytokine gene activation. Figure 3 shows the mechanism of action of CyA, tacrolimus and other immunosuppressant drugs.[44, 45]

As lipophilic drugs, tacrolimus and CyA both are known to bind to lipoproteins.[46-48] Furthermore, tacrolimus and CyA are transported by P-gp and metabolized by CYP3A family and their activities could be altered in HL.[49, 50] Consequently, HL could potentially alter the pharmacokinetics of CyA and tacrolimus. However, there is little if any specific information regarding the impact of HL on the pharmacokinetics of tacrolimus, and perhaps more importantly, the impact that this condition might have on the renal toxicity of these clinically important drugs.

1.7 Adipokines and Cytokines:

White adipose tissue is known to release bioactive peptides, known as adipokines, and also pro-inflammatory cytokines[51]. The protein released from adipose tissue play an important role in insulin activity, energy balance and lipid metabolism.[52] Moreover, a change in mass of white adipose tissue may alter the levels of circulating adipokines. Obesity-related disorders including the cardiometabolic syndrome (with components of diabetes, atherosclerosis, hypertension, and coronary artery disease) are associated with decreased plasma levels of adiponectin.[53] Adiponectin has gained much attention due to its beneficial effects upon obesity-related complications. It is reported that adiponectin stimulates endothelial nitric oxide production, induces vascular

relaxation, inhibits inflammatory response, and reduces oxidative stress.[54] Leptin was discovered as a crucial factor involved in regulation of food intake, body weight, energy expenditure and maintenance of insulin sensitivity; it is also involved in the regulation of blood pressure[51, 55]. Studies have reported that treatment of leptin is beneficial in hypertriglyceridemia and insulin resistance and leptin replacement therapy improves both lipid and glucose levels. However, the increase in leptin in obesity may also be associated with the onset of leptin resistance, which is common in the cardiometabolic syndrome.[56] TNF-α, a pro-inflammatory cytokine, is secreted by adipokines and contributes to insulin resistance. It is also commonly elevated in in cardiometabolic syndrome[32, 57, 58]. TNF-α deficient mice showed beneficial and protective effect on obesity induced insulin resistance on a high fat diet.[51] IL-6 is also positively correlated with body mass and insulin sensitivity.

1.8 Rationale and hypotheses:

P407 induced HL has been widely used model to study the effect HL on the pharmacokinetics and pharmacodynamics of drugs. Thus far there has been very limited information published regarding the duration of HL after an i.p. dose of P407 was given. There is also little to no information regarding its impact on adipokine and cytokine levelss.

Post-transplant HL is commonly present in transplantation patients[59] and HL is known to alter activity of Pgp.[34-36] Moreover, Pgp is an important drug transport protein that is present in several tissues including kidney. HL can result in higher kidney concentrations of tacrolimus and CyA, possibly due to a decrease in Pgp activity.

Based on literature studies our hypotheses are,

1) Poloxamer 407 will induce dose-dependent hyperlipidemia in rats and causes increases in TC, HDL TG, changes in the levels of adipokines and cytokines.

2) Hyperlipidemia will increase renal cellular toxicity of the lipophilic immunosuppressant drugs by decreasing Pgp activity.

1.9 Objectives:

Objective 1: To study the P407-induced HL rat model by investigating dose responsiveness, duration of HL, and effect on adipokine and cytokine levels.

Objective 2: To study the effect of HL on the renal toxicity of tacrolimus and CyA using kidney cell lines.

Objective 3: To develop a high performance liquid chromatography (HPLC) method to measure intracellular concentrations of tacrolimus.

CHAPTER: 2 Experimental Procedures

2.1 Materials:

A kit for quantitative determination of HDL cholesterol was purchased from Wako chemicals (Richmond, VA). Enzymatic assay kits for measurement of TG and TC in serum were obtained from Genzyme diagnostics (Charlottetown, Canada). Calibrator for triglyceride kit, DC-CAL calibrator, was also purchased from Genzyme diagnostics. ELISA kits for adiponectin (ADP) and leptin (LPN) were obtained from Alpco diagnostics (Salem, NH, USA) and the TNF-α kit was purchased from Invitrogen Corporation (Camarillo, California). Sandimmune (CyA formulation, Novartis Pharmaceuticals, Quebec, Canada) and heparin were purchased from the University of Alberta hospital pharmacy. Tacrolimus was purchased from Sigma Aldrich (St. Louis, MO). Powdered CyA was purchased from Wuhan Zhongxin company, China. Amiodarone HCl, rifampin, P407, fetal bovine serum, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and trypan blue (0.4%) were purchased from Sigma Aldrich (St. Louis, MO). Diethyl ether and dimethyl sulfoxide (DMSO) were obtained from Fisher Scientific (Fair Lawn, USA) and normal saline (sodium chloride injection, 9 mg/mL) was purchased from Hospira Healthcare Corporation (Montreal, Canada). Sodium chloride, potassium chloride, potassium dihydrogen phosphate, sodium hydroxide, hydrochloric acid, methanol, acetonitrile, ethyl acetate, ethanol, disodium hydrogen phosphate and ethylene diamine tetraacetic acid

(EDTA), HPLC water and isopropyl alcohol were purchased from Caledon labs (Ontario, Canada). Penicillin-streptomycin, trypsin, TRIzol reagent and Dulbecco's Modified Eagle Medium (DMEM) were purchased from GIBCO, Invitrogen Corporation (Carlsbad, CA, USA). High-capacity cDNA Reverse Transcription Kit, 96-well optical reaction plates and SYBR Green SuperMix with optical adhesive films were obtained from Applied Biosystems (Foster City, CA). Real-time PCR primers were synthesized by Integrated DNA technologies, Inc. (Coralville, IA) as reported by previously published sequences. LLC-PK1 (pig proximal tubular kidney cell line) and NRK-52E (rat renal proximal tubular cell line) were purchased from American Type Culture Collection (ATCC, Manassas, USA). Cytotoxicity was measured by the CytoTox-ONE™ Homogeneous Membrane Integrity Assay kit purchased from Promega (Madison, WI).

2.2 Methods:

2.2.1 Development of high performance liquid chromatography (HPLC) method for measurement of tacrolimus

2.2.1.1 Instrumentation:

The HPLC system consisted of a Waters 712 WISP auto-injector, 610 Fluid unit, 600E system controller (Waters, Milford, MA, USA), and a HP 1012 UV detector (Hewlett Packard, Palo Alto, CA, USA). The UV wavelength was set at 214 nm for detection of drug and internal standard

(IS). The chromatographic separation of tacrolimus and CyA (IS) was carried out using an Alltima C18 analytical column, 5 µm, 150 mm × 4.6 mm (Alltech), attached to a guard column. The column was heated at 40ºC for better separation. The mobile phase consisted of a 65:35 (v/v) mixture of acetonitrile:water. The mobile phase was pumped isocratically at a flow rate of 2 mL/min. Chromatographic data collection was performed using EZChrom software (Scientific Software, Pleasanton, CA).

2.2.1.2 Preparation of standard and stock solutions:

Tacrolimus (5mg) was dissolved in 50 mL of methanol to prepare a 100 µg/mL concentration. Internal standard: CyA stock solution of 50 µg/mL was prepared by dissolving 5 mg CyA powder in 100 mL methanol. All stock solutions were stored at -20ºC. Working solutions of tacrolimus (50, 25, 10, 5 µg/mL concentration) were prepared freshly daily by sequential dilutions.

2.2.1.3 Extraction procedure:

IS (100µL) was added to each glass tube containing 500 µL of cell suspension. To this, 500 µL of HPLC water and 4 mL of ethyl acetate was added. Samples were vortex mixed for 60 sec and cetrifuged for 10 min at ~ 2500 *g*. The organic layer was transferred to clean glass tubes and evaporated to dryness *in vacuo*. A hexane washing step was added to

remove interfering peaks. 200 µL of ACN: Water (35:65) and 1 mL hexane were added to the dries residue and samples were vortexed (30 sec) and centrifuged (5 min) and 150 µL of the aqueous layer was collected. A portion (75 µL) of the aqueous layer was injected into HPLC system.

2.2.1.4 Calibration curves and validation:

Calibration curves were constructed using untreated cell suspensions from 24 well cell culture plates, for a concentration range of 5 - 100 µg/mL of tacrolimus. Calibration curves and quality control samples were prepared by spiking the drugs in the same matrices as the treated cell culture samples. Peak height ratios of drug to IS were used to calculate concentrations of drug. The method was validated for four concentrations of the calibration curve (5, 10, 50, 70 µg/mL) with four replicates of each. Mean % error and percentage coefficient of variance was calculated for validation samples.

2.2.2 Cell culture experiments for tacrolimus

2.2.2.1 Cell lines and growth condition:

The cell lines were ordered from ATCC. The LLC-PK1 call line is an epithelial-like pig kidney cell strain that can be used to study mechanisms of kidney toxicity and activity of membrane transport.[60] A subculture ratio of 1:6 was maintained during their preparation. LLC-PK1 cells were

maintained in medium 199 obtained from Sigma (St. Louis MO) with 10% FBS and 1% of penicillin-streptomycin (100 IU/mL). The other cell line used was NRK 52E which like LLC-PK1 is renal proximal tubular cell line but originating from rat rather than pig. For NRK 52E cells a sub cultivation ratio of 1:4 was maintained. The NRK 52E cells were maintained in Dulbecco's modified eagle's (DME) media with 10% FBS and 1% penicillin- streptomycin. Cells were grown in 75-cm^2 flasks and kept in a 5% CO_2 humidified incubator at 37º C. For normal growth of cells, medium was changed every third day. For splitting cells, a monolayer was washed with PBS and cells were detached with 5 mL of trypsin (in 0.25% solution of PBS EDTA).

2.2.2.2 Induction of Pgp with rifampin (RF):

RF-induced LLC-PK1 cells showed a significant amount of increase in MDR1 gene expression.[61] We measured gene expression of Pgp with NRK-52E cells after rifampin treatment. Both cell lines were treated with rifampin (25 µM) containing media for six days. On day seven cells were treated with 10% of NL/HL/FBS containing media. Both RF and non-RF treated cells were divided in three group and treated with normo-lipidemic (NL), HL, or fetal bovine serum (FBS) containing media. After 24 h of incubation with serum cells were washed properly with PBS and serum-free media. Cells were incubated with drug-containing, serum-free media for the required time.

For drug uptake experiments cells were grown in 24 well plates and for toxicity assays such as the lactate dehydrogenase (LDH) assay and 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT assay), cells were grown in 96 well plates. Gene expression studies were performed on cells grown in 6 well plates.

2.2.2.3 NL and HL serum collection:

For NL serum, blood from normal rats after cardiac puncture was collected in a glass test tube and kept at room temperature for about 30 minutes. The tubes were centrifuged at 3000 *g* for about 15 minutes and NL serum was collected from the top layer. For HL serum rats were treated with P407 (1g/kg) and blood was collected after 36 h (where maximum lipid concentrations were presumed to occur) and serum was prepared.

2.2.2.4 Tacrolimus stock solution preparation:

For cell culture experiments a tacrolimus stock solution was prepared in DMSO. A stock solution of 100 mg/mL was prepared by dissolving 10 mg of tacrolimus powder in 100 µL of DMSO. Further dilutions were prepared in serum-free media on the day of experiments.

2.2.2.5 Toxicity of tacrolimus on LLC-PK1 and NRK-52E cell lines:

A fluorescence based LDH assay was used to determine *in vitro* renal toxicity of tacrolimus. The assay determines LDH enzyme activity from damaged cells by fluorescent measurement of resorufin converted from resazurine in the presence of lactate, NAD^+ and diaphoras. To study the cell damage in tacrolimus-treated kidney cells, various different doses of tacrolimus were selected (0, 1, 5, 10, 25, 50 µg/mL). Cells were grown in 96 well cell culture plates and treated either with RF- or non-RF containing media for 6 days. On day 7 cells were treated with different concentrations of tacrolimus and cytotoxicity measured for 0 and 24 h of treatment. Cytotoxicity was measured using the LDH assay kit ordered from Promega (Madison, USA). Assay solutions were prepared as per directions of the manufacturer. Assay reagent was added in each well to equal volume of cell culture media (100 µL) and after 10 minutes florescence was measured at excitation/emission wavelengths of 560 and 590 nm, respectively. After adding 40 µL of lysis solution in each well, the plate was read again to get approximate maximum LDH release in each well. Percentage cytotoxicity was calculated by using the value of maximum LDH release as 100 %.

2.2.2.6 HL serum effect on toxicity of tacrolimus:

For studying the effect of HL serum on renal toxicity of tacrolimus, LLC-PK1 cells were grown with or without RF media for 6 days. On day 7 cells

in both groups were treated with 10% NL/HL or FBS containing media for 24 h. After 24 h all wells were washed with PBS and fresh serum-free media. Cells were treated with tacrolimus-containing serum-free media. Cytotoxicity was measured using LDH assay as mentioned above in the toxicity measurements of tacrolimus at 0 and 24 h(2.2.2.5).

2.2.2.7 Tacrolimus drug uptake study:

Tacrolimus uptake by LLC-PK1 cells was measured using an HPLC method. For the drug uptake study LLC-PK1 cells were grown in a 24 well culture plates and plates were divided in two groups RF-treated and non-RF treated cells. After 6 days of treatment with media containing RF and lacking RF plates were ready for treatment with 10 % serum (NL/HL/FBS). Each plate was divided in to three different treatment groups of 10% serum either NL or HL or FBS containing media as previously described in section 2.2.2.6. To measure the difference in uptake with HL serum, cells were preincubated for 24 h with NL or HL or FBS containing media. For time-dependent drug uptake, cells were collected at various time points (0, 2,4,6 or 24 h) after treatment.

To collect the cells from 24 well plates cells were washed with PBS solution twice and PBS was discarded after washing. Then, 500 µL of HPLC water was added in each well and the cells were stored at -20º C until extracted. On the day of extraction all cells were found detached and

suspended in water. The cell suspension was collected in clean glass test tubes and wells were rinsed properly with 200 µL of methanol. The samples were extracted as mentioned in the extraction procedure (2.2.1.3) and concentration was calculated using a calibration curve.

2.2.3 CyA Cell culture experiments:

2.2.3.1 Cyclosporine A (CyA) stock solution:

CyA stock solutions for cell culture experiments were prepared in DMSO by dissolving 5 mg of CyA powder in 2 mL of DMSO. Further dilutions were made in serum-free media to obtain 50 µg/mL, 30 µg/mL and 10 µg/mL concentrations. For the CyA formulation (Sandimmune), 50 mg/mL Sandimmune was diluted in normal saline to get 2.5 mg/mL. Sandimmune (25 µL) was dissolved in 475 µL of saline. For treatment, stock solutions were prepared in serum-free media.

For LC/MS experiments stock solutions were prepared by dissolving powdered CyA or Sandimmune in methanol to get 2.5 µg/mL concentrations. Working stock solutions were prepared fresh on the day of extraction. A 3 mg/mL amiodarone solution was prepared by dissolving 6 mg of powder in 2 mL methanol. Further dilutions were made with methanol to obtain 10 µg/mL of working stock solution. All solutions were stored at -4º C.

2.2.3.2 Toxicity study of CyA with LLC-PK1 and NRK-52E cell lines using the MTT assay:

MTT is one of the widely used tests to determine cell proliferation, cell viability, and can be used to assess drug toxicity to cells. The conversion of MTT to purple formazan crystals by mitochondrial dehydrogenase enzyme of metabolically active cells is a measure of viability in the MTT assay. Cyclosporine A toxicity on both LLC-PK1 and NRK 52E cell lines was studied using MTT assay with some modification of a previously published method.[62] As mentioned above with tacrolimus (section 2.2.2.2), cells were divided in two groups and grown with and without RF media for 6 day and CyA treatment was done on day 7. Cells in 96 well plates were treated with 0 µg/mL, 10 µg/mL, 30 µg/mL and 50 µg/mL concentrations of CyA as Sandimmune. Cells were incubated with CyA for 0, 24 and 48 h. After respective hours (0,24 or 48 h) of treatment with Sandimmune, 1.2 mM of MTT solution was prepared in PBS and cells were incubated with 100 µL of MTT solution in serum-free media for 2 h. Then, purple formazan crystals were dissolved in 100 µL isopropanol by shaking for 1 h. Color intensity was measured by a BIO-TEK Instruments EL 312e micro plate reader at 550 nm wavelength. Percentage cell viability was calculated relative to 100% viability of control group wells.

Cell viability was also investigated after incubation with 10% of HL and NL serum for LLC-PK1 and NRK 52E cells treated with CyA treatment. For this experiment cells were pre-incubated with NL/HL- or FBS-containing

media for 24 h and CyA containing serum free media was added after 24 h. Cell viability with the MTT assay was measured as described above for 0 and 24 h.

2.2.3.3 Cytotoxicity study of CyA with NRK-52E cell lines by the LDH assay:

To investigate the magnitude renal cellular damage with CyA treatment. cells were treated with RF and non-RF media for 6 days and pre-incubation of serum was done as mentioned in the MTT experiments (2.2.3.3). Cytotoxicity was measured using LDH assay kit ordered from Promega (Madison, USA). Assay solutions were prepared as per direction of the manufacturer. Equal volume (100 µL) of assay reagent and cell culture media were added and after 10 min the fluorescence was measured at excitation/emission wavelengths of 560 and 590 nm, respectively.

2.2.3.4 CyA measurement by LC/MS method:

A validated LC/MS method developed by Kanduru et al [63] was used to determine *in vitro* CyA concentrations with some modifications. To 100 µL of cell suspension or rat serum 40 µL of amiodarone (10µg/mL) was added as an IS in each tube. Then 500 µL of HPLC water and 80 µL of 1 M NaOH were added. CyA and IS were extracted with 4 mL of 95:5 ether:methanol followed by vortex mixing for 60 s. The samples were then

centrifuged at 3000 *g* for 10 min. The supernatant was transferred into clean glass tubes and evaporated to dryness *in vacuo*. The dried samples were then reconstituted with 300 µL methanol and 10 µL was injected into the LC-MS system. The modified extraction procedure was validated for various concentrations including 50, 100, 250 and 500 ng/mL and a calibration curve was linear over the range of 50 ng/mL to 5000 ng/mL.

2.2.3.5 Drug uptake study of CyA with NRK 52E cells:

To study the effect of HL serum NRK 52E cells were grown with RF and non-RF media and treated with 10% of NL/HL or FBS serum as mentioned above (2.2.3.2). Then, after 24 h of pre-incubation cells were washed with PBS and serum-free media and different concentrations of CyA-containing media was added in each well. After treatment with CyA, the drug containing media was removed and cells were washed with PBS and serum-free media at selected time points (0 ,5 min,10 min, 1 h, 24 h). HPLC water (500 µL) was added in each well and plates were stored at -20º C for extraction. IS was added to each well and samples were extracted as mentioned above.

2.2.3.6 RNA isolation from cells and cDNA synthesis:

Both NRK 52E and LLC PK1 cell lines were treated with RF containing media to induce the level of Pgp. However, Magnarin et al. had previously reported an increase in MDR1 gene expression for LLC-PK1 cells treated

with RF-containing media; this has not been assessed for NRK 52E cells, however. Therefore, NRK 52E cells gene expression with RF and non- RF treatment was measured by real time PCR. For total isolation of RNA, cells were grown in 6-well plates. Total RNA was isolated from cells using TRIzol reagent according to the manufacturer's protocol. RNA concentration measurement was conducted using a UV spectrometer at ration of 260/280 nm wavelengths. First strand cDNA synthesis was performed according to the manufacturer's protocol by using the High-Capacity cDNA reverse transcription kit (Applied Biosystems).

2.2.3.7 Gene expression study with real time PCR:

To measure the level of Pgp with RF treatment, MDR1 gene expression was determined (which is specific to Pgp) after real-time PCR by subjecting the resulting cDNA to PCR amplification in 96-well optical plates by using the ABI Prism 7500 System (Applied Biosystems). The 25 µl reaction mix prepared by 0.1 µl of 10 µM forward primer, 0.1 µl of 10 µM reverse primer, 12.5 µl of SYBR Green, 11.05 µl of nuclease-free water and 1.25 µl of cDNA sample and β-actin gene was used as assay control. No-template controls were added to the test if there is contamination of any assay reagents. The thermocycling conditions were set at 95°C for 10 min, followed by 40 PCR cycles at 95°C for 15 s and annealing/extension at 60°C for 1 min. Determination of melting curve was performed by the end of each cycle to ascertain the specificity of the primers. The real-time

PCR data analysis were performed by using the relative gene expression (i.e., ΔΔ CT) method. The data was reported as the fold change in gene expression normalized to the endogenous reference gene (β-actin) and relative to a calibrator.

2.2.4 P407 induced HL rat model:

2.2.4.1 Animals:

The animal study was approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. The male Sprague–Dawley rats (weight: 250-350 g) were purchased from Charles River Laboratories, Canada.

2.2.4.2 Dose and collection of serum:

A colloidal solution of P407 was prepared by dissolving 7 g in 52 mL of cold normal saline (0.13 g/kg). The solution was stored at 4º C. Rats were anesthetized with isoflurane and oxygen delivery and P407 carefully injected intraperitoneally at a dose of 0.5 g/kg or 1 g/kg. Blood samples were collected from the tail vein of each rat at 0, 36, 60, 84, 108, 132, 156, 180, 282 h after each dose. After 30 minutes, serum was separated by centrifuge and stored at -20º C until assayed for analytes.

2.2.4.3 Lipid, adipokine and cytokine measurement:

The serum concentrations of TG, TC and HDL-C were measured according to the manufacturer's instructions. The lipid concentrations in each sample were calculated using standards provided in the kits, taking into account any dilution factors required. Adiponectin, leptin and TNF-α were measured using ELISA kits as per manufacturer's direction. For analysis of lipids, where necessary, dilution of the serum from P407-treated rats was accomplished using deionized water.

2.2.4.4 Data analysis:

The LDL cholesterol (LDL-C) was calculated by the relationship LDL-C=TC-(HDL-C+TG/5)" but the values were not meaningful (mostly negative values) due to very high concentration of TG.[64] The terminal phase half-lives of TG and TC were determined by analysis of the baseline-corrected log-transformed concentrations during the terminal phase. The increase in mean AUC of each measured lipid component was calculated by $\textit{Fold increase} = \frac{\textit{Total AUC}}{\textit{Total AUC - Baseline corrected AUC}}$.

Significance in difference between serum lipid levels at baseline (predose) and after doses of P407 was measured using the Student's t-test for unpaired samples. Because of difficulties in obtaining the blood from the tail of some rats different rats were used for later time points and to permit determination of the AUC the Bailer's method was used. Significance in

the differences between groups in toxicity (LDH or MTT assay) and drug uptake was assessed by the Student's t-test. Statistical analysis for assessment of dose and time effects on toxicity with CyA for both cell lines using MTT assay was performed by two way ANOVA and P values were used to compare groups.

CHAPTER: 3 Results

3.1 Development of high performance liquid chromatography (HPLC) method for measurement of tacrolimus

3.1.1 HPLC method for measurement of tacrolimus:

An HPLC method was developed to determine tacrolimus concentrations using CyA as an internal standard. The tacrolimus retention time was 5.8 min and the IS appears at about 13 min. Total run time was less than 18 min. The peaks were symmetric and a blank sample in cell culture matrix did not show any interfering peak. (Fig.4)

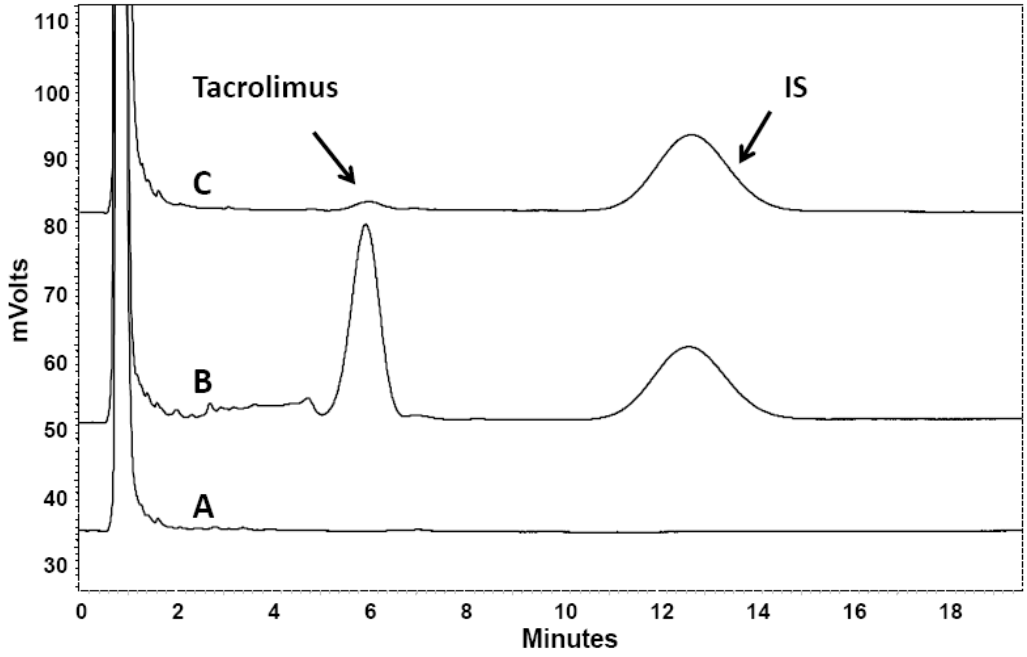

Figure 4: Chromatogram of tacrolimus and CyA (IS); The tacrolimus retention time is about 5.8 min and IS retention time is 13 min,

A. Blank cell suspension, B. Tacrolimus 100 µg/mL, C. Tacrolimus 5 µg/mL

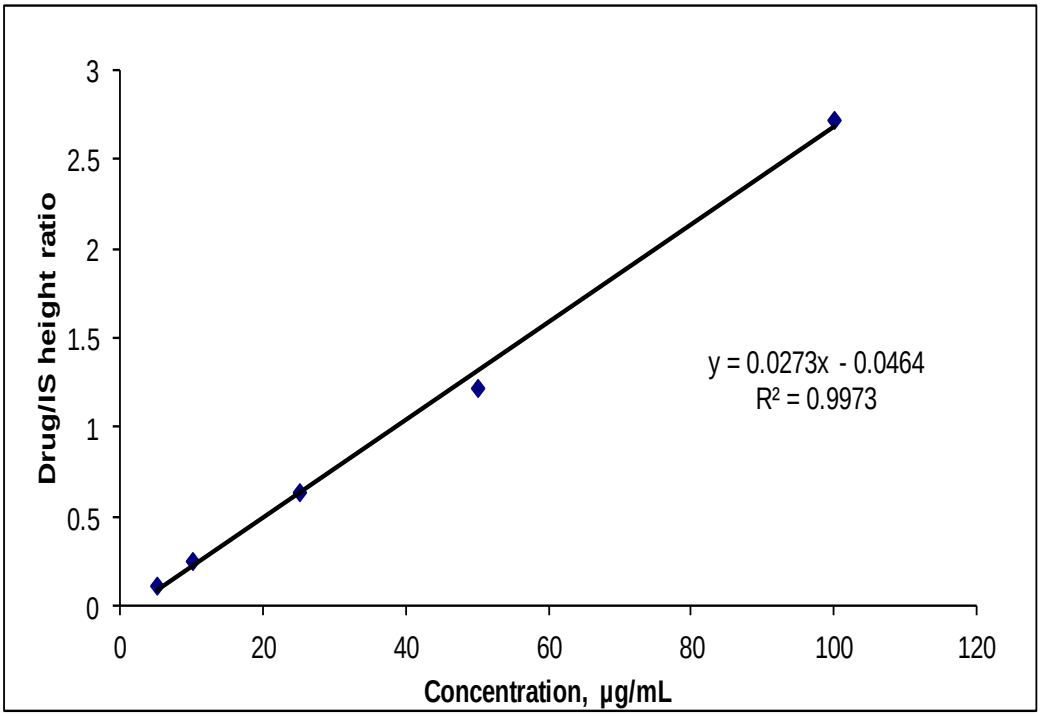

Figure 5: Calibration curve of tacrolimus using CyA as IS on HPLC

Table 1: Tacrolimus validation data in 500 µL of cell suspension

Tacrolimus expected concentration, µg/mL	Tacrolimus mean concentration, µg/mL	% error	CV %
70	73.3	6.7	2.4
50	57.2	14.3	6.1
10	11.6	16.2	3.4
5	5.5	9.5	2.7

As shows in fig.5, the calibration curve was linear (peak height ratio of drug/IS) over a range from 5 to 100 µg/mL of tacrolimus, with an R^2 of 0.9973. The validated lower concentration was 5 µg/mL for 500 µL of cell suspension. Results of one day validation for four replicates are reported in Table 1 and CV percentage and mean error were less than 20 % for the reported method (Table 1).

3.2 Cell culture experiments for tacrolimus

3.2.1 Toxicity of tacrolimus with LLC-PK1 and NRK 52E cells:

Table-2 describes the percentage LDH release in the both cell line calculated from maximum LDH release. NRK 52E cells did not showing toxicity with various concentrations of tacrolimus(0-50µg/mL). LLC-PK1 cells showed comparatively more toxicity with tacrolimus treatment at 50 µg/mL concentration for both RF treated group and non-RF treated group. However, RF treated cells had less LDH release than non-RF cells due to protective effect of increased Pgp level. For NRK 52E cells, the percentage increase in LDH release was less than 30% at 24 h compared to 20 % at 0 h with highest concentration (Fig. 6).

However, LLC-PK1 cells found to have comparatively higher toxic effect of tacrolimus. The graph below demonstrate dose dependent toxicity. Tacrolimus was not causing any damage to the cells till 30µg/mL concentration. For 50 µg/mL concentration the LDH release was about 90%. Table 2 mentioned the difference in LDH release at 24 h from 0 h

for each concentration. As expected, the Pgp-induced cells with RF containing media were showing protective effect compared to control media treated cells as tacrolimus is a substrate of Pgp. For this reason, the rest of experiments for tacrolimus were carried out with LLC-PK1 cell line. Treatment of various dose tacrolimus showed dose dependent toxicity in LLC-PK1cell line but not with NRK 52E cells.

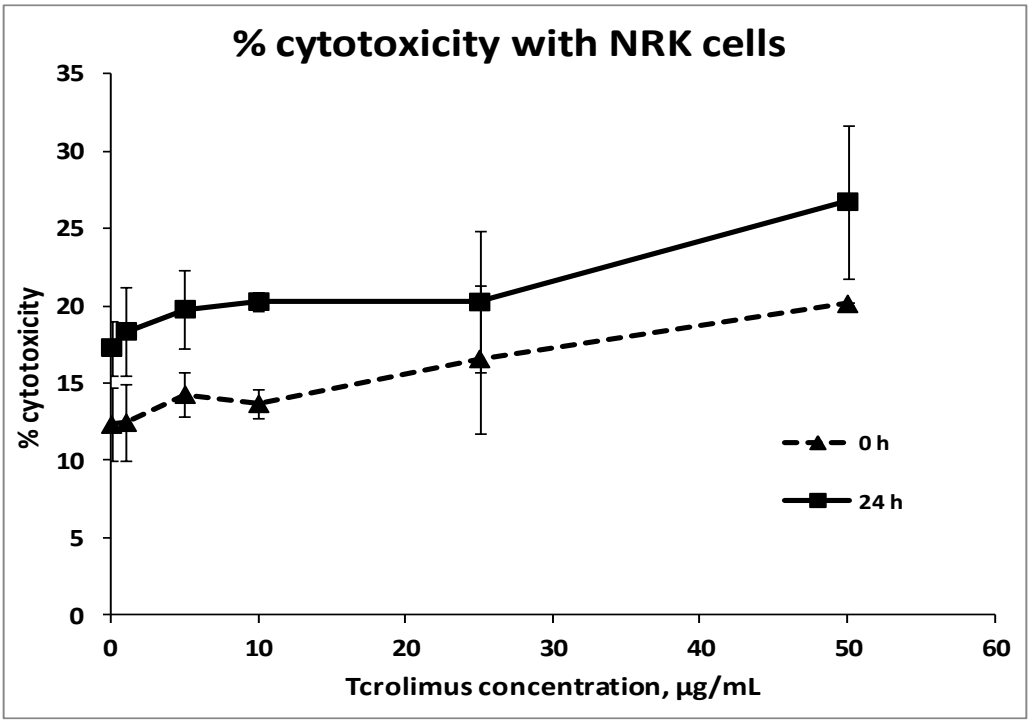

Figure 6: LDH release after 24 h of incubation of different concentrations of tacrolimus (0-50 µg/mL) with NRK 52E cells for 2 different time points, 0 and 24 h.

Percentage cytotoxicity calculated by comparing with maximum LDH release in each well.

Table 2: % LDH release in different cell lines with different concentrations of tacrolimus

Difference in LDH release compared to 0 h (Mean % LDH release)						
Concentration, µg/mL	**0**	**1**	**5**	**10**	**25**	**50**
LLC-PK1, Non-RF	3.5	3.5	2.9	4.8	3.6	75.8
LLC-PK1, RF	0.5	1.0	0.9	0.6	1.4	21.9
NRK 52E	4.9	5.9	5.5	6.6	3.6	6.6

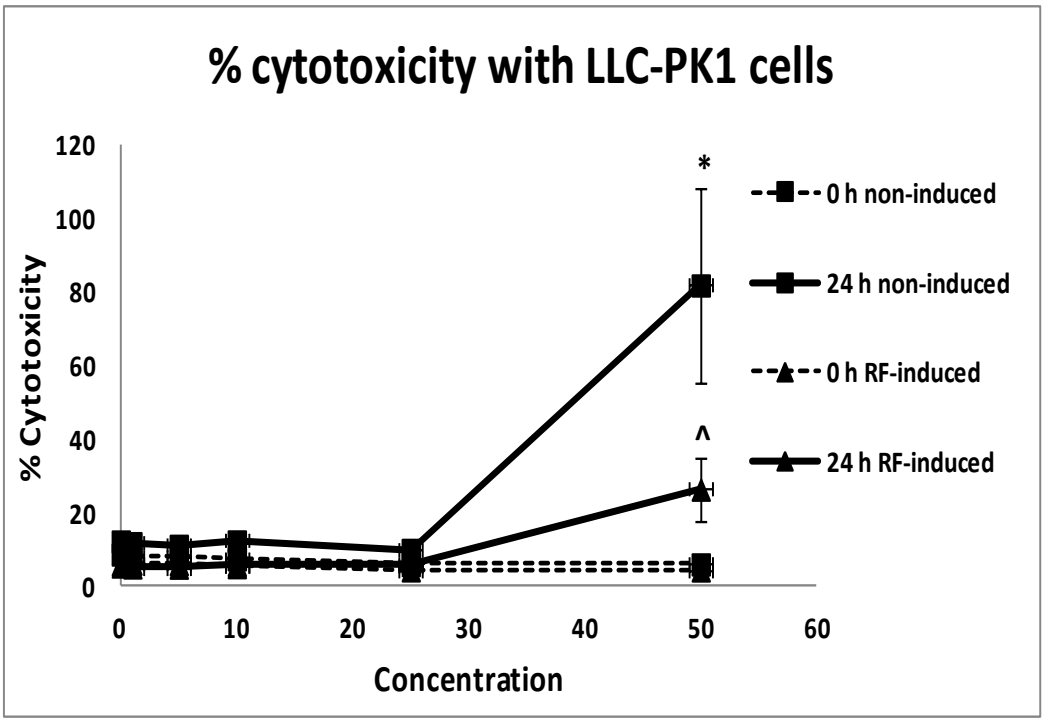

Figure 7: Tacrolimus cytotoxicity measured for different concentrations in LLC-PK1 cells after 0 and 24 h incubation.

Percentage cytotoxicity calculated by comparing with maximum LDH release in each well. * P< 0.05 compared to 0 h of incubation for non RF-induced cells, ^ P<0.05 compared to 0 h of incubation for RF-induced cells

3.2.2 Tacrolimus toxicity after hyperlipidemic serum treatment:

LLC-PK1 cells were pre-incubated with 10% of NL/HL/FBS containing media to study the effect of HL on renal toxicity with tacrolimus. LDH release was measured at 0 h and 24 h after treatment of tacrolimus. Surprisingly, the results were opposite to our expectation. As described in Fig.8, HL-treated cells showed no toxicity (~30% LDH release) followed by some toxicity with FBS- (~55 %) and most with NL- (~80%) serum. The LDH release in HL group did not differ as the dose increased. However, there was dose dependent increase in LDH release in NL- and FBS-preincubated cells. Compared to HL treatment, NL-treated cells were more susceptible to toxicity.

Moreover, the results showed a similar trend in RF-treated cells but a difference in toxicity of tacrolimus compared to the non RF-treated group in dose escalation study (Fig.6 and Fig.7). The % LDH release for the RF-treated group at 24 h for the highest concentration was around 27% for HL preincubated cells, 34 % for FBS and 42% for the NL group (Fig.9). The difference in percentage LDH release in RF treated cells from non RF cells was about 21, 40 and 1 for the FBS, NL and HL groups, respectively. Results match with the dose escalation study and the RF treated group showed a protective effect due to induction of Pgp in cells. As described in Figures 8 and 9, 30 µg/mL concentration of tacrolimus was not toxic for the RF or non RF group. The trend was similar to the toxicity studies with different dose of tacrolimus, where 25 µg/mL concentration was not toxic

(Figures.6 and 7). A 30 µg/mL concentration was not significantly toxic for any group except NL for non-RF treated cells. For the HL group, the LDH release values were ~ 32% at 0 µg/mL, ~26% at 30 µg/mL and ~28% at 50 µg/mL. The % LDH release was significantly different at 24 h for all groups compared to 0 h including the group with 0 µg/mL concentration of tacrolimus. The cytotoxicity studies showed no significant toxicity with HL treated cells compared to NL for RF and non-RF treated cells.

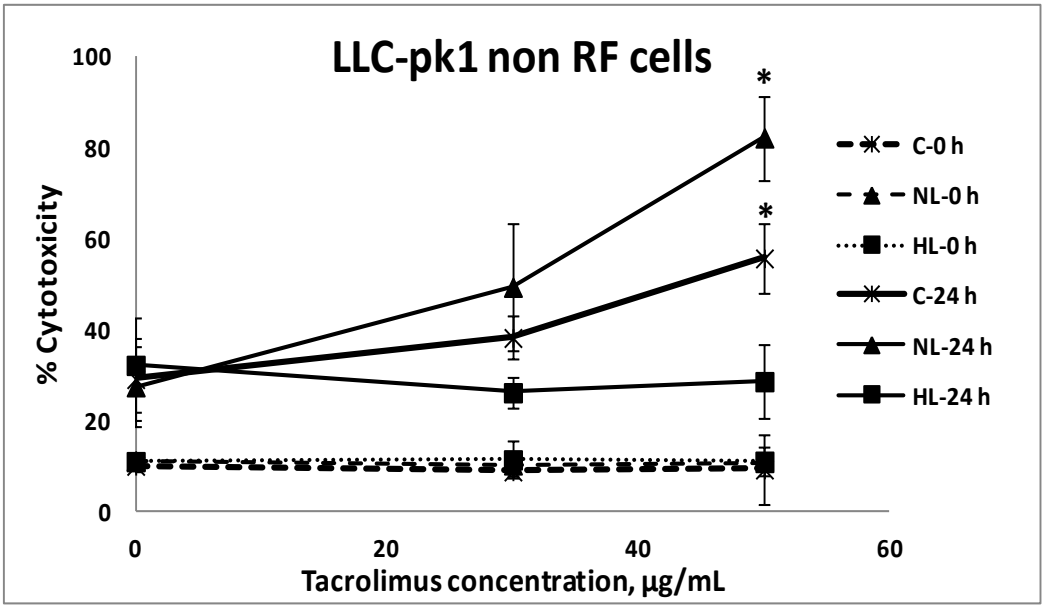

Figure 8: Cytotoxicity of tacrolimus with NL/HL/FBS-preincubated cells measured by LDH assay in non RF LLC-PK1 cells.

Percentage cytotoxicity calculated by comparing with maximum LDH release in each well. C-0 h= Control 0 hour; NL-0 h= NL 0 hour, HL-0 h= HL 0 hour, C-24 h= control 24 hour, NL-24 h= NL 24 hour, HL-24 h= HL 24 hour, *P<0.05 significant difference from 0 µg/mL tacrolimus concentration group at same time point

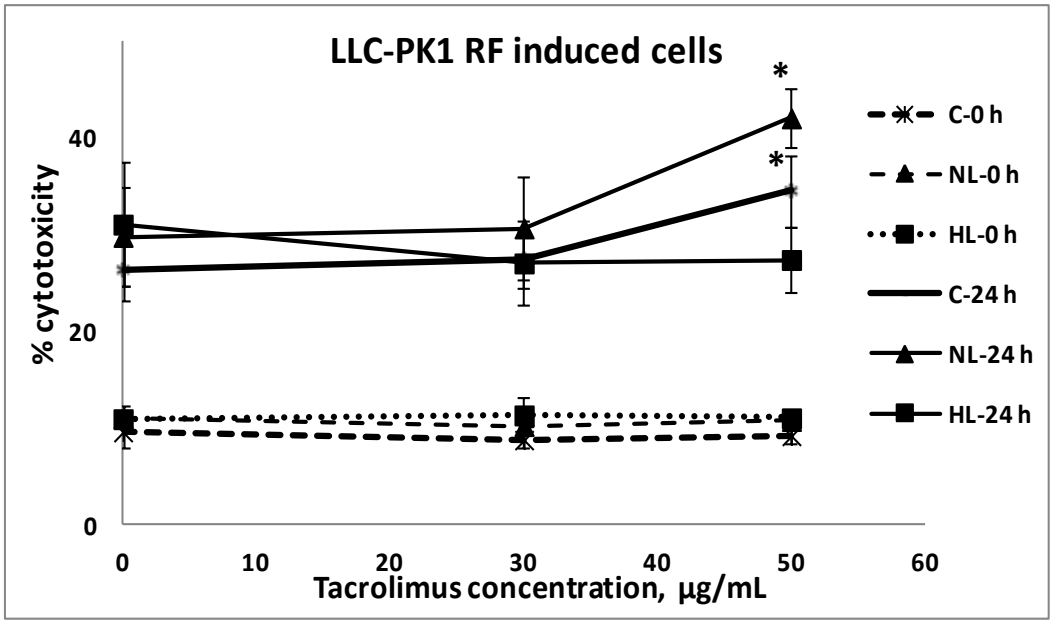

Figure 9: Cytotoxicity of tacrolimus (0,30,50 µg/mL) with NL/HL/FBS preincubated cells measured by LDH assay in non RF LLC-PK1 cells. The cytotoxicity was calculated by comparing supernatant to maximum LDH presence in each well. C-0 h= Control 0 hour; NL-0 h= NL 0 hour, HL-0 h= HL 0 hour, C-24 h= control 24 hour, NL-24 h= NL 24 hour, HL-24 h= HL 24 hour, *P<0.05 significant difference from 0 µg/mL tacrolimus concentration group.

After removal of (NL/HL/FBS) serum containing media, the cells were washed with PBS and serum free media. Despite this there was still some visual turbidity in HL group compared to NL and FBS treated cells. The reason may have been the presence of some residual HL serum remaining in the wells. This might have been associated with a some lipoprotein binding of drug that could have had a protective efffect. Another possible reason for less toxicity in HL is might be metabolism of drug, and in HL CYP enzyme activity is known to decrease. In addition to an upregulation in P-gp, the protective effect with RF group could have also been enhanced by an induction of CYP enzyme activity.[61]

3.2.3 Drug uptake studies:

LLC-PK1 cells were treated with tacrolimus and cells were collected from plates at different time points. The samples were extracted and tacrolimus concentrations were determined using HPLC (Fig.10). The drug uptake for 2 and 4 h showed no time dependent increases in intracellular concentrations. Results from 2 and 4 h treatment suggested that cells were saturated with drug and that in order to see differences in flux, shorter time periods would be needed for assessment. Unfortunately, the assay was not sensitive enough to study early time points. From 2 and 4 h treatment results, there was no significant difference in intracellular tacrolimus concentration of NL-, HL- or FBS-treated cells (Fig.10). The results at 2 h with 50 µg/mL tacrolimus showed a similar trend in drug concentrations as toxicity studies with tacrolimus (Fig. 8 and 9). The intracellular concentration was higher in NL treatment followed by FBS and HL serum treated cells.

For detection of earlier time points in drug uptake we needed a more sensitive method, which might also be useful for *in vivo* PK studies with tacrolimus in HL and NL rats. We tried to develop more sensitive LC/MS method for tacrolimus. An attempt to develop a suitable LC/MS method was not workable, however with respect to its reliability (bias and precision) at concentrations of less than those used in the HPLC assay.

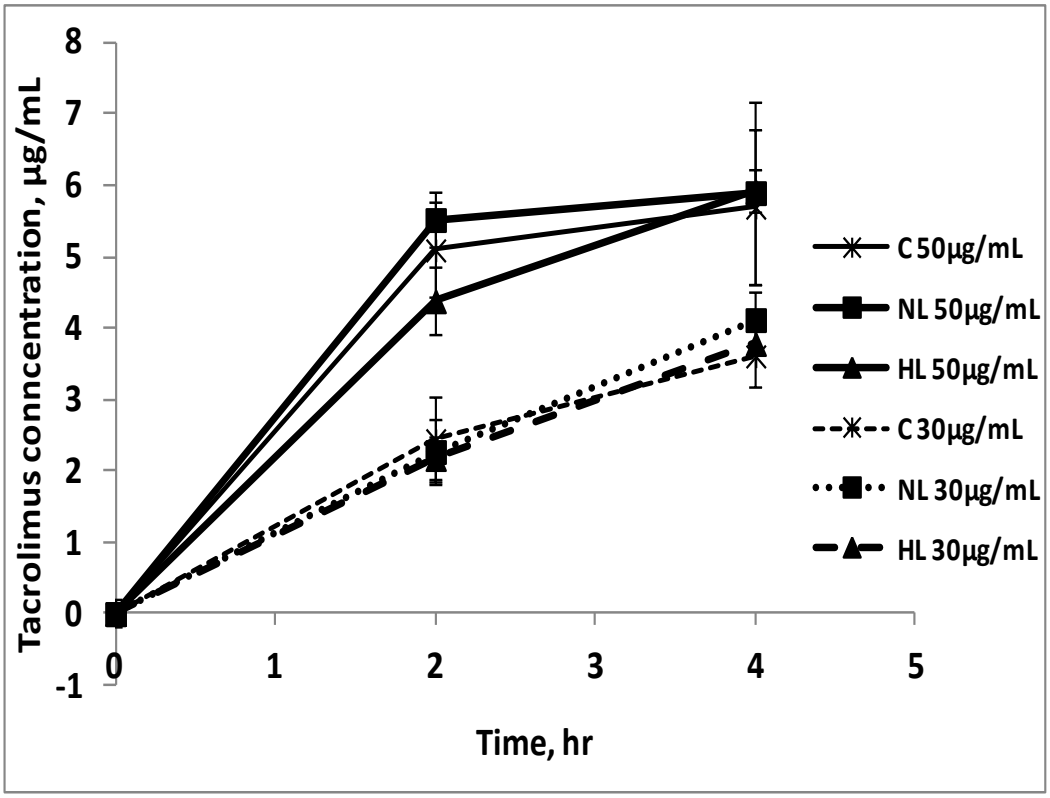

Figure 10: Drug uptake study with LLC-PK1 cells pre-incubated with 10% NL,HL or FBS and treated with tacrolimus.

C 50= Control 50 µg/mL; NL 50= NL with 50 µg/mL, HL 50= HL with 50 µg/mL, C 30= Control 30 µg/mL, NL 30= NL with 30 µg/mL, HL 30= HL with 30 µg/mL

3.3 Cell culture experiments:

3.3.1 Cyclosporine A viability study with NRK 52E and LLC-PK1 cell lines:

Both cell lines were treated with 3 different concentrations of CyA (10, 30 and 50 µg/mL) and cell viability was measured with MTT assay. There were differences in viability after 24 and 48 h of incubation with CyA in both cell lines. As described in Figure11 , NRK 52E cells after incubation

with CyA for 48 h, resulted in significant decreased in viability. The decrease in percentage viability with CyA treatment was about 20% with the highest drug concentration (50 µg/mL) followed by about 30% with the 30 µg/mL concentration and 80% with the smallest concentration. This means that as concentration increase the viability decreases due to the toxic effect of CyA. For the 24 h time point viability was about 40%, 70% and 82% for 50, 30 and 10 µg/mL concentration, respectively. NRK 52E cells showed dose-dependent and time dependent toxicity with CyA treatment. Unlike tacrolimus, CyA produced more significant toxicity with NRK 52E cells. As time progresses within each concentration there was increase in toxicity. At 0 h there was no significant difference in viability within NL, HL or FBS group for any concentration. As concentration increases the toxic effect was higher with time for NRK cells.

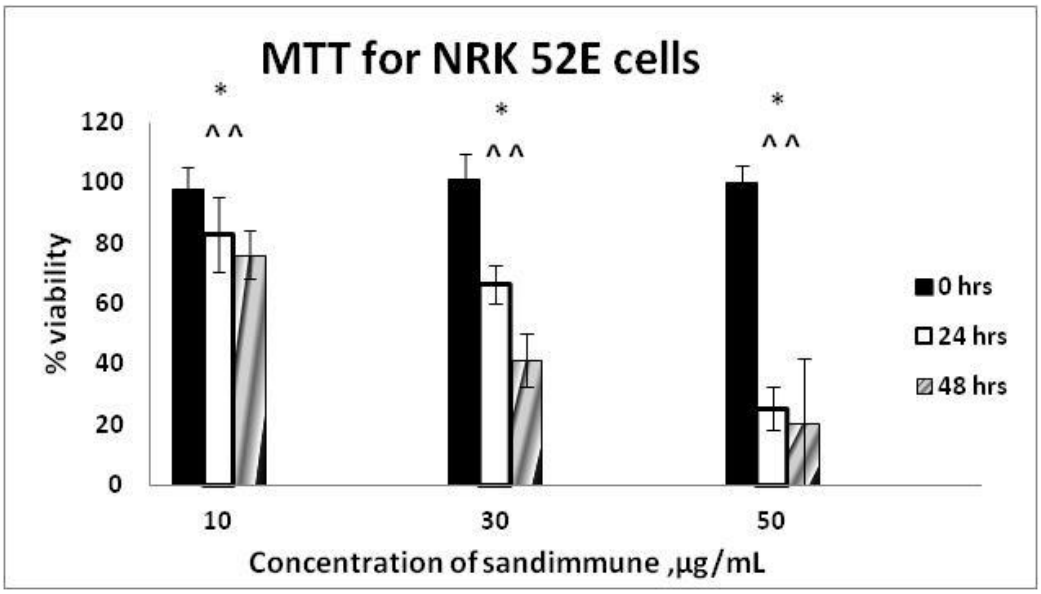

Figure 11: Cell viability measured by MTT assay (% to control group) with CyA (10,30,50 µg/mL) at 0, 24 and 48 h of incubation

* P< 0.05, significant difference between each time point, at the same concentration,

^ P< 0.05, significant difference between each concentration at the same time point

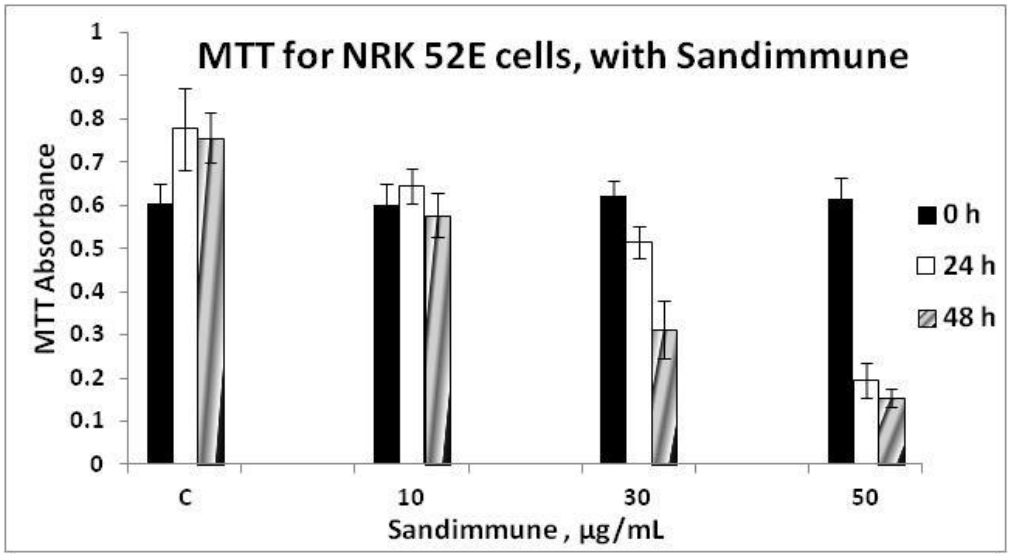

Figure 11 a: Cell viability measured by MTT assay (absorbance values) with CyA (0,10,30,50 µg/mL) at 0, 24 and 48 h of incubation

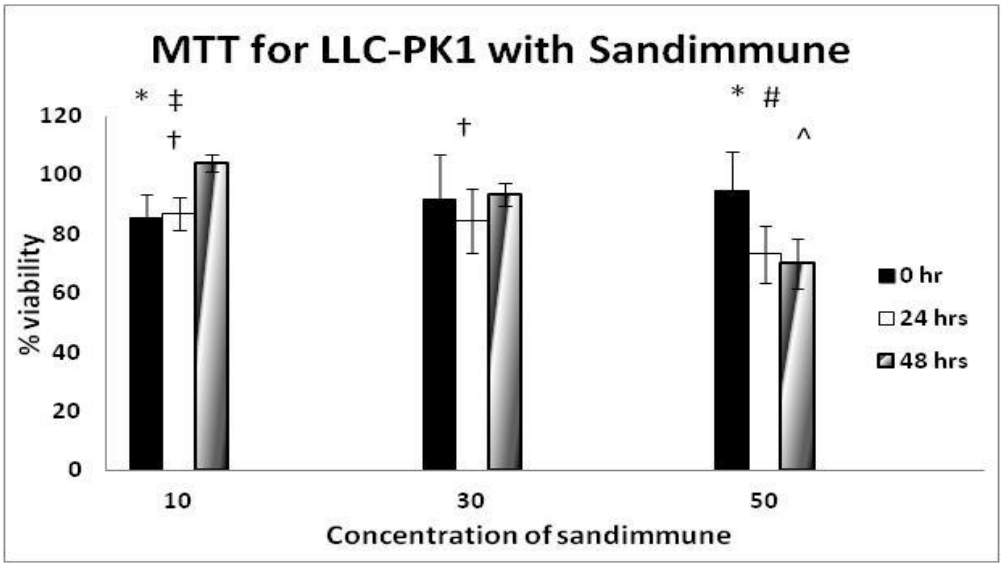

Figure 12: Cell viability with LLC-PK1 cells(% to control) determined by MTT assay after incubation with CyA (0,10,30,50 µg/mL) for 0, 24 and 48 h

*, P<0.05 at that concentration between time 0 and 48 h; #, P<0.05 at that concentration between time 0 and 24 h; ‡, P< 0.05 at that concentration between time 24 and 48 h; ^, P< 0.05 at that time between each of the three concentrations

†, P< 0.05 at that time with 50 µg/mL concentration

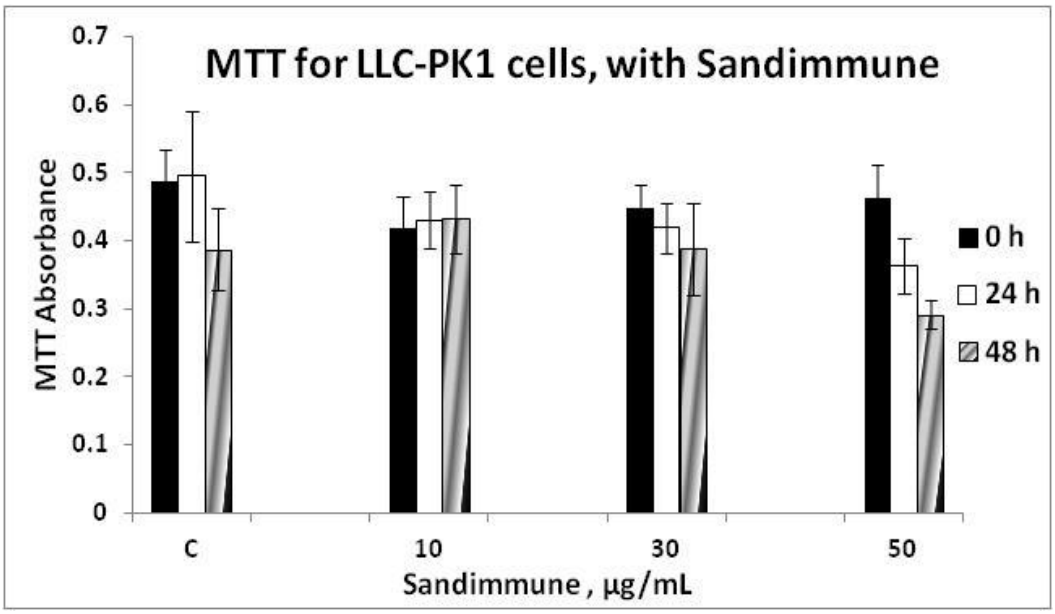

Figure 12 a: Cell viability with LLC-PK1 cells (absorbance values) determined by MTT assay after incubation with CyA (0,10,30,50 µg/mL) for 0, 24 and 48 h

For the LLC-PK1 cell line the decrease in viability was much less than compared to the rat kidney cell line (NRK 52E). For the pig kidney cell line (LLC-PK1) 48 h of incubation of CyA decreased viability about 70% with highest concentration. For 30 µg/mL concentration the viability was more than 90% at 48 h and approximately 85 % at 24 h. (Fig. 12) Similarly, the smallest concentration at 48 h of treatment showed almost 100 % viability compared to about 80% for both other time points (0 and 24 h). This trend might be result from variability but the difference in viability at 30 and 10 µg/mL at different time points was not significant. LLC-PK1 cell line might be more resistant to cell damage with CyA compared to the NRK 52E cell line. Thus, the rest of the experiments were continued with the NRK 52E cell line.

Figure 11a and 12a describes as the mean of absorbance measured after MTT assay. Statistics is not done for those graphs as the purpose was simply to to depict the absolute absorbance with treatment of CyA in both cell lines; statistical analysis was performed using the data normalized to similarly treated control (drug-free) incubations.

3.3.2 MDR 1 gene expression in NRK 52E cells after treatment with RF:

Pgp was induced by treatment of RF-containing media for 6 days. MDR1 gene expression was measured in NRK 52E cells on day 7. RF treatment with NRK cells showed an approximate 14-fold increase in MDR1 mRNA. Fig.13 shows the increase in MDR1 gene expression in NRK-52E cells compared to the control group. β-actin was used as a housekeeping gene and there was no effect of RF treatment found on the housekeeping gene. Cells grown with normal media (without RF) for a similar time were used as control.

The purpose of the RF induced group was to understand the effect of Pgp in drug uptake, and if HL could interact with Pgp in its toxicity to cells in the presence of the immunosuppressive drugs. However, RF is not only inducer for Pgp but also CYP enzymes, which were not measured in

present study. However, the protective effect from drug toxicity in RF group might be result of drug metabolism by CYP enzymes.

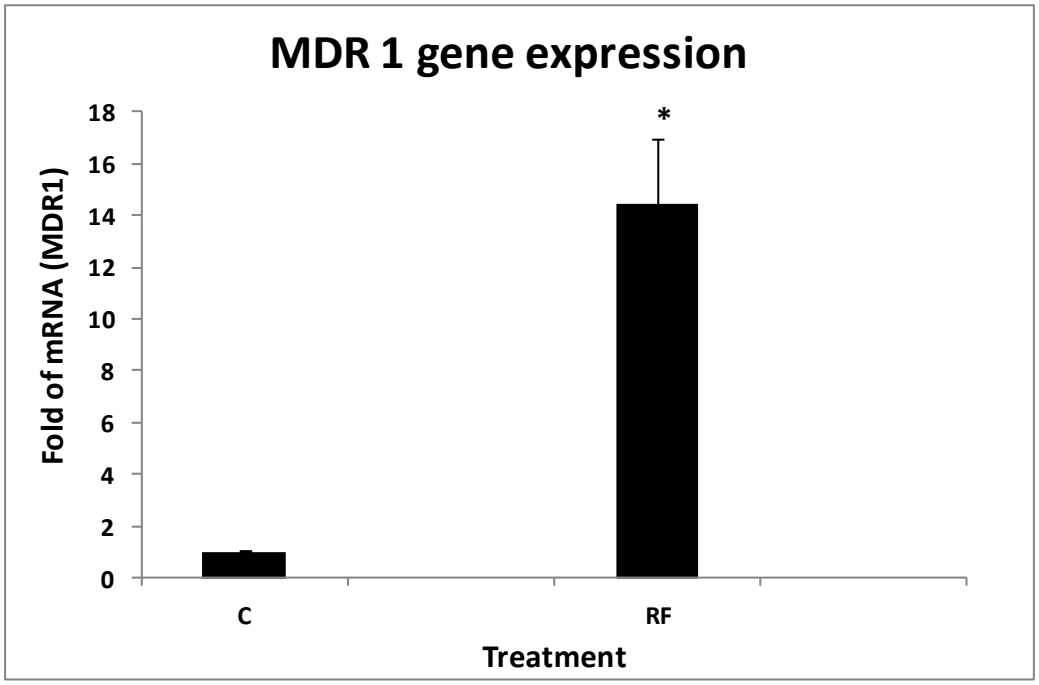

Figure 13: MDR 1 gene expression using β-actin as a housekeeping gene after treatment of RF containing media.

C= Control group, RF= rifampin-induced group * P< 0.05 significant difference from control group

3.3.3 HL serum treatment effect analyzed with LDH assay:

The effect of serum on toxicity was studied for 0, 24 and 48 h of incubation of CyA. Figure 14 describes the LDH release in non RF treated NRK cells after 24 h pre-incubation of cells followed by CyA treatment. There was no significant difference between NL, HL or FBS-treated cells. RF-treated cells showed less LDH release due to increased Pgp expression. However, both groups did not show any difference due to serum treatment.

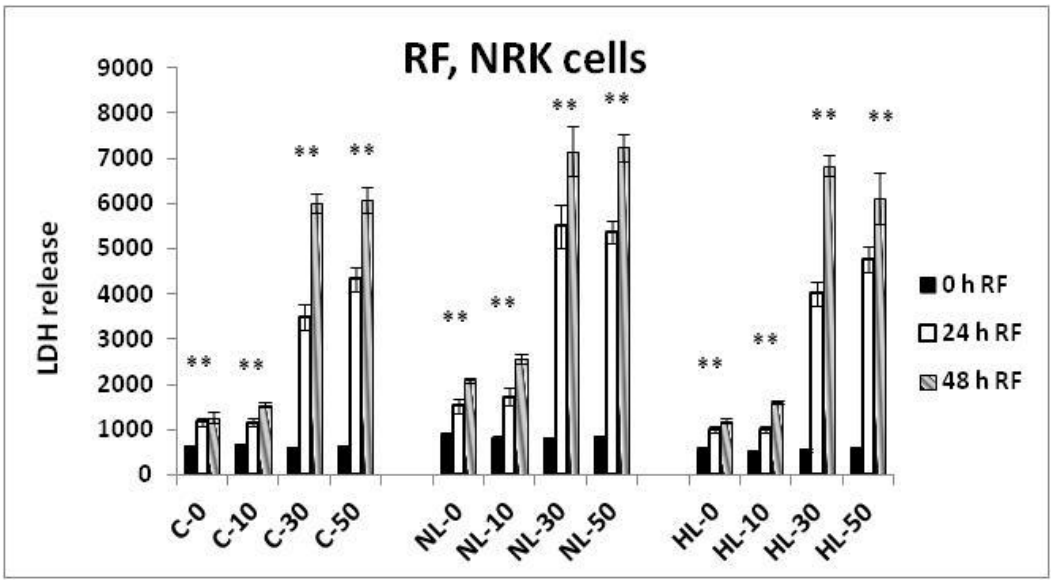

Figure 14: CyA (0, 10, 30 and 50 µg/mL) cytotoxicity measured in NRK 52E cells using LDH assay in non-RF treated cells. X axis represent groups and Y axis represent total LDH released measure by fluorescence.

*, P<0.05 significantly different from 0 h treatment in same group

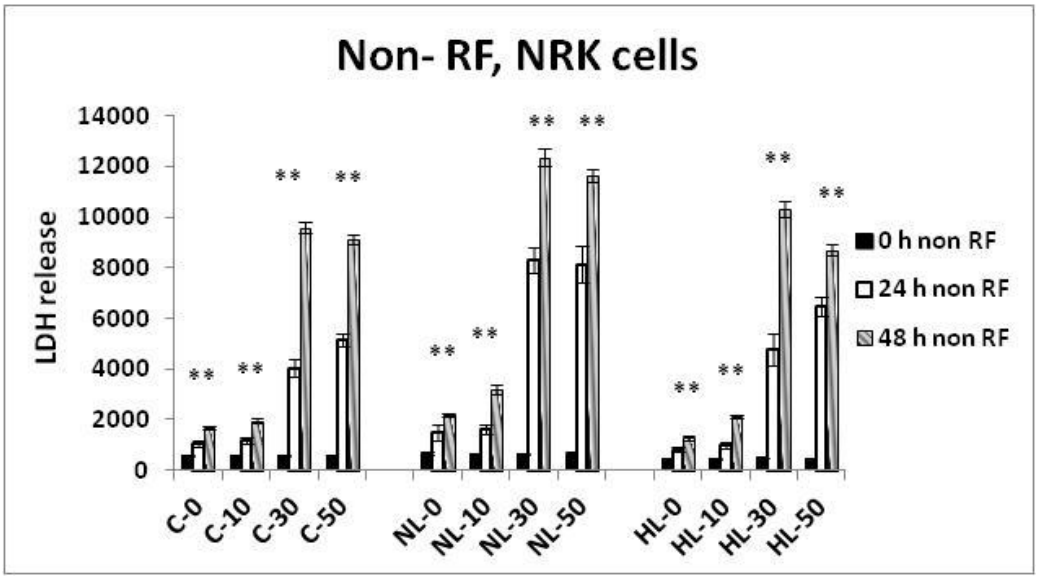

Figure 15: CyA(0,10,30,50 µg/mL) cytotoxicity measured in NRK 52E cells using LDH assay in RF treated cells. X axis represent groups and Y axis represent total LDH released measure by fluorescence.

*, P<0.05 significantly different from 0 h treatment in same group

The results were not similar with tacrolimus treatment for the HL group. Nevertheless, there was no higher toxicity in the HL pre-incubated group with either of the immunosuppressant drug (Tacrolimus or CyA) as we expected. For some reason, we were not able to calculate maximum LDH release here as tacrolimus. So, the results reported as LDH release fluorescence reading. Stability studies with CyA were performed with LDH assay kits reagent and there was no interference in fluorescence by CyA.

3.3.4 Cyclosporine A measurement by LC/MS method:

The previously published LC/MS method was validated after some changes in the extraction procedure. The method was specific and sensitive and the validated lower limit of quantification was 50 ng/mL. The calibration curve was linear for the range of 50-1000 ng/mL with R^2 of 0.998.

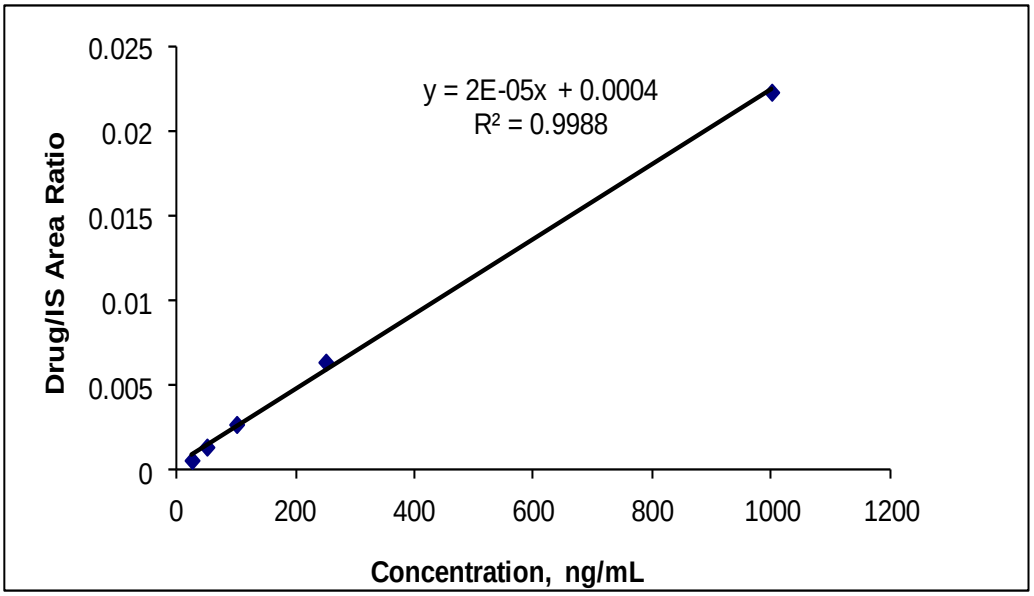

Figure 16: CyA LC/MS calibration curve for concentration range of 0-1000 ng/mL

Table 3: Validation of CyA LC/MS method (n=4)

CyA expected Concentration, ng/mL	CyA Mean concentration, ng/mL	% error	CV %
500	551.1	10.2	14.2
250	258.5	3.4	7.5
100	109.2	9.2	13.2
50	48.2	-3.5	10.8

3.3.5 Drug uptake study of CyA with NRK 52E cells:

The drug uptake study was performed by pre-incubation of NRK 52E cells with NL or HL or FBS serum. The intracellular concentration of CyA was measured by LC/MS. The cells were collected after 0, 5 min, 10 min, 1 h and 24 h to measure intracellular concentrations. The intracellular concentrations between groups (NL or HL or FBS) were not significantly different except at some time points. For RF-treated cells and non-RF treated cells the trend was similar. The following graphs demonstrate the intracellular concentrations of CyA in NRK 52E cells after incubation with three different concentrations of CyA for RF and non-RF treated cells. In some groups, there was a trend where higher concentrations were found in HL followed than in NL and control groups. (fig.18, 20, 21,22) However, the difference was only significant in between NL and HL groups in non-RF group with 50 µg/mL concentration at 60 mins. Figure 22 shows intracellular concentrations of CyA in NRK 52E cells after 50 µg/mL treatment with Sandimmune. The intracellular concentration of CyA in HL serum preincubated group was significantly different than control (FBS preincubated group) but not different from NL serum treated cells.

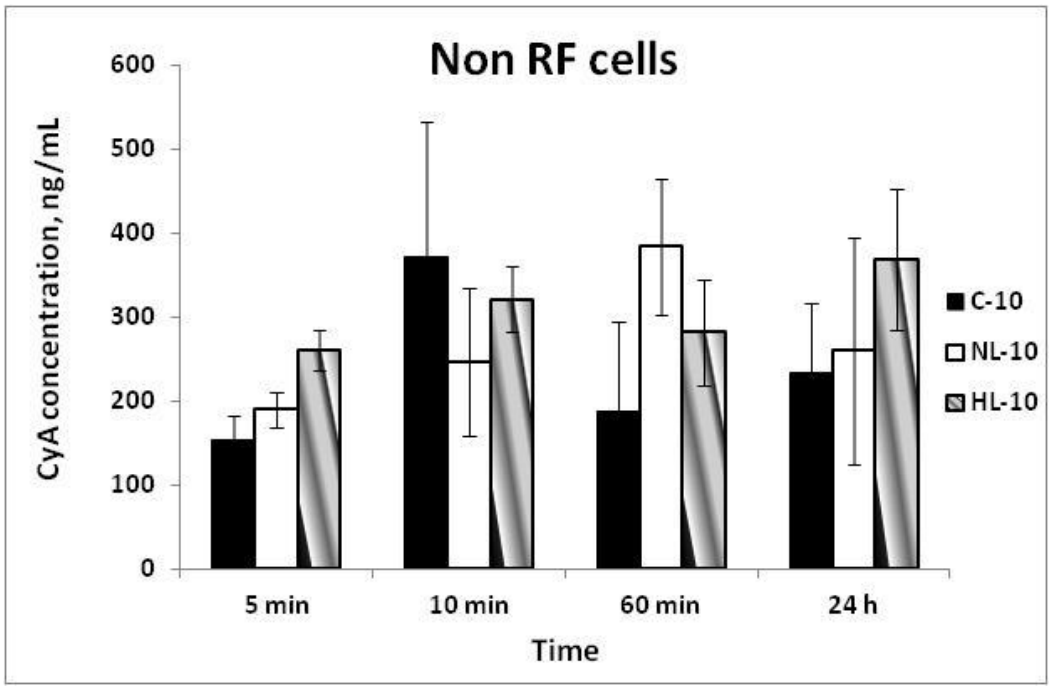

Figure 17: Intracellular concentration in NRK 52E cells after 10 µg/mL treatment with Sandimmune with C/NL/HL serum-preincubated cells.

The difference between intracellular concentration of CyA in NL and HL groups were not significant

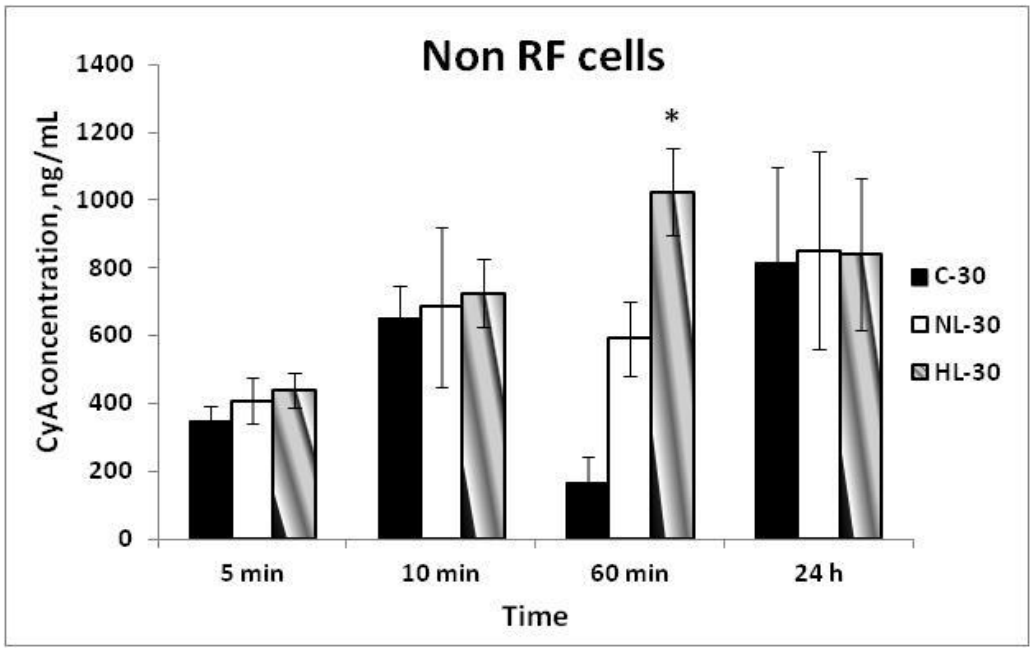

Figure 18: Intracellular concentration in NRK52E cells after 30 µg/mL treatment with Sandimmune with C/NL/HL serum preincubated cells. * P< 0.05,CyA concentration is significantly different in HL than control group

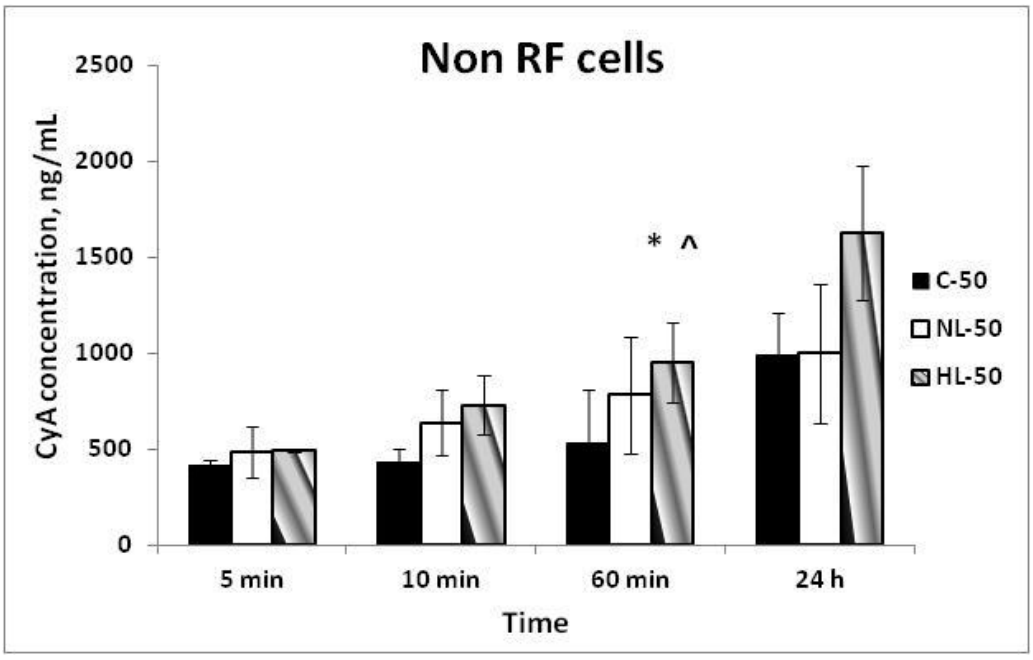

Figure 19: Intracellular concentration in NRK 52E cells after 50 µg/mL treatment with Sandimmune with C/NL/HL serum preincubated cells. * P< 0.05, CyA concentration in HL group is significantly different than control group, ^ P< 0.05, CyA concentration is significantly different in HL than NL group

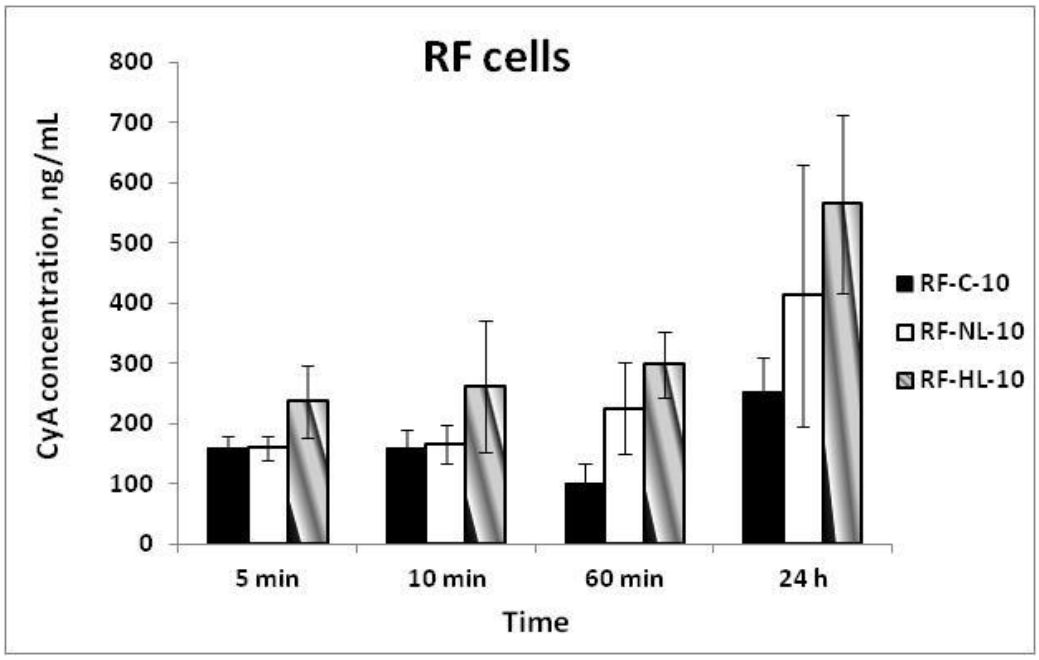

Figure 20: Intracellular concentration of CyA in NRK 52E cells after 10 µg/mL treatment with Sandimmune with C/NL/HL serum-preincubated cells.

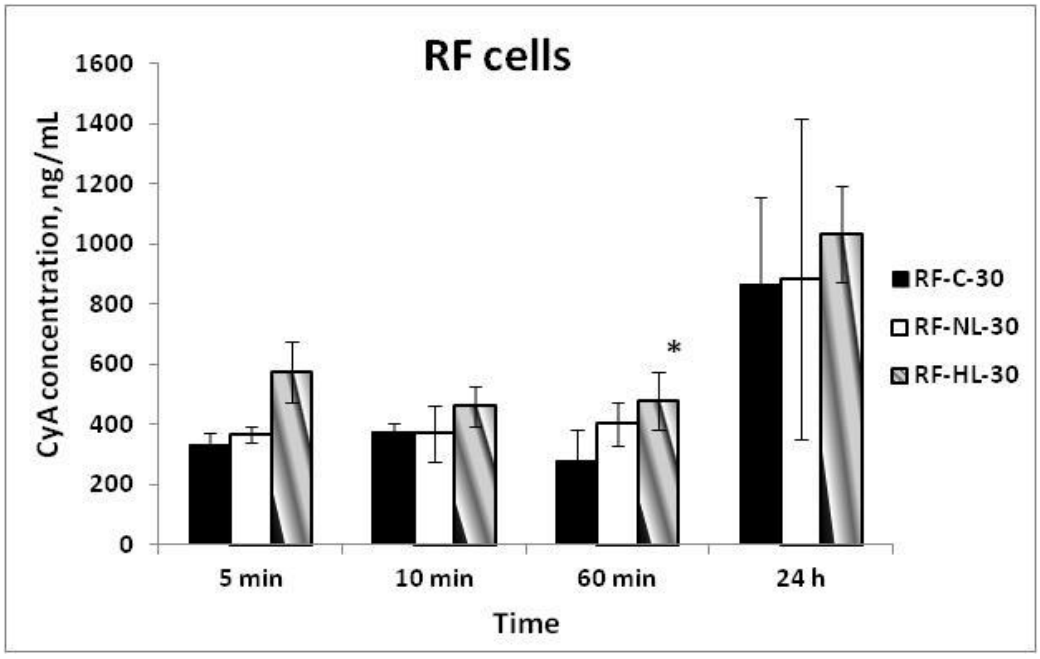

Figure 21: Intracellular concentration in NRK 52E cells after 30 µg/mL treatment with Sandimmune.

* P< 0.05, HL concentration is significantly different than control group

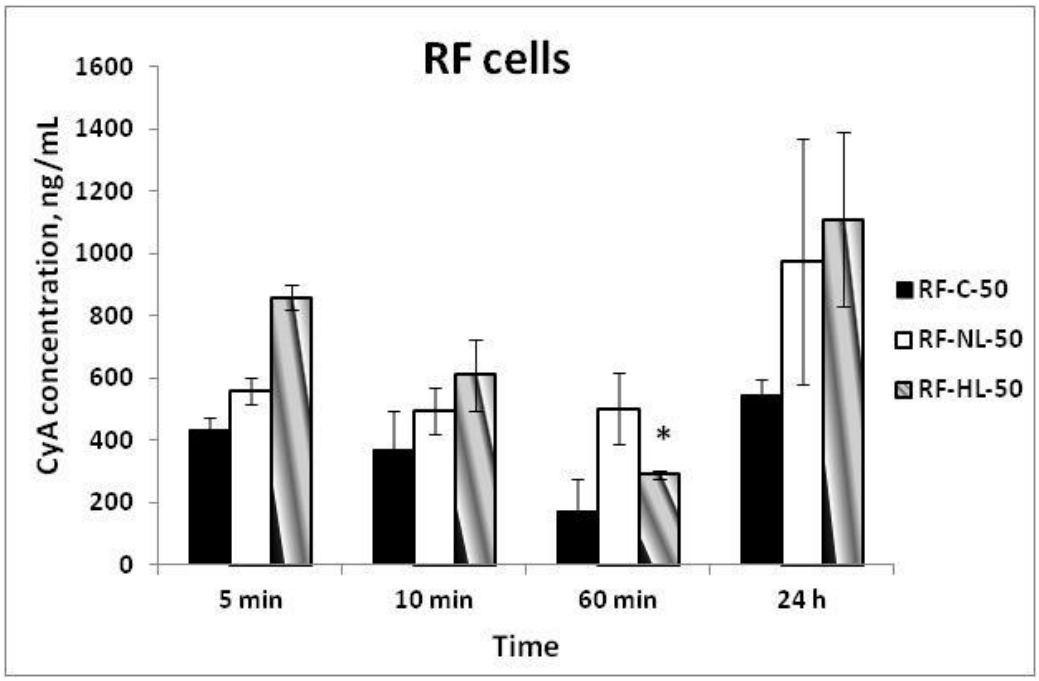

Figure 22: Intracellular concentration of CyA in NRK 52E cells after 50 µg/mL treatment with Sandimmune.

***** P< 0.05, CyA concentration is significantly different in HL than control group

3.4 P407 induced HL rat model:

3.4.1 Lipid concentrations

All values of Cmax were significantly higher after P407 compared to baseline values. As expected, both doses of P407 caused rapid and substantial increases in plasma TC and TG (Figures 23 and 24). For both dose levels, the tmax of both lipids occurred at 36 h after dosing. For the Cmax, the maximum fold increases in serum TC for the 0.5 and 1 g/kg doses were 20 and 44, respectively, increasing in a nearly dose-proportional fashion. The TG also increased substantially, by 41 and 48-fold after doses of 0.5 and 1 g/kg P407, respectively, although not in a

dose proportional fashion (Table 4). The Cmax of HDL-C also increased but not as much as for the other two classes of lipids, with TC increasing 6.7 and 17-fold for the 0.5 and 1 g/kg doses of P407, respectively.

Using the difference between total and baseline corrected values of mean AUC, for TC the increase in AUC was found to be 4.5 and 16-fold for 0.5 and 1 g/kg doses, respectively, an increase greater than proportional to the increase in dose. In contrast, for TG the increase in baseline corrected AUC was nearly proportional to the difference in dose (6.5 and 15 for the 0.5 and 1 g/kg doses, respectively). For HDL-C the increase in AUC was somewhat higher than the increase in dose (3.1 vs 8.8-fold increase in AUC for the 0.5 and 1 g/kg P407 dose levels, respectively).

In all rats a reversible nature of HL was seen, although there were some differences in the duration of time over which elevated lipid levels presented with the lower and higher dose of P407. The HDL-C had returned to baseline conditions (defined as <150% of the initial lipid levels) within 5.5 and 6.5 days for the 0.5 and 1 g/kg doses, respectively). The TC took 7.5 days to return to baseline conditions after both dose levels. The increase in TG was more sustained. With a dose of 0.5 g/kg, TG levels took ~11 days to return to baseline TG. With the dose of 1 g/kg, mean TG concentrations were still 1.9-fold higher than baseline in rats at ~12 days after the dose of P407 was given.

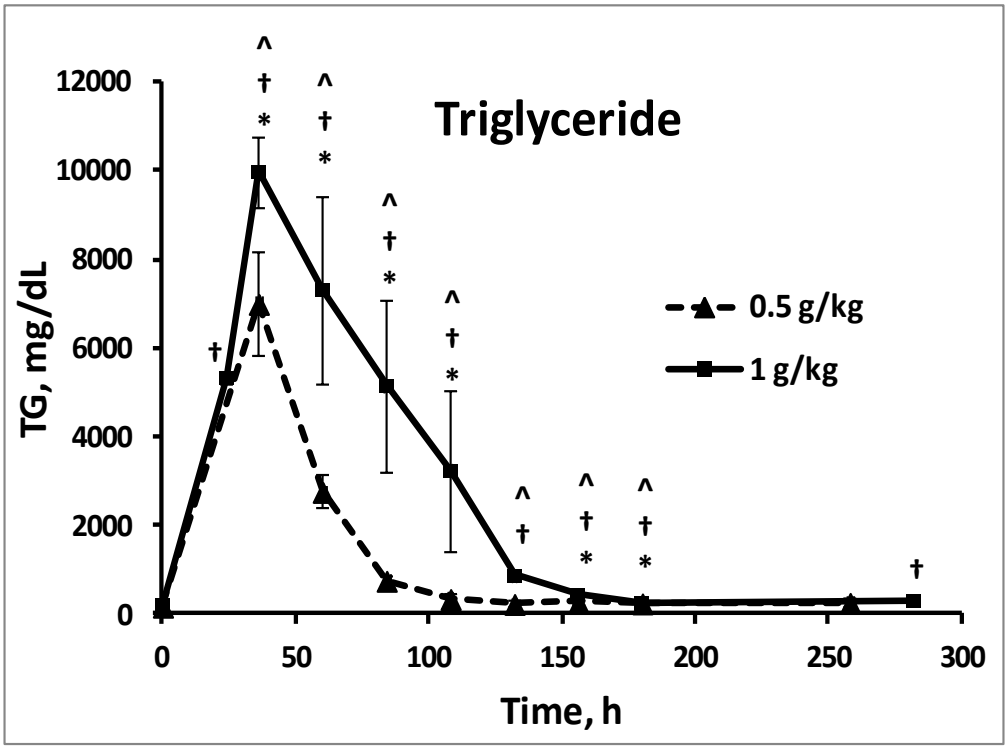

Figure 23: Triglyceride serum concentration vs time curve.

* P<0.05 significant difference from baseline concentration (0 h) for 1 g/kg.

† P<0.05 significant difference from baseline concentration (0 h) for 0.5 g/kg.

^ P<0.05 significant difference between 1 g/kg and 0.5 g/kg.

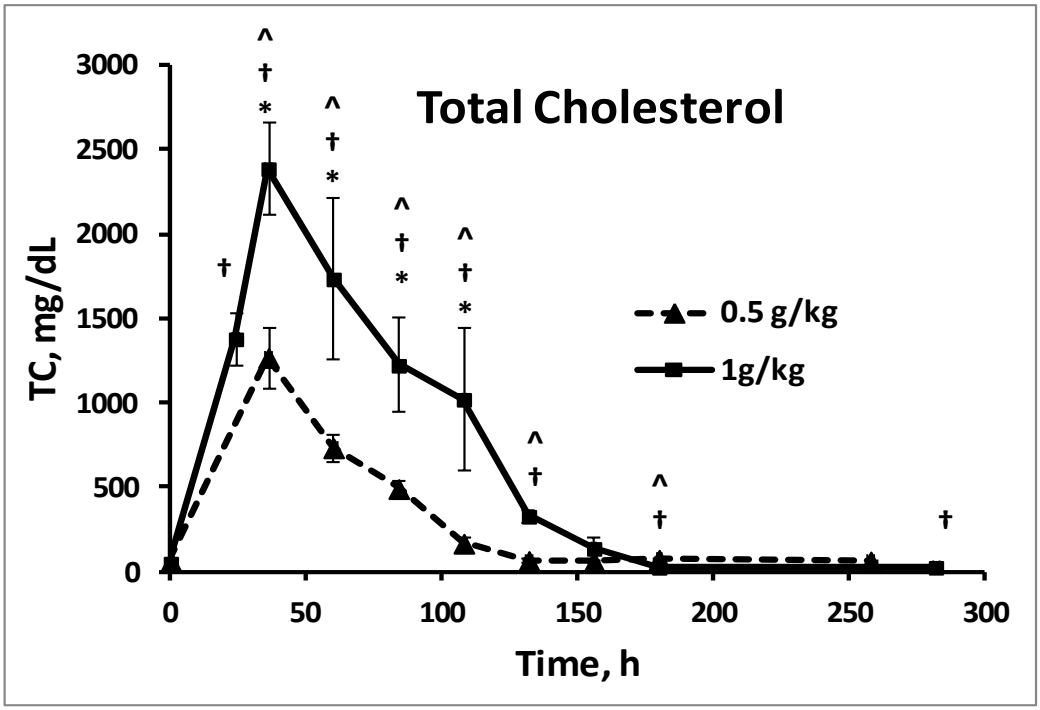

Figure 24: Total cholesterol serum concentration vs time curve.

* P<0.05 significant difference from baseline concentration (0 h) for 1 g/kg

† P<0.05 significant difference from baseline concentration (0 h) for 0.5 g/kg.

^ P<0.05 significant difference between 1 g/kg and 0.5 g/kg.

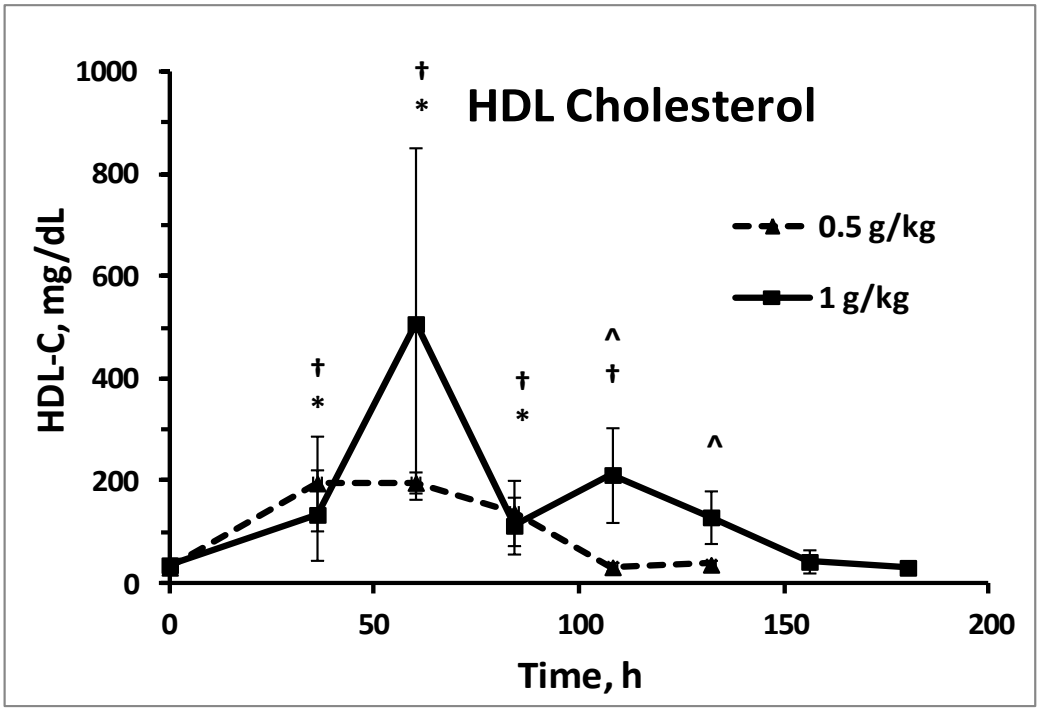

Figure 25: HDL cholesterol serum concentration vs time curve.

* P<0.05 significant difference from baseline concentration (0 h) for 1g/kg.

† P<0.05 significant difference from baseline concentration (0 h) for 0.5 g/kg.

^ P<0.05 significant difference between 1 g/kg and 0.5 g/kg.

Table 4: P407 altered levels of total cholesterol, triglyceride and HDL cholesterol (mean ± SD) and other calculated parameters after P407 administration

Parameters	TC	TG	HDL-C	TC	TG	HDL-C
	0.5 g/kg			1 g/kg		
C_0, mg/dL	60.7±10	179±40.5	29.5±8.24	60.7±10.8	179±40.5	29.5±8.24
C_{max}, mg/dL	1272±182* ^	7005 ±1176* ^	197±22.0*	2391±273*	9973±1004*	508±344*
BC C_{max}, mg/dL	1211±173* ^	6826±1142* ^	168±20.7*	2330±273*	9794±1006*	479±341*
$AUC_{0\text{-}tlast}$, mg·h/L	82691±2695^	340962±16519^	15598±1456^	197429±8121	716537±37269	29796±4086
BC $AUC_{0\text{-}tlast}$, mg·h/L	67307±2688^	295147±16531^	11797±1454^	185453±8486	676873±57216	25566±4176
$t_{1/2}$, h	12.6	13.2	ND	18.3	15.0	ND

C_0 -Baseline concentration at time 0 h, C_{max}- maximum serum concentration, BC C_{max}- baseline corrected C_{max}, $AUC_{0\text{-}tlast}$- area under the concentration vs time curve, BC AUC $_{0\text{-}tlast}$ -baseline corrected $AUC_{0\text{-}tlast}$.

^ Significant difference between 0.5 g/kg and 1g/kg dose ($P<0.05$).

* Significant difference in concentration from 0 h (baseline) concentration ($P<0.05$).

3.4.2 Adipokines and cytokines:

Serum adiponectin, leptin and TNF-α concentrations were measured using ELISA kits. P407 was found to transiently decrease serum concentrations of adiponectin, but increase those of leptin (Figures 26 and 27). Adiponectin concentrations decreased significantly with P407 dose but returned to normal after more than 84 h. Doses of 0.5 g/kg P407 increased leptin concentrations up to about 84 h. There was ~2 fold decrease in adiponectin and leptin (after 0.5 g/kg). Leptin levels with the higher P407 dose led to more variability, although the mean increases seemed to be higher than the lower dose.The baseline measure for the lower dose group was lower than for the higher dose group. When this was taken into account, the relative maximal increase in leptin seemed to be similar for the two dose groups.

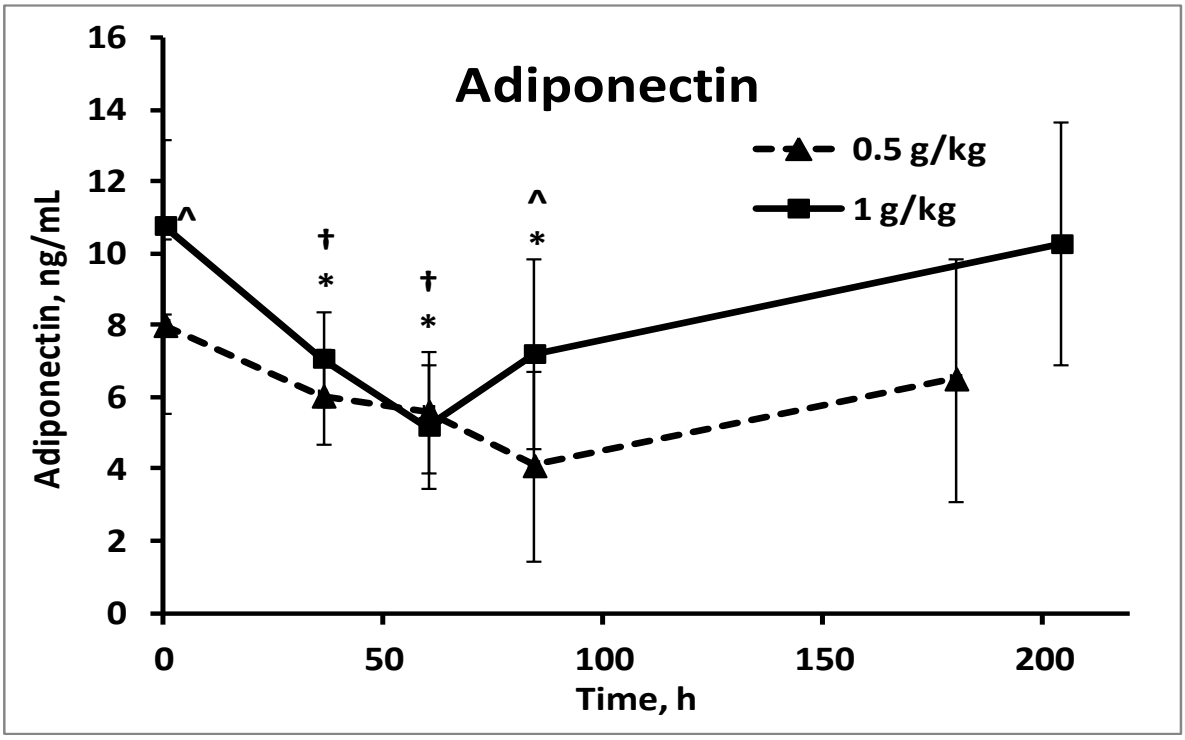

Figure 26: Adiponectin concentration vs time curve.

* P<0.05 significant difference from baseline concentration (0 h) for 1g/kg,

† P<0.05 significant difference from baseline concentration (0 h) for 0.5 g/kg,

^ P<0.05 significant difference between 1 g/kg and 0.5 g/kg

Leptin levels had high variability with higher dose. Levels remained higher even at 204 h but the difference was not significantly higher from baseline levels due to variability. There was small a increase in TNF-α levels but the difference was not significant.

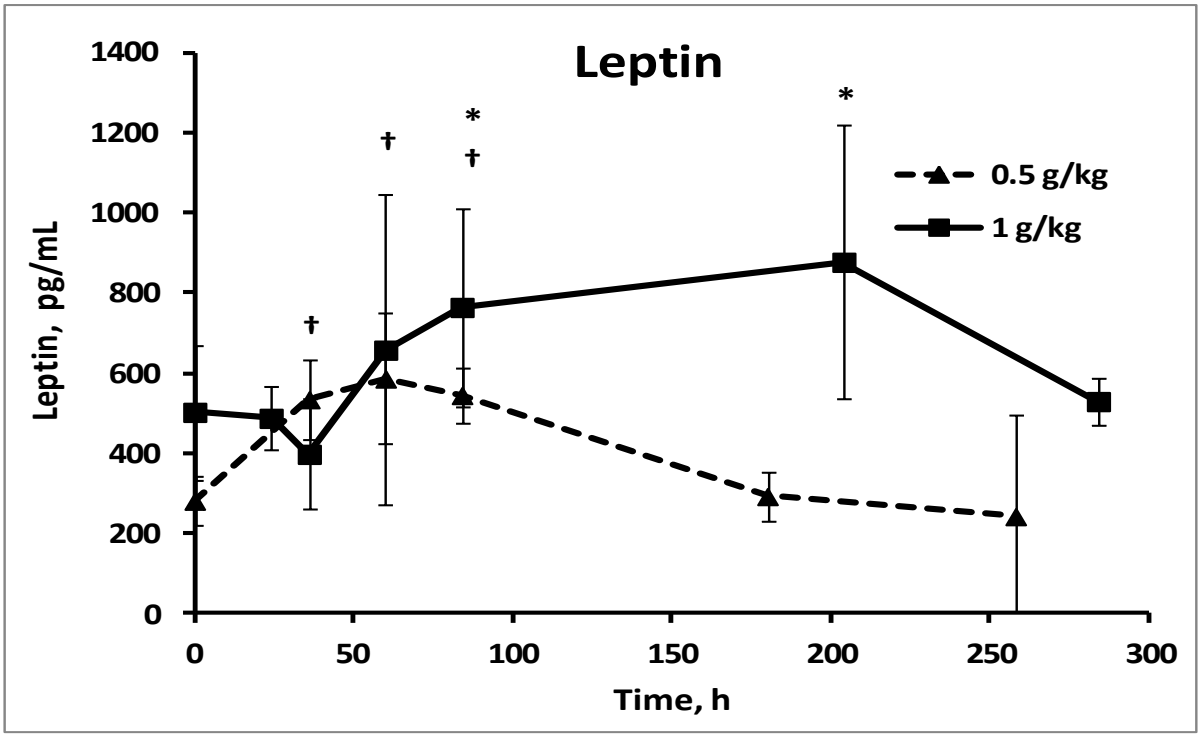

Figure 27: Leptin concentration vs time curve.

* P<0.05 significant difference from baseline concentration (0 h) for 1g/kg,

† P<0.05 significant difference from baseline concentration (0 h) for 0.5 g/kg

Table 5: Altered levels of adiponectin, leptin and TNF-α (mean ± SD) and other calculated parameters

Parameters	Adiponectin,	Leptin	TNF-α	Adiponectin	Leptin	TNF-α
	0.5 g/kg			**1 g/kg**		
C0	7.99±0.72 ng/mL	281±62 pg/mL	2.6±1.6 pg/mL	10.7±2.4 ng/mL	281±62pg/mL	2.1±1.17
BC Cmax	-	586±163pg/mL	2.6±1.7 pg/mL	-	537±217pg/mL	3.3±1.28pg/mL
Cmax	-	305±163pg/mL	5.7±1.6 pg/mL	-	268±199pg/mL	3.9±0.6 pg/mL
BC AUC	409±43 ng×h/mL	32463±3226 pg×h/mL	324.4±62.7 pg×h/mL	611±121 ng×h/mL	40789±11351 pg×h/mL	375.6±31 pg×h/mL
AUC	1042±43ng×h/mL	81874±3226 pg×h/mL	814.6±62.6 pg×h/mL	1666±159 ng×h/mL	86020±12820 pg×h/mL	133.9±20 pg×h/mL

C_0 -Baseline concentration at time 0 h, C_{max}- maximum serum concentration, BC C_{max}- baseline corrected C_{max}, $AUC_{0\text{-}tlast}$- area under the concentration vs time curve, BC AUC $_{0\text{-}tlast}$ -baseline corrected $AUC_{0\text{-}tlast}$

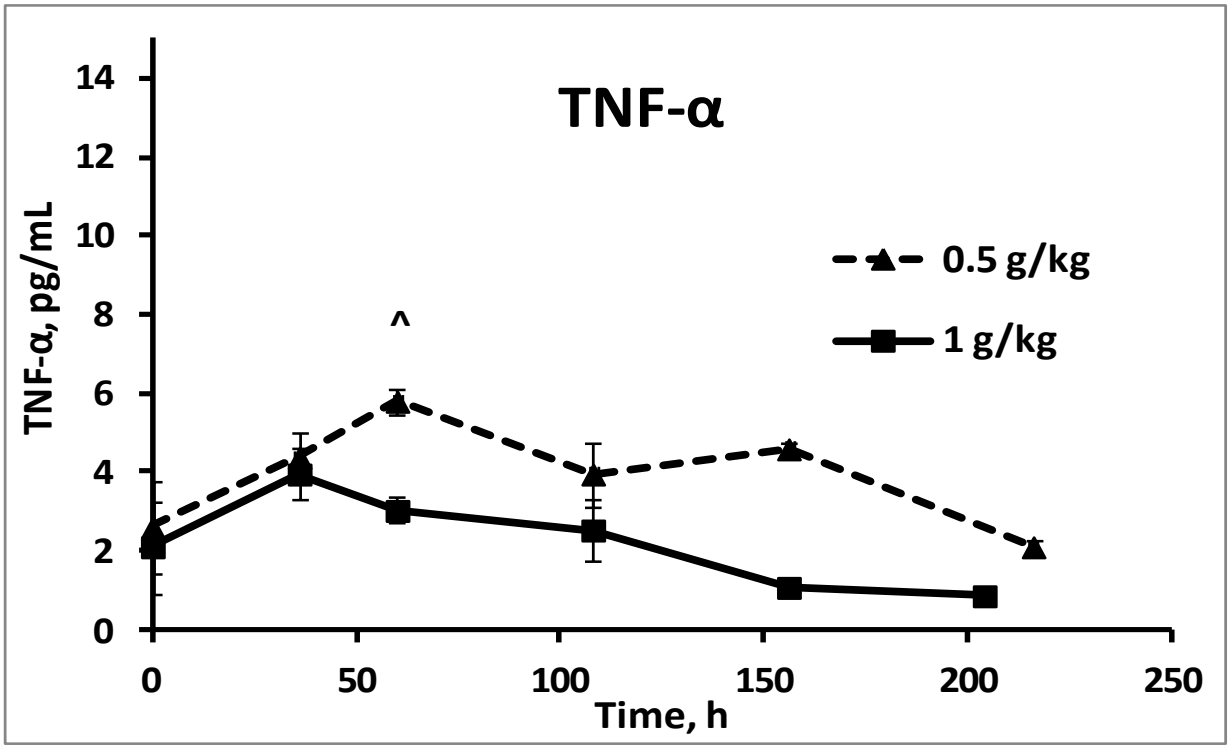

Figure 28: TNF-α concentration vs time curve.

^ P<0.05 significant difference between 1 g/kg and 0.5 g/kg

CHAPTER:4 Discussion

4.1 Cyclosporine A, Tacrolimus and nephrotoxicity

Since CyA was introduced in clinical practice in the early 80s there has been a large decrease in the incidence of acute rejection in renal transplantation, with corresponding increases in solid organ transplant graft surgeries and patient survival. Subsequently, its use was extended as an immunosuppressant for other organ transplant and for the treatment of a variety of autoimmune diseases [42, 65, 66]. The combination of efficacy, minimial risk of acute rejection and late graft failure, combination therapy with other newer drugs and decreasing cost makes CyA still widely used in clinical practice.[67]

In 1989 tacrolimus, a second calcineurin inhibitor, was approved for clinical use as an immunosuppressant.[68] Tacrolimus has about 100-times more potent immunosuppressive effect than CyA and is an effective immunosuppressant for acute rejection in renal, liver and heart transplantation.[67] Tacrolimus use has increased significantly and in fact, it has become the calcineurin inhibitor of choice for the prevention of rejection in solid organ transplantation due to the facility of drug monitoring by trough levels, less cosmetic side effects and a putative better profile in preventing acute rejection, in the majority of centers.[67]

Steroids have been a part of any immunosuppressive regimen after transplant because of their anti-inflammatory, immunosuppressive property and their ability to reverse acute rejection. [69] However, it was recognized early on that the long-term use of steroids was associated with a wide range of adverse effects such as cardiovascular events,

cataracts, osteopenia and skin and Cushingoid changes which may develop within the first year of treatment.[66, 69] With the introduction of other immunosuppressive agents such as CyA, tacrolimus, mycophenolate mofetile (MMF) and molecular targets of rapamycin (mTOR) inhibitors (sirolimus and everolimus), steroid-free immunosuppression in kidney transplantation has been gaining popularity over the past decade.[66, 69]

However, calcineurin inhibitors are associated with many serious side effects including acute and chronic renal dysfunction, hemolytic-uremic syndrome, hypertension, electrolyte disturbances (hyperkalemia, hypomagnesemia and hypocalcemia), renal tubular acidosis and defects in urinary concentrating ability.[67] To minimize its toxicity and maximize efficacy, combination drug therapy with drugs such as CyA and sirolimus are used.[66] Immunosuppressant drugs are selected according to the type of transplant, disease state and risk of acute or chronic rejection over time.

4.2 Hyperlipidemia and calcineurin inhibitors:

Hyperlipidemia is a well recognized risk factor in transplant patients. [59] Transplant patients showed increase in TG and TC levels with slight increases in HDL cholesterol. One study reported 61% of incidence of HL in 205 renal transplant patients.[59] A study comparing lipid levels in chronic haemodialysis patients and stable renal allograft recipients found increases in levels of TG and very low density lipoprotein (VLDL) in both groups. However, TC and LDL cholesterol levels were only elevated in transplant patients.[70] Another study with CyA and tacrolimus with kidney transplant patients

reported effects of CD36 on peripheral blood monocytes in progression of HL. The study found over-expression of CD36 on blood monocytes, which is involved in the uptake of oxidized LDL as well as in the initiation and progression of atherosclerosis.[71]

Indeed, studies have reported effects of HL on CyA pharmacokinetics.[19, 30] One study on P407 induced HL rats reported about a 65% reduction in unbound fraction (*fu*) and decrease in volume of distribution at steady state (Vdss) compared to fasted rats. However, clearance (Cl) of CyA remains the same in HL and NL rats. In the same study rats were also fed with high fat meal and CyA PK parameters were not significantly changed compared to fasted rats. [19] P407-induced HL rats have higher concentrations of CyA in plasma, blood, kidney and liver compared to NL rats. The increased kidney concentrations of CyA in HL rats may lead to nephrotoxicity with long term treatment is not clear.[30, 72] An *In vitro* study with CyA uptake by LLC-PK1 cells found TG and TC, the respective major components of VLDL and LDL, did not alter CyA uptake or toxicity; however, the study reported LDL and apoA-I as the major effectors of CyA toxicity and uptake in LLC-PK1 cells. [73] In present studies we found similar results as there was no significant difference in CyA uptake in HL serum treated group compared to NL serum treated cells.

Tacrolimus has a macrolide ring structure. Amphotericin B, which is a macrolide antibiotic, has also been reported to have increased renal toxicity in HL condition. A previous study found that patients with higher plasma LDL cholesterol levels are more susceptible to amphotericin B-induced kidney toxicity compared to patients with lower plasma LDL-cholesterol levels.[74] Koldin et al reported that high cholesterol specifically

LDL cholesterol, in blood induced the nephrotoxicity of amphotericin B in rabbit.[75] In vitro studies also reported that increased association of amphotericin B with plasma LDL can enhance the ability of the amphotericin B to damage renal cells.[74] Tacrolimus renal toxicity in HL condition hass not been studied so far. However, liver transplant patients reported to have higher blood concentrations of tacrolimus with HL and hypercholesterolemia and hypertriglyceridemia.[76] Liver transplant patient were divided in two groups as hyperlipidemic and non-HL based on plasma lipid levels measured up to six months after transplant. The concentration of tacrolimus in the HL group was significantly higher than non-HL group.[76]

Conversely, in the present study, we observed no significant toxicity of tacrolimus with pre-incubation of kidney cells with HL serum. P407-induced HL is found to alter activities of many enzymes and transporters such as CYP and some studies reported an effect of cholesterol on activities of transporter such as Pgp.[31, 34, 36] If drugs are metabolized by the cells then there may be an effect of HL serum treatment on the activity of CYP enzymes. However some studies reported less activity of CYP enzymes in LLC-PK1 cells[77]. Another possible reason may be that *in vitro* studies are not directly relatable to the *in vivo* situation.

4.3 Tacrolimus studies

4.3.1 HPLC method for measurement of tacrolimus and drug uptake study:

LC/MS/MS and microparticle enzyme immunoassay (MEIA) and enzyme multiplied immunoassay technique (EMIT) are widely used methods to measure tacrolimus concentration.[78, 79] An HPLC UV method was developed to measure tacrolimus

concentrations. The drug uptake study for 0, 2 and 4 h found no significant difference in uptake of tacrolimus between NL, HL or FBS-treated cells. The concentrations at 2 h and 4 h was almost the same. So, the later time points were not measured. As the assay is not sensitive enough to measure concentrations for earlier time points, we did not repeat the study. We tried to develop a more sensitive an LC/MS method. Unfortunately, the drug had very low sensitivity on LC/MS as well and due to poor results we were not able to develop LC/MS method.

4.3.2 Toxicity of tacrolimus with LLC-PK1 and NRK 52E cells:

LLC-PK1 and NRK-52E cell lines are widely used for *in vitro* studies of tacrolimus as well as CyA.[80-83] Tacrolimus and CyA were studied previously with LLC-PK1 cells for endotheline release.[82] Tacrolimus showed a significant toxicity at 50 µg/mL after 24 h of incubation. Moutabarrik et al reported that tacrolimus and CyA exposure caused dose and time-dependent cell injury with LLC-PK1 cells.[84] The toxic concentrations with LLC-PK1 reported for tacrolimus were 1.0, 0.1 and 0.01 µM (~ 804, 80.4, 8.04 µg/mL) measured by endothelin secretion. CyA also caused endothelin-1 secretion at 10, 1, 0.1 and 0.01 µM (~ 1202, 120.2, 12.02, 1.202 µg/mL).[84] Endothelin-1 is known to play an important role in acute renal vasoconstriction and glomerular dysfunction and its increased levels might affect normal kidney functions.[85, 86]

In present study, the results showed significant toxicity with tacrolimus for LLC-PK1 cells. However, the percentage of LDH release with NRK cells was much less compared to LLC-PK1 cells. For that reason further experiments including NL/HL serum treatment and tacrolimus uptake by cells were continued with LLC-PK1 cell line for tacrolimus. 50

µg/mL was found as a significant toxic concentration for LLC-PK1 cells. Interestingly, RF treated cells showed much less LDH release compared to normal media treated cells. The reason for the protective effect of tacrolimus with RF might be induced Pgp levels which increase efflux of tacrolimus. [87]

4.3.3 Tacrolimus toxicity after hyperlipidemic serum treatment:

Our hypothesis was that HL treated cells will show more toxicity with tacrolimus. Li et al reported that higher concentrations of tacrolimus, 3 and 6 months after transplantation is associated with HL[76]. However, the results of our *in vitro* study with LLC-PK1 found no toxic effect of HL treatment with tacrolimus. Surprisingly, the results showed more LDH release in NL and FBS treated group compared to the HL serum treated group which is opposite to our hypothesis and there is no explanation about it at this time. The possible reason may be involvement of other transporter in tacrolimus uptake present in kidney cells. However, the drug uptake after preincubation with different serum was not significantly different. So, the involvement of other transporters might not be the reason for no cell death in HL treated cells. Moreover, no information was found in the literature about another transporter of tacrolimus. Calcineurin inhibitors inhibit various genes including interleukins, interleukin 2 receptor, nitric oxide synthase, transforming growth factor β (TGF-β), endothelin, collagen I and IV and bcl-2, responsible for protein Bcl-2. Inhibition of these genes which are likely implicated in cellular protection against apoptosis and possibly calcineurin inhibition at the same time that it blocks immune cell-mediated reaction against the transplanted tissue may trigger renal injury.[88] HL might be affecting one of the gene and possibly protect the cells against toxicity of tacrolimus that was found in FBS and NL serum treated cells. However, the reason for the

protective effect of HL preincubated cells is not clear yet and further studies are required to understand the effect of HL.

As noted in our results, there was some small amount of turbidity in the HL treated cells after washing out the HL serum (Fig. 8 and 9). This might perhaps have caused HL treatments to display less toxicity with tacrolimus. On the other hand, this should have been expected to occur for CyA as well (Fig 12 and 12a), but for that drug toxicity was apparently the same in NL as in HL serum. These unusual findings for tacrolimus cannot be explained based on the current data.

4.4 Cyclosporine A studies

4.4.1 Cyclosporine A viability study with NRK 52E and LLC-PK1 cell lines:

CyA toxicity was measured with LLC-PK1 and NRK 52E cells.[89, 90] Our findings showed more toxicity with CyA in NRK cells compared to LLC-PK1 cells. However previous studies with LLC-PK1 and NRK 52E cells with CyA did not show difference in CyA toxicity between these cell lines after 24 h exposure.[90] In present study, cells were incubated with CyA for longer time up to 48 h. Due to high magnitude of CyA toxicity with NRK 52E cells compared to LLC-PK1 cells, we used NRK 52E cells for the rest of the experiments.

4.4.2 CyA LC/MS method and drug uptake studies:

A previously published LC/MS method was used to measure intracellular CyA concentrations. Ho0wever, we made some changes in the extraction procedure and validated the new method; the reported method by a former student (Srividya Kanduru) used water in the last step, which did not work in our hands; use of methanol corrected the problem. It is possible that an error was made in recording the solvent used The assay sensitivity (50 ng/mL) remains the same as the previous method after modification. The drug uptake study did not show significant differences between the NL and HL groups except the non RF group with the 50 µg/mL concentration at 60 min time point showed a significant difference between NL and HL groups. At some time points there was a trend to higher concentrations in the HL group followed by NL and FBS.

4.4.3 MDR 1 gene expression in NRK 52E cells after treatment with RF:

For cytotoxicity measurement with NL/HL/FBS serum treatment NRK 52E cells were also treated with RF containing media. Treatment of LLC-PK1 cells with RF containing media reported in an increase in Pgp levels.[61] We measured expression of the MDR 1 gene with RF treatment for 7 days with NRK-52E cells. The RF treatment showed a significant increase in MDR 1 gene expression in NRK-52E cells. The purpose of inducing Pgp level by rifampin is to get better idea about effect of HL with altered Pgp level.

4.5 P407 induced HL rat model:

A previous study in rabbit measured the P407 effect for 14 days and the levels of TG and TC were at baseline after 14 days.[17] Blonder et al reported lipid levels in rabbit after different doses of P407 (5.5 mg/kg, 27.5 mg/kg and 137.5 mg/kg) and showed significant increases in levels of serum lipoproteins after 2 days only with the highest dose. The level of TG and TC were about 550 mg/dL and 140 mg/dL on day 2. The purpose of this study was to determine the lowest dose of P407 that can be safely used in control release drug delivery applications without HL effect.[17] Similarly, in the present study we found that rats achieved maximum concentration of TG and TC after 36 h after P407 administration.

Previous studies in rats reported the effect of P407 on lipids for 96 hrs with doses ranging from 1mg to 300 mg.[26] Plasma TG concentrations with higher dose increased up to 6000 mg/dL and TC was 550 mg/dL 24 h after i.p. injection of the poloxamer. The poloxamer effect of i.m. and i.p. both route was compared in this study and the lipid levels were significantly higher 48 h after dosing with both route. However, at a later time point (96 h), the effect was appeared to be dependent on route of administration. P407 by i.p. route was showed a higher magnitude in increased levels of TG and TC due to larger fraction of poloxamer dose available to liver, which results in increased activity of HMG-CoA.

Joo et al used the P407 rat model to evaluate the effect of lipid lowering and anti-inflammatory agents. In their study HL was induced by repeated doses of P407 (500 mg/kg) at 3 day intervals and blood samples were collected on day 21. The rats were fasted for 6 h after P407 injection while in our study rats had free access to food after

dose. The levels of TC, TG and HDL cholesterol were 470.2 ± 52.0 mg/dL, 1034.5 ± 141.2 mg/dL and 38.6 ± 7.3 mg/dL respectively. Moreover, the study reported no significant effect of repeated doses of poloxamer on levels of TNF-α and IL-1.[64] In present study, there was no significant effect of a single dose of P407 on TNF-α. Interestingly, the increase in HDL cholesterol was not significantly higher than our results. In the present study we found significant increase in HDL cholesterol concentration (38.08 to 197.09 mg/dL) even with 0.5 g/kg dose of P407, which is similar to the dose given to rats by Joo et al. [64] The difference in cholesterol level might be result of either different type of rats or food consumption after P407 dosing, which was different in both experiments or some other unknown differences in experiments. Johnston et al reported the consumption of food resulted in significantly higher concentrations in TG and TC levels. One group of their rats were fasted for 48 h after dose and other group had free excess to rat chow with i.p. dose of P407.[26]

Release of adipokines and cytokines has been found to be affected by various metabolic syndromes including obesity, hyperglycemia, hypertriglyceridemia and hypertension. A previous study reported an important role of adipokines in formation and progression of atherosclerosis.[91] Another study was done with HL rats with and without diabetes and HL was induced by an atherogenic diet[92]; the study reported decreases in levels of leptin and adiponectin in the high fat diet group and increase in TNF-α. The change in adiponectin does matches with our results. Surprisingly, with the P407 model we found increases in leptin levels and no effect on TNF-α. The difference in results may be explained by different ways of inducing HL as rats were fed with a high fat diet for 12 weeks that might result in inflammation and increase TNF-α levels.

Interestingly, the HL group showed decrease in leptin levels too, which is opposite of our finding.

Other studies in humans reported a relationship between adipokines and dislypidemia.[52, 93] Gannag-Yared et al investigated the association between both adiponectin and leptin and the metabolic syndrome, lipid parameters, insulin sensitivity and sex steroids in healthy non-diabetic Lebanese men and the results found a negative correlation between adiponectin levels and waist size and TG. However, HDL cholesterol was positively correlated with adiponectin.[52] The correlation between leptin and the lipid profile was poor. Matsubara et al investigated the effect of dyslipidemia on adiponectin levels in nondiabetic women; the results indicated high TG and low HDL cholesterol levels were associated with low plasma adiponectin concentrations.

4.6 Conclusion:

The HPLC method developed for measurement of tacrolimus was validated for a 5μg/mL concentration. The method was specific and validated for measurement of intracellular concentrations in drug uptake studies during early time points.

Tacrolimus induced cell death in LLC-PK1 cells at 50 μg/mL; however, no effect of HL was observed on tacrolimus induced cytotoxicity in LLC-PK1 cells. Interestingly, the HL group showed a protective effect and less toxicity compared to NL and FBS-treated cells. No significant differences were observed in Tacrolimus uptake between HL and NL group0 at 2 and 4 h time points.

CyA was toxic when measured with three concentrations (10, 30 and 50 µg/mL) in NRK 52E cells and it was found to be significantly toxic to cells during incubation with the 30 and 50 µg/mL concentrations. We did not observe significant toxicity of CyA in HL serum pre-incubated NRK 52E cells compared to NL treated cells. The drug uptake study and LDH assay further supported the cytotoxicity observation. A commercially available formulation of CyA, Sandimmune is more toxic to the cells compared to powdered CyA in kidney cell lines. The reason might be toxicity of Cremophore EL and alcohol (2:1), which is vehicle used in Sandimmune formulation.[94] The toxicity of Cremophore EL was also reported on human primary proximal tubular epithelial cells by Bakker et al at higher than 10 µg/mL concentrations. [94] The studies with both calcineurin inhibitors found no significant role of P-glycoprotein efflux mechanism on renal toxicity with rat and pig kidney cell lines.

P407 induces HL and increases TG, TC, HDL cholesterol and LDL cholesterol levels in rats in an approximate dose proportional manner between 0.5 and 1 g/kg. P407 alters level of the serum adipokines (adiponectin and leptin); however, it does not appear to have any effect on proinflammatory cytokines (TNF-α and IL-6).

CHAPTER:5 Future directions

Previous studies in animals found effects of HL on PK of various lipophilic drugs.[2] Li et al reported increased blood concentrations of tacrolimus with HL in transplant patients.[76] However, in the present *In vitro* studies with the LLC-PK1 cell line tacrolimus did not show significant cell damage after 24 h of HL serum treatment. *In vivo* studies might explain the effect of HL on tacrolimus blood concentrations, kidney toxicity as well as other pharmacokinetic parameters. For measurement of tacrolimus a more sensitive LC/MS/MS method is needed. HL can be induced in animals using the P407 model and the effects of tacrolimus on blood concentrations and kidney toxicity might be explained. The sensitive LC/MS/MS method might also be useful in drug uptake studies as well in the LLC-PK1 cell line.

Effects of HL on various other genes involved in renal toxicity of tacrolimus and CyA might be studied using real time PCR and western blot analysis. These studies might be helpful in understanding the mechanism of the protective effect of HL serum treatment with tacrolimus.

CyA was not found to have any significant toxicity with HL preincubated NRK cells. However, previous studies in HL animals found damage in kidney cells with CyA and also reported altered PK parameters with increased lipoprotein levels.[19, 30] Further studies might be done with fluorescent microscopy to understand CyA and tacrolimus-induced nephrotoxicity and cellular mechanisms.

HL or induced lipid levels are known to alter various metabolizing enzymes as well as activity of transporters such as Pgp.[31, 34, 35] In future, the P407 induced HL model can be studied to understand the effect of HL on various genes in kidney and other important organs such as liver, heart and intestine using real time PCR and western blot analysis. Effect of HL on transport activity may explain altered PK and PD of various lipophilic drugs in HL conditions. Activity of other kidney transporters might be studied in HL animals to explain the effect of tacrolimus and CyA on renal toxicity in HL conditions.

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