A New Model of Drug-Disease Interaction in Rabbits

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Abstract: Background: Drug-infection interaction should be considered in drug prescribing, particularly if potentiated by co-occuring drug-drug interactions. The effects of Candida and e-coli infections separately, and fluconazole administration were tested on cyclosporine blood level in rabbits. Methods: Three study designs were carried out, crossover single-dose-fluconazole-cyclosporine study testing fluconazole-cyclosporine interaction, multi-dose candida-fluconazole-cyclosporine and multi-dose escherichia coli-cyclosporine study designs involving each rabbit acting as its control; cyclosporine was given daily in both studies. Candida and e-coli infections were inoculated on day 5. In the Multi-Candida-Fluc-CyA, fluconazole-cyclosporine interaction was also considered, and fluconazole was administered daily from day 9-18. In all three studies, Cyclosporine trough levels and serum creatinine were measured by CMIA and enzymatic assay respectively, pre, during, post-infection and after fluconazole administration in the Candida study. Results: Both infections resulted in significant rise in cyclosporine trough level ($p = 0.018$) and ($p = 0.005$) in fungal and bacterial studies, respectively. The median rise in CyA level reached 52% (range 9-426%) in the Candida infection and reached $60 \pm 47.5\%$ (mean) with the e-coli infection. Fluconazole also increased mean cyclosporine trough levels in the single-dose study by 70.3 ± 45.3 and the concomitant rise in the multi-dose study reached 76% (median, range 22-665%). Conclusions: Cyclosporine trough levels increased during Candida and e-coli infections and also during fluconazole administration in the single and multi-dose studies. Fluconazole exerted an additive effect to the Candida inhibitory effect. Type of infection and inoculum size affected CyA levels differently. Monitoring cyclosporine level during episodes of infection as well as in therapy regimen involving interacting drugs is advisable.

Key words: Candida, cyclosporine, CYP450, disease-interaction, e-coli, rabbits.

1. Introduction

Disease-alteration of drug metabolism is of clinical importance [1]. Many studies have looked at the depression of cytochrome P450 (CYP450)-dependent hepatic drug metabolism during inflammatory reactions & infectious diseases [2]. The present animal study addresses the impact of infection on cyclosporine exposure and in presence of drug interactions.

Patients with *P.falciparum* malaria showed slower clearance rates of quinine and caffeine suggesting depressed drug metabolism during malaria [3]. Viral and bacterial infections as well as influenza and BCG vaccinations decreased the clearance of theophylline

and antipyrine, secondary to decreased activity of multiple isoforms of the CYP450 [4].

Alteration in drug metabolism in infection was also tested in animal models. Coxsackievirus B3 have been shown to increase the toxicity of dioxin, in dioxin-exposed infected mice [5]. Alteration in enzyme CYP450 activity has also been measured after *in vivo* exposure of animals to immunostimulatory agents, including viruses, lipopolysaccharides [2].

The list of immunomodulators reported to alter CYP450-mediated metabolism includes several bacteria such as *Corynebacterium parvum [6], Listeria monocytogenes [6], Mycobacterium butyricum, Chlamydia trachomatis [6], Citrobacter rodentium [7]* and various types of *Schistosoma* [6]. Viruses like *Coxsackievirus B3 (CBV3)* [5], parasites

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like *Toxoplasma gondii, Fasciola hepatica, Trypanosoma brucei [6], and* malaria infection by *Plasmodium berghei [3]* were also implicated.

These organisms were either inoculated in the tested models such as mice [3, 5-7]; or lipopolysaccharides of *Proteus [8], Escherichia [8], Salmonella, Bacteranoides* or *Coxiella* strains [8], were injected in rodents [8]. Alternatively, isolated hepatocytes were cultured *in vitro* with lipopolysaccharides where transcription factors and protein of the metabolizing enzymes were significantly decreased [9].

From bench to bedside, patients on a stable drug regimen who experience an infection or inflammatory episode may experience decreased clearance, resulting in possible increased adverse events. The clinical consequences are likely to be more significant for drugs with a narrow therapeutic index [10]. A notable case was when an influenza epidemic resulted in decreased clearance of theophylline in asthmatic children [11].

Studies looking at the effect of fungal (Candida) infection on drug levels in animals or humans are lacking. In Candida infection, an antimicrobial agent is often prescribed (such as Fluconazole), which itself has an inhibitory effect on drug metabolism. In an earlier study, e-coli lipopolysaccharides were shown to inhibit liver CYP450in mice [8], but no studies tested the effect of inoculating e-coli on the metabolism of hepatically metabolized drugs.

The narrow therapeutic index drug, cyclosporine (CyA), is still used in clinical practice in Egypt for the prevention of allograft rejection in solid organ transplant recipients [12]. CyA is extensively metabolized *via* CYP3A4 in the liver and gut to multiple metabolites [13]. The clearance of CyA indicates that it is a drug with a low extraction ratio [12].

We have recently reported data describing the effect of active infection on CyA blood level in renal transplant patients [14]. To better understand the effect of infection on drug handling in immunosuppressed patients, we have since then evaluated effects of a fungal (*Candida*) and a bacterial (e-coli) infection on CyA blood level in rabbits. The study was also designed to investigate whether the CYP inhibitory effect of Fluconazole (Fluc) has an additive effect, on CyA level in the Candida experiment.

It is anticipated that this model of Candidemia and Bacteremia in rabbits administered CyA daily could reflect drug-disease interactions encountered in the clinical study.

2. Materials and Method

2.1 Materials

Drugs: CyA amp 50mg/mL (Sandimmun®, Novartis), Fluc vial 2mg/mL (Diflucan®, Pfizer). Microbiological media: for the Candida*: sabouraud dextrose agar (Oxoid®) and sabouraud dextrose broth (Difco^TM),* for the e-coli: luria bertani (LB) broth (Oxoid®), macconkey agar (Oxoid®). Microorganism: *candida albicans* (ATCC10231). *The* candida was obtained from the microbiological department in the Faculty of Pharmacy, Pharos University, while in the *Escherichia coli* study, a well characterized clinical isolate from a proven case of e-coli septicemia was used.

2.2 Methods

2.2.1 Animal Study Design

Three studies were carried out. Different rabbits were used in each study. A total of twenty-three rabbits were used.

Animals from the animal house facilities in Faculty of Pharmacy, Pharos University in Alexandria were used. The animals were individually housed in single metal cages and maintained according to National Institutes of Health guidelines for animal care and in fulfillment of the criteria of the American Association for Accreditation of Laboratory Animal Care [15]. The procedures followed were approved by the Ethics

committee for animal studies of the Faculty of Medicine, Alexandria University.

2.2.2 Animal Management

Animals were allowed one week for acclimatization prior to inclusion in the study [16]. They had access to water and food ad libitum. The temperature of the room ranged from 25°C to 15°C in spring and 19°C to 8°C in winter, the light-dark cycle was almost 12 hours light/dark cycle.

The animals were immobilized in a restraining box when drugs were administered and blood samples were taken [16].

2.2.3 CyA Doses in Rabbits

CyA doses in rabbits were the calculated human equivalent dose and the administered doses were similar to those reported by others in similar studies [16]. For scaling doses from human to rabbit, Eq. (1) was used:

$$\text{Animal dose (mg/kg)} = \text{HED(mg/kg)} \times \frac{\text{Human Km}}{\text{Animal Km}}$$

Eq. (1)

Where HED (human equivalent dose) is the Human Equivalent Dose; values used in the equation were 5-6mg/kg/day. Km was calculated by dividing weight (kg) by body surface area (m^2) [17].

2.2.4 Single-Dose Fluconazole-Cyclosporine Study

Aim

The aim was to assess the inhibitory effect of fluconazole on cyclosporine metabolism in rabbits and to assess whether the inhibition occurs with a single fluconazole dose. The study also aimed to test the use of cyclosporine trough level as a possible marker of CYP metabolism inhibition, instead of AUC.

Animals

Six New Zealand white male rabbits, weighing from 2 to 3kg, were used.

Study Design

The design was a crossover single dose study. In the control arm of the study, each rabbit received a single IV dose of cyclosporine (diluted in saline and administered slowly) through the marginal ear vein.

The HED used in calculating the doses was 5mg/kg; administered CyA doses were 11.6-14.0 mg/kg. A one-week wash-out period was allowed.

In the intervention arm of the study, fluconazole was administered as a single IV dose of 10mg/kg [18], infused slowly and followed by the cyclosporine dose.

Only one rabbit (rabbit # 6) died before completing both study arms, its data were excluded from the experiment.

Parameters Measured

Blood samples were collected from a central ear artery at 1, 2, 3, 4, 6, 8, and 12 h, following cyclosporine administration. Cyclosporine levels were determined. Area under the blood level curve (AUC_{0-12h}) was calculated. Total body clearance (TBC)(L/h/kg) was calculated by dividing dose (mg/kg) by AUC (mg/L.h) [19].

Measurement of Cyclosporine Level

Whole blood was collected in EDTA tubes and was analyzed using a CMIA (chemiluminescent microparticle immunoassay) on the ARCHITECT i System (Abbott, USA) [20].

Controls and standard CyA prepared in blank blood were measured with samples in addition to necessary blanks. Control readings were used to calculate inter-day precision. Precision data (%CV) were 12.2% for low, 9.6% for medium and 8.3% for high controls.

2.2.5Multi-DoseCandida-Fluconazole-Cyclosporine Study

Aim

The aim was to assess the inhibitory effect of Candida infection on CYP450 as reflected on CyA steady-state blood level, and to investigate whether the inhibitory effect of the antifungal agent, administered daily to treat Candida infection, was additive.

Animals

Seven New Zealand White male rabbits weighing between 2.2-3.2kg at the time of inoculation were used in this study.

Inoculation and Induction of Candida Infection

Lyophilised Standard Strain Candida albicans

ATCC 10231 was transferred by a sterile swab into 3 mL Sabouraud glucose broth and incubated at 37°C for 48 h. Cells from the suspension of Candida albicans were streaked onto SGA (Sabouraud glucose agar) plates, incubated at 37 °C for 24 h, and maintained during the course of these experiments at 4 °C.

For preparation of the inoculum, three well-isolated colonies were sampled from freshly grown culture plates and suspended in 50 mL of Sabouraud glucose broth in a 250 mL Erlenmeyer flask. The suspension was incubated in a shaker incubator at 80 oscillations per min at 37 °C for 16-18 h [21, 22]. 1 mL of this overnight culture was subcultured in 49 mL Sabouraud glucose broth (i.e. 2% of Candida suspension) for 2.5 h in a shaker incubator at 80 oscillations per min at 37 °C to generate log-phase growth [23].

The Candida suspension was then centrifuged at 3,000 g for 10 min and washed three times with sterile normal saline. Counts were adjusted to 1×10^7 to 2×10^7 CFU/mL with a spectrophotometer (OD_{600} nm 0.4) [21], which coincides with mid-log phase (OD_{600} nm 0.4-0.6) [24].

One mL of the adjusted Candida suspension was diluted with 4 mL 0.9% normal saline and was slowly administered to the rabbits via the intravenous catheter on day 5 of the experiment, followed by 5 mL 0.9% normal saline to push the Candida suspension into the circulation and to avoid cannula localized infection. The inoculum size was confirmed by plating cultures of a 10 fold serial dilutions onto SGA plates [21, 25].

The pattern of infection of disseminated Candidiasis permitted survival of nearly all rabbits (7 out of 8) throughout the experiment [21, 25]. The inoculum was prepared fresh prior to each use [26].

Study Design

Each rabbit acted as its own control. Cyclosporine was given daily as an IV dose (diluted in saline and administered slowly) through the marginal ear vein for 23-24 days (to avoid CyA toxicity in rabbits reported by 60 days) [27]. Cyclosporine HED (human equivalent dose) used in calculating animal doses was 6mg/kg/day; administered doses were 13.5-16.6 mg/Kg. Cyclosporine trough levels were measured at 24 h prior to the daily dose.

Candida infection was inoculated on day 5 after reaching cyclosporine steady state concentration, half-life of terminal elimination phase is 4.25-9.17 h in rabbits [28]. Candida count in inoculum was adjusted to $1\text{-}2 \times 10^7$ CFU/mL with a spectrophotometer ($OD_{600\ nm}$ 0.4) [21].

Once inhibition of CYP 450 due to infection was evident, by comparing cyclosporine trough level to baseline value, IV Fluconazole was slowly infused daily (10mg/kg), before withdrawing the morning blood sample. Fluconazole was started on day 9 and was given for 10 subsequent days. Additive inhibitory effect of Fluconazole on CYP450 was assessed by measuring cyclosporine trough level. IV doses of CyA were continued for 4-5 days beyond day 19.

Cyclosporine, in the present study, acted both as a probe drug [29] for monitoring inhibition of CYP450 and as immunosuppressant to avoid the risk of infection clearance from blood [30].

2.2.6 Parameters Measured

Measurement of Cyclosporine Level

Cyclosporine trough level in whole blood was measured as discussed above.

Assessment of Infection Induction

Mannan level [31] was measured in serum on day 10 using ELISA. Mannan concentrations ≥ 125 pg/Ml were considered positive, concentrations between 62.5 to 125 pg/mL were intermediate and concentrations 62.5 pg/mL were negative for Candida. Rabbits were also monitored for signs of infection: behavioral and food intake changes.

Assessment of Immune Suppression

Immune suppression, resulting from cyclosporine administration, was monitored by measuring lymphocyte proliferation [30]. WBCs and lymphocytic counts were recorded at baseline, and at

day 5 prior to induction of infection [30]. Total and differential WBCs were counted using Automated Hematology Analyzer (Sysmex®xp, Sysmex Corporation, Japan).

2.2.7 Multi-dose e coli-Cyclosporine study

Aim

The aim was to assess the inhibitory effect of Escherichia coli infection on CYP450, through monitoring steady-state CyA trough levels, and to compare it to the inhibitory effect of the fungal infection and the fluconazole in the respective studies.

Animals

Six New Zealand White male rabbits weighing between 2.20-3.36kg at the time of inoculation were used in this study.

Inoculation and Induction of E-coli Infection

A single colony of e-coli was inoculated into 10 mL of LB (luria-bertani) broth and grown overnight at 37 °C; 5mL of the overnight broth culture was inoculated into 200 mL of prewarmed LB broth and incubated at 37 °C for 2 h to reach logarithmic growth phase (OD660, 0.2). The broth culture was centrifuged (10,400 g for 20min) [32] and E. coli were washed twice and resuspended in sterile normal saline for intravenous administration [21]. One mL of the adjusted e-coli suspension was diluted with 4 mL 0.9% normal saline and was slowly administered to the rabbits via the intravenous catheter on day 5 of the experiment, followed by 5 mL 0.9% normal saline to push the e-coli suspension into the circulation and to avoid cannula localized infection [21]. Serial 10 fold dilutions of E. coli were plated and incubated overnight at 37 °C to calculate the number of viable bacteria [32].

Two counts were used; the first 7×10^8 CFU/mL that permitted the survival of 2 out of 6 rabbits, so experiment was repeated with a count of 4×10^6 CFU/mL in 4 rabbits which permitted survival of all rabbits throughout the experiment. The inoculum was prepared fresh prior to each use [26].

Study Design

Each rabbit acted as its own control. IV

Cyclosporine was given daily (diluted in saline and administered slowly) through the marginal ear vein for 9-12 days, cyclosporine HED used was 6 mg/kg/day and cyclosporine trough levels were measured at 24 h prior to the daily dose. CyA administered doses ranged from 14.0-16.2 mg/kg. *Escherichia coli* infection was inoculated on day 5 of the experiment. For e-coli, two inoculum counts were used; the first $(7 \times 10^8$ CFU/mL) permitted the survival of only 2 out of 6 rabbits. The experiment was repeated with a lower count of 4×10^6 CFU/mL in four more rabbits (all four survived). Counts were adjusted by means of a spectrophotometer (OD $_{660nm}$, 0.2).

Inhibition of CYP450 was monitored by measuring cyclosporine trough level during the different stages of the experiment.

Parameters Measured

Measurement of Cyclosporine Level

Cyclosporine trough level in whole blood was measured as discussed above.

Assessment of Infection Induction

Automated blood culture using BacT/ALERT® 3D Select blood culture system (bioMérieux Inc.; l'étoile, FRANCE) was used. The bottles showing a positive signal in the 3D were subjected to growth on blood agar plates and/or MacConkey agar. The colonies were identified with the Vitek system (bioMérieux Inc.) [33].

Assessment of Serum Creatinine

Serum creatinine level was measured by enzymatic assay [34] during both Candida and e coli studies to assess possible cyclosporine-induced nephrotoxic effect.

2.2.8 Statistical Analysis

Data generated were analyzed using IBM SPSS software package version 20.0. Normal distribution was tested using Kolmogorov-Smirnov test, Shapiro-Wilk test and D'Agstino test [35]. Parametric test (paired *t*-test) was applied to normally distributed data and non-parametric test (Wilcoxon signed ranks test) was applied otherwise. Significance test results

(t or Z values) were quoted as two-tailed probability. Statistical significance was judged at the 5% level [35].

3. Results

3.1 Single-Dose Fluc-CyA Study

Mean CyA trough level showed a rise from 184.38 ± 42.13 ng/mL in the control arm to 308.22 ± 77.62 ng/mL in the Fluc arm, (t value 3.77, $p = 0.02$, n = 5) (Table 1) and (Fig. 1A). There was no significant difference in the weights of the rabbits between the control arm (2.55 ± 0.38 kg) and the Fluc arm (2.58 ± 0.37 kg), (t value 0.388, $p = 0.718$, n = 5 in each arm).

Mean AUC increased from 16.67 ± 4.14 mg/L.h in the control arm to 25.61 ± 5.68 mg/L.h in the Fluc arm, (t value 4.481, $p = 0.011$, n = 5) (Table 1) and (Fig. 1B). Calculated clearance values revealed a significant decrease from 0.782 ± 0.155 L/h/kg in the control arm to 0.498 ± 0.086 L/h in the Fluc arm, (t value 4.521, $p = 0.011$, n = 5) (Table 1) and (Fig. 1C).

CyA trough levels in both control and Fluc arms were tested as a possible measure of CyA systemic exposure, and consequently of CYP metabolism inhibition, instead of AUC. The regression equation correlating trough levels with AUC_{0-12h} (r^2 0.837, $p < 0.001$, $n = 5$) indicated good correlation (Fig. 2).

3.2 Multi-dose Candida-Fluc-CyA Study

The Candida inoculum size chosen and the doses of CyA and Fluc, were not life threatening to the rabbits during the experiment (21-23days); only one rabbit out of seven died on day 14 (rabbit # 2) and another rabbit (# 3) lost 16% of its weight (Table 2).

3.2.1 CyA Levels

CyA trough level increased from median baseline level 58 ng/mL (range 39-99 ng/mL) on day 3-5 before the infection to 88ng/mL (range 53-402 ng/mL) on day 7, two days after induction of Candida infection(Z value 2.366, $p = 0.018$) (Table 2, $n = 7$ and Fig. 3).

In Table 2, a drop in median CyA level was noticed in six rabbits, (Table 2, drop in level column), before Fluc administration, from 88 ng/mL (53-402 ng/mL) to a median level 23 ng/mL (11-41 ng/mL) (Z value 2.201, $p = 0.028$, $n = 6$).

Table 1 CyA levels, calculated AUC_{12}, total body clearance of the control and intervention arms of the Single-dose Fluc-CyA study design in rabbits.

Rabbit #		Cyclosporine level (ng/mL)							AUC_{0-12} (mg/L.h)	TBC (L/h/kg)	% change in trough[a]
		1 h	2 h	3 h	4 h	6 h	8 h	12 h (Trough)			
Rabbit 1	Control	4,630	2,365	3,030		566	332	246	21.45	0.65	28
	Interv.	6,035	3,360	3,805	2,025	1,755	488	315	26.87	0.50	
Rabbit 2	Control	3,415	1,820	1,070	1,085	650	339	208	13.45	0.89	63
	Interv.	6,580	3,530	2,030	1,735	840	650	338	27.30	0.45	
Rabbit 3	Control	2,555	1,365	1,770	735	446	289	144	12.05	1.00	42
	Interv.	4,680	2,675	2,865	1,815	562	327	203	20.05	0.59	
Rabbit 4	Control	4,960	2,115	1,160	1,100	459	304	156	16.06	0.71	75
	Interv.	4,335	2,230	1,600	815	1,255	460	272	20.19	0.57	
Rabbit 5	Control	3,420	2,275	2,525	910	533	377	169	20.34	0.66	145
	Interv.	6,525	3,330	3,320	2,705	1,545	1,210	413	33.64	0.38	
Statistical Analysis	Mean ± SD				Control			184.38 ± 42.13	16.67 ± 4.14	0.782 ± 0.155	
					Interv.			308.22 ± 77.62	25.61 ± 5.68	0.498 ± 0.086	
	t^b							3.766	4.481	4.521	
	p (two-tailed)[*]							0.020	0.011	0.011	

[a] Calculated from the control & intervention trough levels; [b] Paired t-test; [*] Statistically significant at $p \leq 0.05$; Shaded area: value not determined; Control arm (single dose CyA alone); Intervention arm (single dose of each of CyA and Fluconazole).
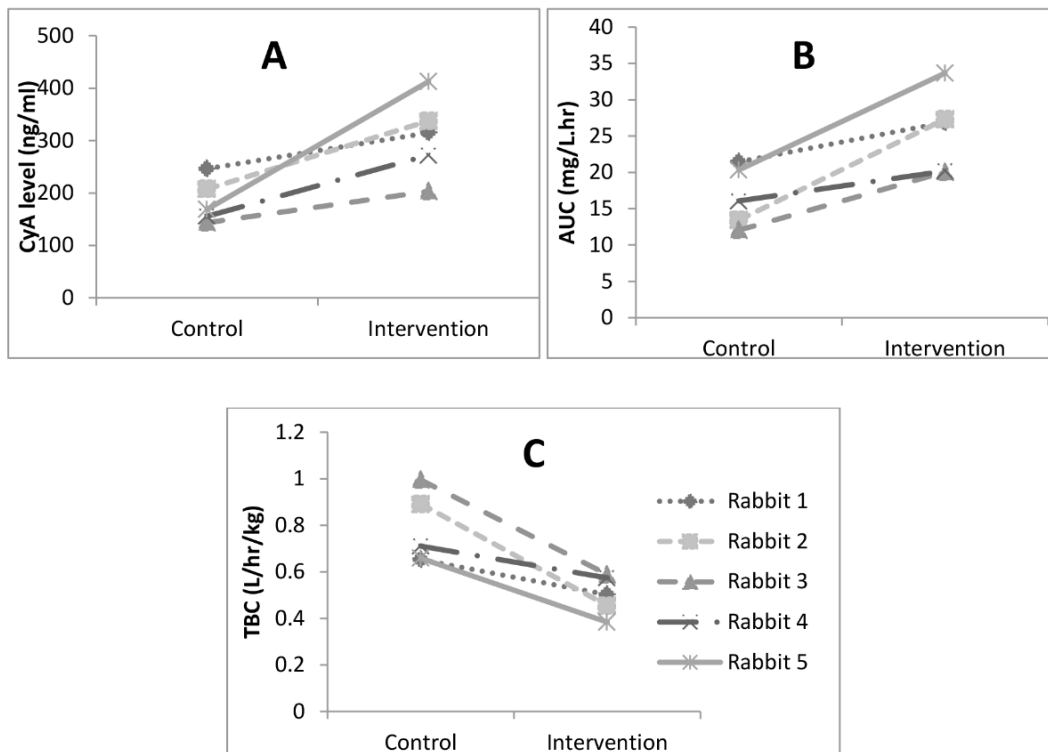
Fig. 1 CyA trough (A), AUC (B) and TBC (C) in the control arm (CyA only) and the intervention arm (CyA-Fluc) in Single-dose Fluc-CyA study in rabbits.
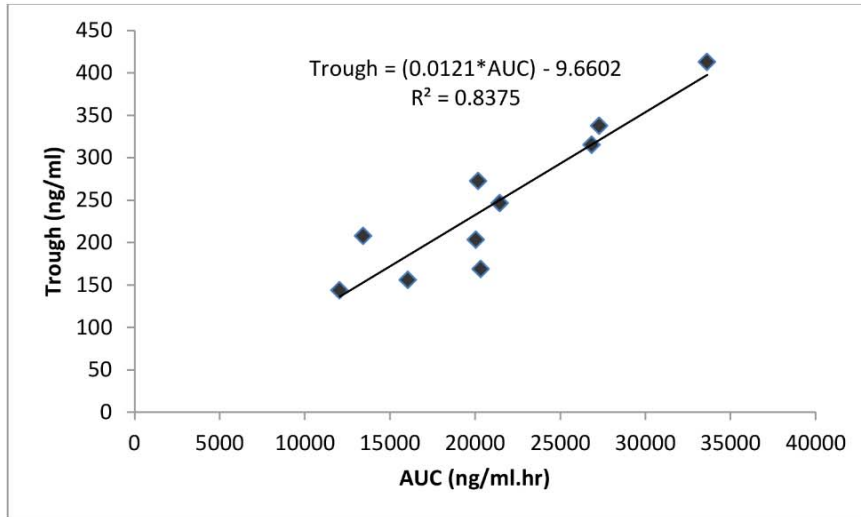

Fig. 2 Relation between CyA trough levels and corresponding AUCs in control and intervention arms in the Single-dose Fluc-CyA study in Rabbits.

Table 2   CyA trough levels (ng/mL, 24 h data) following multi doses of CyA & Fluc in the Multi-dose Candida-Fluc-CyA study in rabbits.

Rabbit #	Study Stages						% change	
	Pre-infection	During infection	Drop level[a]	During fluconazole administration		Post-infection	% change due to Candida[b]	% change due to Candida & Fluc[c]
				Initial rise	Peak CyA level			
Rabbit 1	66	84	28	59	80	31	27	22
Rabbit 2	39	80	11	57	Died	--------	107	---------
Rabbit 3	77	402	41	218	585	938	426	665
Rabbit 4	99	108	26	165	199	187	9	102
Rabbit 5	58	88	19	75	87	72	52	50
Rabbit 6	43	53	16	42	57	16	24	32
Rabbit 7	51	115	-----	150	351	289	126	590
Median (range)	58 (39-99)	88 (53-402)	23 (11-41)	75 (42-218)	143 (57-585)	72 (16-289)[d]	52 (9-426) (n = 7)	76 (22-665) (n = 6)
Statistical Analysis[*]	Z= 2.366 $p = 0.018$		Z= 2.201 $p = 0.028$		Z = 2.023 $p = 0.043$			
		Z = 2.201 $p = 0.028$		Z = 2.201 $p = 0.028$				
	(Pre- & Post-infection) Z = 0.674, $p = 0.5$							

[a] Unexpexpected drop in CyA trough levels in six rabbits; [b] Calculated from difference between pre-infection and during infection data; [c] Calculated from difference between pre-infection data & peak CyA level after Fluc administration data; [d] Excluding rabbit # 3 value; [*] Wilcoxon signed ranks test (Z values) were quoted as two-tailed probability, statistically significant at p ≤ 0.05;
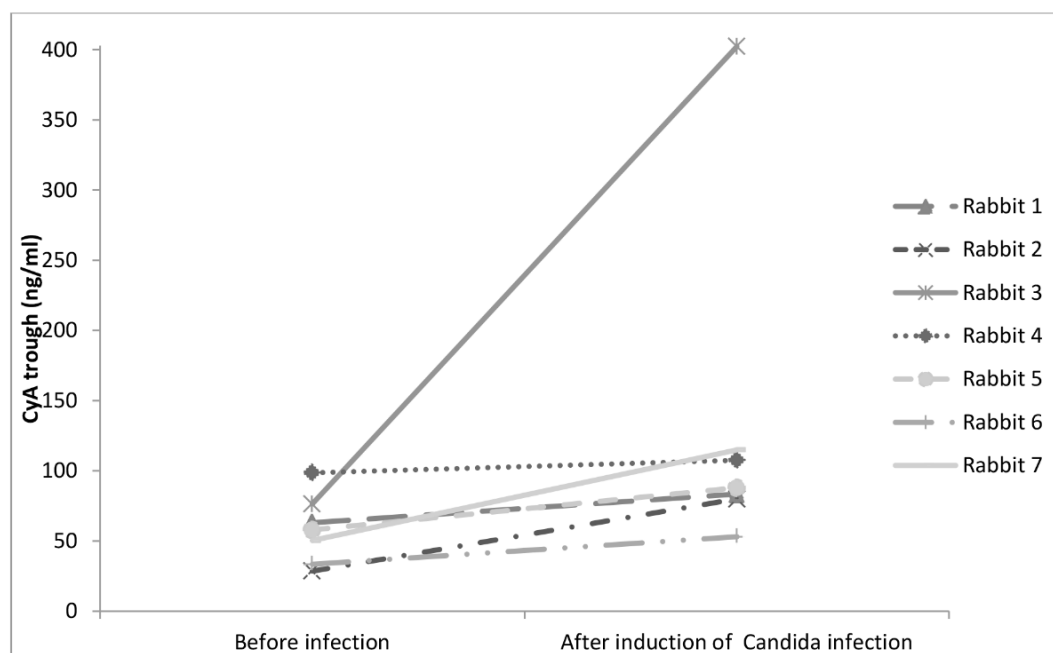
Fig. 3   CyA trough level before the infection and after induction of infection in the Multi-dose Candida-Fluc-CyA study in Rabbits.

After starting Fluc (on day 9), rabbits experienced initial rise in median CyA trough level from 23 ng/Ml (11-41 ng/mL) to 75 ng/mL (42-218 ng/mL) 2-7 days following Fluc administration (Z value 2.201, $p = 0.028$, $n = 7$), Table 2.

Peak CyA trough elevation was seen 5-7 days after Fluc initiation in three rabbits and, the other three rabbits was seen 2 days after Fluc discontinuation.

Trough level elevations reached a median level of 143 ng/mL (57-585 ng/mL) (Z value 2.201, $p = 0.028$, $n = 6$), Table 2.

After Fluc discontinuation, median CyA trough level returned back from 143 ng/mL (57-585 ng/mL) to almost baseline levels 72 ng/mL (16-289 ng/mL) by day 19 ($n = 1$), day 21 ($n = 2$), day 23 ($n = 2$). Only rabbit #3 showed persistent elevation in CyA level, reaching almost 9 fold, possibly due to weight reduction (0.5kg, 16%); at this stage, rabbit #3 was withdrawn from the study. Decrease from peak CyA level to post-infection level was statistically significant (Z value 2.023, $p = 0.043$, $n = 5$). The final median trough level 72 ng/mL (16-289 ng/mL) was not statistically different from baseline 58 ng/mL (range 39-99 ng/mL) (Z value 0.674, $p = 0.5$, $n = 5$).

The percentage change in median CyA level attributed to Candida infection was 52% (range, 9-426%) ($n = 7$). The percentage change in median CyA level attributed to concomitant Candida and Fluc was 76% (range 22-665%, $n = 6$) (Table 2), indicating an additive effect of Fluc to the Candida inhibition of CyA metabolism.

3.2.2 Serum Creatinine

There was no significant difference between mean serum creatinine level before (1.2 ± 0.24 mg/dL) and after the induction of Candida infection (1.21 ± 0.4mg/dL), (t value 0.063, $p = 0.952$, $n = 7$).

3.2.3 Proof of Infection

Candida infection, checked by mannan test five days following infection, gave positive results in rabbits # 1, 3, 4, 5, 6; rabbit # 2 (before it died) revealed two intermediate test results but had physical signs of infection (feverish and decreased appetite); in rabbit # 7 mannan value was not determined. All rabbits showed decreased appetite and mild weight loss during the period of infection; however, they regained appetite after infection resolution (except rabbit #3 that experienced 16% weight loss).

3.2.4 Immunosuppression

The differential lymphocytic count in peripheral blood on days 0 and 4 was not statistically different (Z value 1.57, $p = 0.116$). CyA may require 7 days &/or higher blood levels (100-400 ng/mL) to alter lymphocytic count as described elsewhere [30].
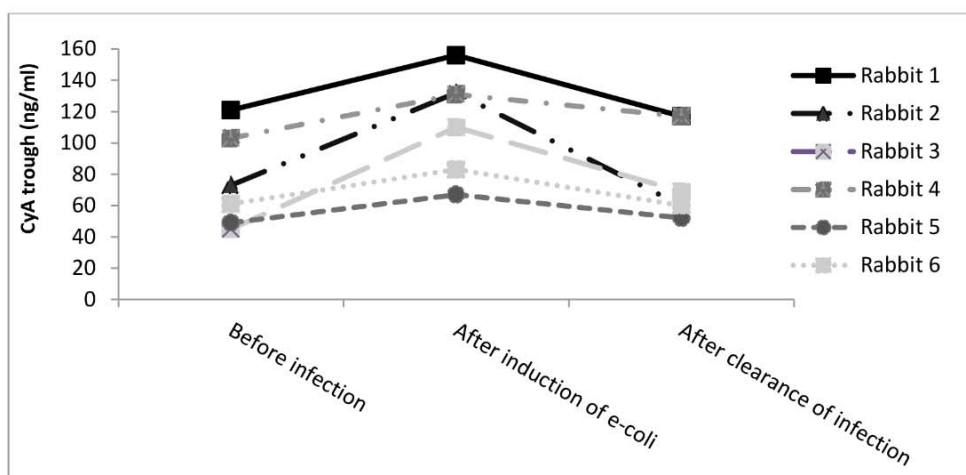
3.3 Multi-dose e coli-CyA Study

3.3.1 CyA Levels

Mean CyA trough level increased from baseline level 75 ± 30.6 ng/mL on day 4 before infection to 113 ± 33.3 ng/mL after induction of infection on day 6 ($n = 2$, inoculum size 7×10^8 CFU/mL) and on day 8 (n = 4, inoculum size 4×10^6 CFU/mL); (t value 4.69, $p = 0.005$, $n = 6$) (Table 3 and Fig. 4).

Table 3   CyA trough levels (ng/mL, 24 h data) following multi-doses of CyA in Multi-dose e coli-CyA study in rabbits.

Rabbit #	Study stages			% change due to e-coli[a]
	Pre-infection	During infection	Post-infection	
Rabbit 1[b]	121	156	117	29
Rabbit 2[c]	73	132	60	81
Rabbit 3[c]	45	110	69	148
Rabbit 4[c]	103	131	117	27
Rabbit 5[c]	49	67	52	36
Rabbit 6[b]	61	83	60	37
Mean ± SD	75 ± 30.6	113 ± 33.3	79 ± 29.8	60 ± 48
Statistical Analysis[*]	$t = 4.69$ $p = 0.005$ (Pre- & Post-infection) $t = 0.76$, $p = 0.482$	$t = 3.83$ $p = 0.012$		

[a] Calculated from difference between pre-infection and during infection data; [b] Inoculated e-coli count 7×10^8 CFU/mL; [c] Inoculated e-coli count 4×10^6 CFU/mL; [*] Paired t-test, statistically significant at p ≤ 0.05 (two-tailed probability).

Fig. 4 CyA trough level before infection, after induction of infection, and after clearance of infection in rabbits in the Multi-dose e coil-CyA study.

Two inoculum sizes were used in this study 7×10^8 CFU/mL in rabbits # 1 & 6 (rabbits # 2-5 died and were replaced by new rabbits) and 4×10^6 CFU/mL in new rabbits # 2, 3, 4, 5 (Table 3). The rise in CyA level in the higher inoculum size appeared 24 h following the induction of e-coli infection while in the lower inoculum size, the elevation appeared 72 h following induction.

At the end of the experiment, mean CyA trough level returned back from 113 ± 33.3 ng/mL to almost baseline levels 79 ± 29.8 ng/mL on day 8 ($n = 2$) and day 12 ($n = 4$); (t value 3.83, $p = 0.012$, $n = 6$). The final trough level 79 ± 29.8 ng/mL was not statistically different from baseline 75 ± 30.6 ng/mL; (t value 0.76, $p = 0.482$, $n = 6$) (Table 3 and Fig. 4). The percentage change in mean CyA level attributed to e-coli infection was $60 \pm 48\%$ ($n = 6$).

3.3.2 Serum Creatinine

There was no significant difference between mean serum creatinine level before and after the induction of e-coli infection, (t value 2.23, $p = 0.076$, $n = 6$). However, there was a small but significant rise from 0.77 ± 0.09 mg/dL at baseline to 0.92 ± 0.18 mg/dL at the end of the experiment (t value 2.65, $p = 0.045$, $n = 6$).

3.3.3 Proof of Infection

Positive blood cultures of BacT/ALERT 3D blood culture system and the subsequent plate subcultures were obtained 24 h following infection in the 7×10^8 CFU/mL inoculum size; the lower inoculum size of 4×10^6 CFU/mL gave positive culture on day 9 (96 h post infection). All rabbits showed decreased appetite and were feverish following the infection and they regained their appetite (judging by quantity of food consumed) after infection resolution.

4. Discussion

Changes in drug handling capacity during inflammation/infection will continue to complicate therapy [36]. A limited number of studies appear to have discussed infection-mediated inhibition of drug metabolism. Most of them were for bacterial infection, and few were for parasitic or viral.

Studying infection effect on drug metabolism *per se* and not simply measuring liver enzymes appears to have only been studied clinically but not experimentally and, to our knowledge CyA was not included. As far as we know, only one study reported decreased CYP3A4-dependent CyA metabolism in bone marrow transplantation patients, and interestingly an association was found between high interleukin 6 (IL-6) plasma concentrations and increased CyA concentrations [37], which may partly explain the results of the present study as IL-6 is reported to be

released by human cells in response to Candida cells [38].

4.1 Suitability of the Animal Model

Rabbit was chosen due to the similarity in metabolism of CyA in humans and in rabbits. M-17 is the only CyA metabolite with significant *in vitro* immunosuppressive activity, and it is the major metabolite of CyA in liver transplant patients and in rabbits [39]. In addition, the rabbit isoforms CYP3A6 and the human isoform CYP3A4 have similar predominance and substrate specificity [40].

Possible CyA toxicity in rabbits, reported with long term administration (60 days) [27] was avoided in the present study by limiting the duration of the experiment to not more than 23 days.

4.2 Agreement with Reported Data

Single-Dose Study

The results indicated that Fluc-mediated inhibition of CyA metabolism occurs with a single Fluc dose which is parallel to reports by others [41]. Similar to AUC, trough CyA values proved to be a good indicator of changes in CyA metabolism, (r^2 for trough/AUC_{0-12h} correlation = 0.837).

The baseline CyA mean trough levels, AUC, and TBC in the control arm (184 ± 42.1 ng/mL, 16.67 ± 4.14 mg/L. h, and 0.78 ± 0.16 L/h/kg respectively) agreed with published values produced elsewhere in the same model generated with comparable doses [16].

Multiple-Dose Studies

The multiple dose studies were designed to simulate the situation in patients administering daily doses of CyA and to test the suitability of the rabbit model for investigating drug-infection interaction on the CyA steady state.

Baseline CyA median trough levels (24 h) in the Multi-Candida-Fluc-CyA study were 58 ng/mL and in the Multi-e coli-CyA study mean corresponding level was 75 ± 31 ng/mL.

The rise in CyA level after Fluc administration in the second leg of the Multi-Candida-Fluc-CyA study followed a similar pattern in all rabbits, an initial rise then a peak rise several days after; this pattern was also observed in a multi dose Fluc-CyA interaction study in renal transplant patients [42]. It was reported, as well, that the inhibition occurs with the first dose and takes approximately one week for peak inhibition [41].

4.3 The Effect of Fluc, Candida-Infection and Concomitant Infection-Fluc on CyA Blood Level

The percentage rise in CyA level varied in the different studies. In the Single Fluc-CyA study, CyA trough level elevation due to Fluc interaction reached a mean of 70 ± 45 % with a maximum elevation of 145 % (*n* = 5); calculated from Table 1. The percentage change in median CyA level attributed to Candida infection was 52% (range 9-426%) (*n* = 7), in the Multi-Candida-Fluc-CyA study (Table 2).

Data generated allowed assessment of the possible additive inhibitory effects by both Candida infection and Fluc administration on CyA metabolism. This additive effect was particularly evident in four out of six rabbits (Rabbits #3, 4, 6, 7, Table 2). Overall, the percentage change in median CyA level was 76% (range 22-665%) (*n* = 6) (Table 2).

Using concomitantly two inhibitory factors to the CYP450 may exhibit stronger inhibition of the specific enzyme. In a study of the impact of multiple, CYP2D6, inhibitors on plasma risperidone levels, the data proved that an increase in the number of concomitant inhibitors may be associated with a lower CYP2D6 activity [43]. This additive inhibition was also apparent in our results in the Multi-Candida-Fluc-CyA study.

4.4 The Effect of Candida and e-coli Infection Inhibition on CyA Metabolism

In the Multi-e coli-CyA study, CyA trough level elevation due to e-coli interaction reached a mean of

$60 \pm 48\%$ with a maximum elevation of 148% ($n = 6$) (Table 3), in agreement with the study performed by injecting lipopolysaccharides of e-coli (10 micrograms/mouse) that decreased CYP450 level 56-69% [8]; while in the first leg of the Multi-Candida-Fluc-CyA study, the elevation reached a median of 52% (range 9-426%) due to Candida-CyA interaction ($n = 7$) (Table 2)

In relation to infection, the effect of an immunological response on enzyme inhibition is difficult to predict, since the effect is dependent on the degree of inflammation, on the inflammatory mediators released and may be reversed with successful treatment of the disease [44].

Positive correlations were found between CFU and inflammatory mediators [45]. From what we observed, the low inoculum bacterial size in the e-coli study was associated with delayed increase in CyA level compared to the high inoculum size. Consequently, the time of assay of the CyA blood level to judge the effect of infection varies depending on severity of infection.

A persistent drop in CyA level below baseline after an initial rise following the Candida infection appeared in CyA profiles of 6 out of 7 rabbits (Table 2). Several reports suggested factors that may be implicated, most of them involving cytokines, mainly Il-10 and Il-4. These cytokines Il-10 and Il-4 were found to have a stimulatory effect for the CYP3A4 isoform [44], which may be the reason of the sudden temporary drop in CyA level due to induction of CYP3A4 isoform; CyA levels were then, raised again after further Fluc inhibition of CyA metabolism.

4.5 Comparing Results of the Clinical and Animal Studies

The present data followed a clinical study in patients, which looked at the effect of active infection on CyA blood level in immunocompromised renal transplant patients [14]. The animal studies were designed to assess the effect of infection on CyA systemic exposure in rabbits.

The infection studies resulted in significant rise in CyA trough level after infection in clinical (p < 0.001, $n = 20$) [14] and in experimental either fungal ($p = 0.018$, $n = 7$) or bacterial ($p = 0.005$, $n = 6$) studies respectively, which subsided down to near baseline levels after infection was resolved. The CyA levels after infection resolution were not statistically different from baseline ($p = 0.382$) in patients [14], and ($p = 0.5$) in the fungal experiment and ($p = 0.482$) in the bacterial experiment in rabbits.

However, the increase in CyA blood level was not associated with a significant rise in serum creatinine in the animal, contrary to the clinical study [14].

CyA is mildly nephrotoxic in rabbits and serum creatinine rises only slightly and mainly after weight loss starts to occur [27], also noted with rabbit # 3 in the Candida study where a rise in serum creatinine reached 2.25 folds in this rabbit possibly related to weight loss and dramatic rise in CyA level.

In this study, we demonstrated two types of interactions in rabbits, drug-drug interaction (Fluc-CyA interaction in a single and multi-dosage regimen) and drug-disease interaction (Candida-CyA and e-coli-CyA interactions both in multi dosage regimen).

The results of the present animal studies, and the clinical study [14] are in agreement with the statement: "Narrow therapeutic index drugs that are metabolized by CYP450, pose a significant risk in placing patients in a position where an infection or inflammatory response might lead to aberrant drug handling and an adverse drug response [46]."

Both clinical and experimental animal infection studies agreed on the fact that hepatically metabolized narrow therapeutic indexed drugs should be monitored for drug level to guide dose adjustment after infection and if a potentially interacting drug is initiated (Fluc in this study) in inhibitory doses.

The time at which the rise in CyA level starts, depends on the initial count and severity of infection

as evident in the e-coli study.

5. Conclusions

Candida and e-coli infections increased CyA trough levels, as well as Fluconazole in the single-dose study. Fluconazole, used for the treatment of Candida infection, exerted an additive rise to CyA levels in the multi-dose study.

Based on the data suggesting that by the resolution of infection, CyA level will subside down to pre-infection values, and there may be no urgent need for dose adjustment of CyA unless infection persists &/or CyA-induced nephrotoxicity is detected.

Acknowledgements

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