Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Cyclosporine A versus tacrolimus monotherapy. Comparison on bile lipids in the first 3 months after liver transplant in humans

Leonardo Baiocchi,[1] Mario Angelico,[1] Linda De Luca,[2] Domenico Ombres,[1] Alessandro Anselmo,[2] Claudia Telesca,[1] Giuseppe Orlando,[2] Daniele D'Andria[2] and Giuseppe Tisone[2]

1 Chair of Gastroenterology, Faculty of Medicine, University of Rome 'Tor Vergata', Rome, Italy
2 Chair of Transplant Surgery, Faculty of Medicine, University of Rome 'Tor Vergata', Rome, Italy

Keywords
bile acids, biliary lipids, cyclosporine A, liver transplantation, tacrolimus.

Correspondence
Leonardo Baiocchi MD, PhD, Faculty of Medicine, Chair of Gastroenterology, Dipartimento di Sanità Pubblica, University of Rome 'Tor Vergata', Edificio F, Torre Nord, Stanza F-575, Via Montpellier 1, 00133 Rome, Italy. Tel./fax: +39-06-72596875; e-mail: baiocchi@uniroma2.it

Received: 9 November 2005
Revision requested: 9 December 2005
Accepted: 31 January 2006

doi:10.1111/j.1432-2277.2006.00296.x

Summary

Biliary lipids output is reduced after liver transplantation and tends to normalize thereafter. Cyclosporine A (CyA) is reported to interfere with the normal bile-restoring process after liver grafting, but data are inconclusive, in particular regarding the comparison with the other widely used calcineurin inhibitor tacrolimus (TCR). Furthermore, previous researches were conducted in patients taking multiple immunosuppressive therapies and with a short follow up. In this study we readdressed this issue by comparing biliary lipids in the first 3 months after liver transplant, in 20 patients randomized to receive immunosuppression with CyA or TCR monotherapy. Bile samples, harvested through a T-tube at days 1, 3, 7, 15, 30, 60 and 90 were assessed for cholesterol, phospholipids, and total and individual concentrations of bile acids (BA). Liver and kidney function tests were evaluated as well. We found no differences between CyA and TCR in biochemical findings or in total biliary BAs, cholesterol, and phospholipids. However, CyA-treated patients showed lower levels of glycochenodeoxycholic acid at day 15, compared to those treated with TCR ($P < 0.04$). This difference normalized thereafter, without any biochemical or clinical effect at 3-month follow up.

Introduction

Bile production is a major function of the liver, and qualitative or quantitative impairment of this process invariably determines cholestasis [1]. After orthotopic liver transplantation (OLT) bile secretion is always empirically monitored as an early indicator of graft function. In this setting, some authors have suggested that measurement of bile lipids concentration is helpful in discriminating patients with early graft dysfunction or rejection [2–6]. These studies have consistently reported a decrease in biliary lipids concentration in the first phases after OLT with a progressive recovery after 2 weeks from surgery. Impairment of normal bile secretion shortly after transplant is mainly attributed to ischemic damage of the graft, but several authors have claimed that immunosuppressive drugs may also interfere with the bile restoration process. Cyclosporine A (CyA) in fact has been demonstrated to exert hepatotoxic and cholestatic effects in animals [7–9] as well as in humans [10,11]. CyA-related impairment of bile production has been attributed to its interference with normal enterohepatic cycling of bile acids (BA), through the direct inhibition of their hepatic uptake and secretion [12,13]. In addition, CyA in rat and human hepatic cell cultures exerts a direct inhibition of cholesterol 7α-hydroxylase, considered to be the rate-limiting enzyme in BA synthesis [8,14]. Tacrolimus (TCR) is another calcineurin inhibitor commonly used for immunosuppression after transplant. Although chemically different from CyA, its mechanisms of action are similar; they include inhibition of gene transcription for interleukin (IL)-2, interferon-γ, and other immunomodulatory

factors [15,16]. An *in vitro* study has shown TCR to be an immunosuppressant 10 to 100 times more effective than CyA. Limited data are present on the comparative effect of CyA and TCR on bile lipid production after OLT. Data coming from human studies are potentially confounded by the fact that patients received multiple immunosuppressant regimens (steroids, azathioprine, etc.), thus not allowing definitive conclusions on the exclusive effect of CyA or TCR [17,18]. On the other hand, data from animal studies were mainly conducted with the bile-fistula rat model on animals that did not undergo liver transplantation [19]. Current trends in immunosuppression after OLT show that azathioprine is being progressively abandoned [20]; furthermore, data from our own as well as other groups question the usefulness of corticosteroids [21,22]. Based on these observations, since 1999 our liver transplant center has adopted an immunosuppressive protocol, starting from the first day after OLT, consisting of either CyA or TCR monotherapy. This allowed us to study the comparative effects of CyA or TCR on biliary lipids after OLT. In addition, while previous research limited bile lipids analysis to the first 2–3 weeks after grafting, in the current study we extended our follow up until the third month. Finally, our study includes a detailed analysis of BA in bile, as concentrations of cholic acid (CA) and chenodeoxycholic acid (CDCA), the main primary BA in humans, are reported individually according to their degree of conjugation with taurine or glycine.

Patients and methods

Twenty patients (15 M/5 F mean age: 50.6 ± 17 years) were enrolled in the study. Inclusion criteria were elective OLT and age >18 years. Exclusion criteria were: multiorgan transplantation; re-transplantation; previous history of immunosuppressive therapy; primary nonfunction of the graft. Indications for OLT were viral cirrhosis in 16 patients [eight hepatitis C virus (HCV), four hepatitis B virus (HBV), four HBV + HCV]; alcoholic cirrhosis in three; primary biliary cirrhosis in one. The operation was performed according to standard technique, with the use of veno-venous bypass and the position of a T-tube to protect the bilio-biliary anastomosis. The T-tube was removed 3 months after grafting, in compliance with the standard protocol of our as well as other Liver Units [23,24]. After surgery, patients were randomly assigned to receive either CyA or TCR. The study protocol was approved by the Ethics Committee of our Institution and the research was conducted according to the principles of the Declaration of Helsinki. Patients gave their informed written consent to participate in the study. The two groups were homogeneous for several individual and donor variables (Table 1). CyA was administered to achieve blood levels of 250 ng/ml within 1 week. TCR was adjusted to reach blood levels ranging between 5 and 10 ng/ml within 1 week. Patients did not receive any other immunosuppressive drug according to the protocol of our center. HBV patients received standard immunoprophylaxis plus lamivudine 100 mg/day. Ursodeoxycholic acid was not administered in any patients as no clear clinical indication for using it after OLT has been demonstrated [25]. Few other drugs were administered in the early phases after liver transplant in the intensive care unit: none of them had documented choleretic or cholestatic effects and are comparable with those used in previous studies. During the 3-month follow up, liver function tests were evaluated every day for the first 2 weeks, and weekly thereafter. Bile samples were harvested from the T-tube at days 1, 3, 7, 15, 30 and 90 after transplant. Bile specimens (approximately 5 ml) were diluted in three volumes of isopropanol, centrifuged at 3,300 *g* for 10 min and then stored at −20 °C. Total BA, phospholipids, and cholesterol concentrations were assessed in the supernatant with colorimetric enzymatic methods, using commercially available kits, following the instructions of the vendor (Sigma-Aldrich, Milan, Italy). Qualitative analysis of individual BA in bile was obtained by high-performance liquid chromatography (HPLC), using an isocratic

Table 1. Patient, donor, surgical, and biochemical variables according to type of immunosuppression.

	CyA	TCR
Mean age	49.1 ± 8.1	48.5 ± 11.8
Gender (M/F)	8/2	7/3
Cause of transplant		
C hepatitis	3	5
B hepatitis	3	1
Alcoholic hepatitis	2	1
C + B hepatitis	1	3
Primary biliary cirrhosis	1	
Warm ischemia time (min)	43.8 ± 8	46.6 ± 20
Cold ischemia time (min)	402 ± 220	394 ± 116
Glutamic Pyruvic transminase (GPT) (U/l; mean value during the study)	230.4 ± 473.2	259.5 ± 672.3
Glutamic Oxalacetic transminase (GOT) (U/l; mean value during the study)	326.8 ± 626	258 ± 487.7
Bilirubin (mg/dl; mean value during the study)	8.1 ± 10	6.5 ± 14
Gamma Glutamy transpeptidase (GGT) (U/l; mean value during the study)	118.8 ± 133.1	116.2 ± 89.2
Donor age	44.9 ± 17.9	56.4 ± 18.5
Donor gender (M/F)	6/4	6/4

Data are mean ± SD for continuous variables. Differences between groups were not statistically significant.
CyA, cyclosporine A; TCR, tacrolimus.

acidic method, as previously described [4,5,26]. We employed a Gilson HPLC system (Villers La Belle, France) equipped with a 5 μm ODS2 Spherisorb column (Phase Sep, Norwalk, CT, USA) and a diode array detector (Applied Biosystem, Foster City, CA, USA) set at 205 nm. Statistical analysis was carried out with unpaired *t*-test using the NCSS software package (Kaysville, UT, USA). A *P*-value of <0.05 was considered statistically significant.

Results

Patient outcome and bile lipid concentrations

The mean hospital stay was 12.3 ± 7.2 days. After discharge patients were followed in outpatients clinic at weekly intervals during the first month and twice a month thereafter. During the 3 months of the study there were no differences in routine liver function tests between patients receiving CyA or TCR (Table 1). Biliary lipid concentrations (total BA, cholesterol, total phospholipids) at the various time intervals are summarized in Table 2. As previously described both by our group and others [2–6], we observed a profound decrease in total BA biliary concentration in the first days after transplant. However, in 2 weeks time, grafts were able to restore an almost normal bile lipid production. There were no differences in terms of BA, phospholipids, and cholesterol biliary concentrations between the two groups, demonstrating the lack of a specific effect of CyA or TCR on these parameters. Calculated values of total BA–phospholipids molar ratio, showed fluctuations during the study but were not different between the two groups (Table 2).

Individual BA analysis

High-performance liquid chromatography quantitative estimation of primary CA and CDCA, either tauro- or glyco-conjugated, is illustrated in Fig. 1. The increase of individual BA paralleled the enhanced total BA production, involving each separate class. There were no major differences in the recovering profile of each single BA between patients treated with CyA or TCR. However, at day 15 patients receiving TCR treatment showed higher biliary levels of glycochenodeoxycholic acid (GCDCA) compared with those receiving CyA ($P < 0.04$), suggesting a possible specific inhibitory effect of CyA on the synthesis of this primary bile salt. This difference was also evident in the 30- and 90-day bile samples but did not reach statistical significance. Total CA or CDCA concentrations in bile (sum of Tauro- plus glyco-conjugated forms) were not statistically different between CyA and TCR during the 3-month study (Table 3). Deoxycholic acid (DCA) was not present in bile until day 30; after that its concentration moderately increased reaching a maximum of around 15% of total BA (day 90), with no differences between CyA and TCR. We did not observe litocholic acid in our samples.

Discussion

Bile acids represent the main driving force in bile formation. Their reported biliary concentration in normal cholecystectomized patients is around 10–15 mM [17]. However in the OLT setting, BA pool is greatly reduced in the first days after surgery, showing a progressive restoration thereafter due to the synthesis of primary CA and CDCA in the liver [2–6]. Secondary BA formation (such as DCA) is usually counteracted in this phase by T-tube bile diversion, which interferes with a normal BA enterohepatic circulation. Several studies focused on the possible effects of immunosuppressive drugs on bile lipid production. CyA inhibition of BA synthesis for instance, has been demonstrated in several experimental models including: (i) rat and human hepatocyte cell cultures [14,27];

Table 2. Comparison of biliary lipid concentrations and bile salt–phospholipid molar ratio at different time points, according to type of immunosuppression.

	Total bile salts (mM)		Phospholipids (mM)		Cholesterol (mM)		Bile salt–phospholipid molar ratio	
Day	CyA	TCR	CyA	TCR	CyA	TCR	CyA	TCR
1	2.3 ± 2.1	2.5 ± 2.4	0.8 ± 0.6	0.3 ± 0.3	0.1 ± 0.1	0.1 ± 0.2	22.6 ± 30.2	13.6 ± 11.3
3	3.4 ± 2.9	3.3 ± 3.4	1.2 ± 1.5	1 ± 1.8	0.8 ± 1.3	0.3 ± 0.5	4.6 ± 3.2	6.8 ± 4.2
7	4.5 ± 3.7	6.6 ± 7.1	0.8 ± 1.2	0.7 ± 0.8	0.8 ± 1.2	0.8 ± 0.7	18.8 ± 8.6	12.1 ± 8.2
15	12.1 ± 9	17 ± 11.3	0.7 ± 0.4	1.4 ± 1.2	0.3 ± 0.3	0.5 ± 0.5	13.5 ± 11.2	13.5 ± 1
30	15.3 ± 12.8	20.6 ± 8.4	1.4 ± 1	0.8 ± 0.5	0.8 ± 1.3	0.6 ± 1.1	15.3 ± 14	21.1 ± 9.5
90	17.1 ± 12.7	14.6 ± 8.6	1 ± 0.5	1 ± 0.9	0.3 ± 0.3	0.5 ± 0.3	13.6 ± 5.9	8.5 ± 7.6

Data are mean ± SD. Differences between groups were not statistically significant.
CyA, cyclosporine A; TCR, tacrolimus.

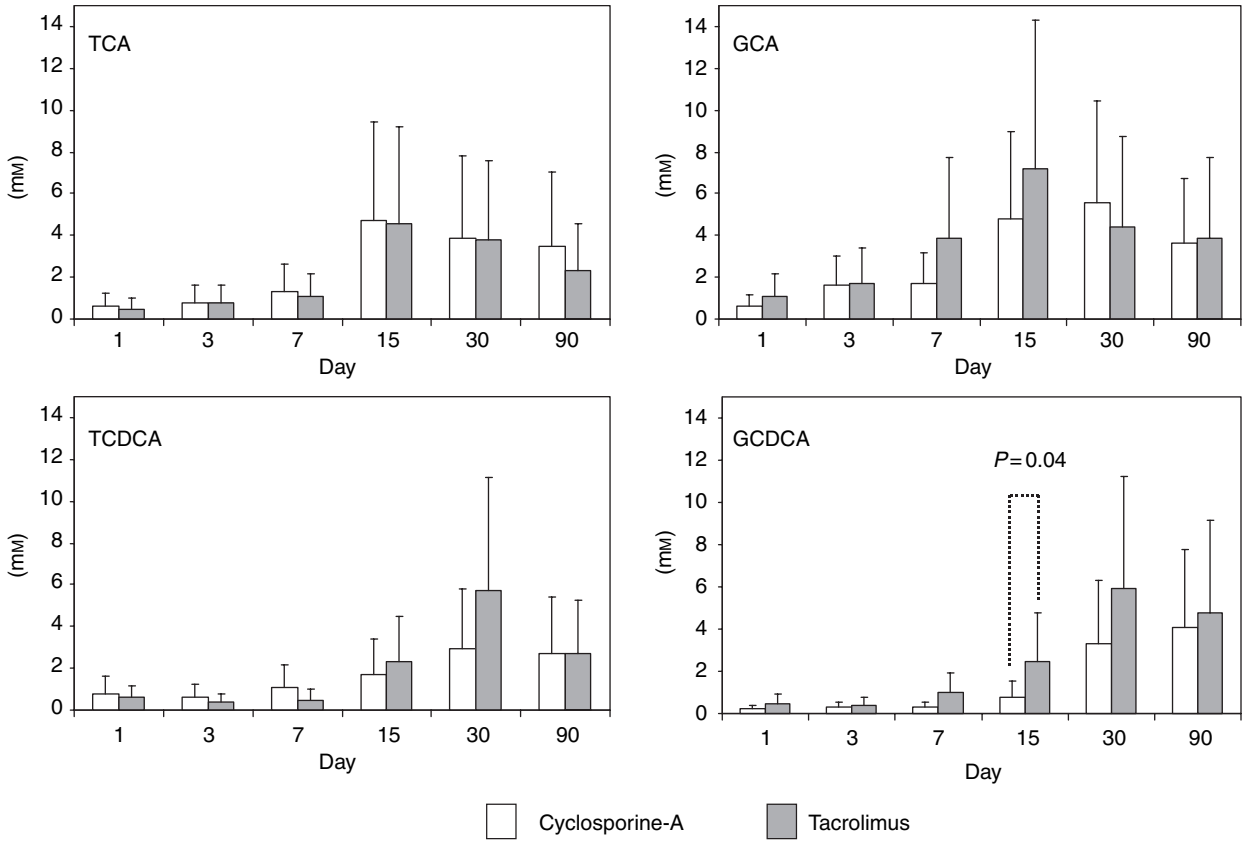

Figure 1 Comparison at different time points of individual biliary bile acids between patients receiving cyclosporine A (white bar) or tacrolimus (grey bar) monotherapy. TCA, taurocholic; TCDCA, taurochenodeoxycholic; GCA, glycocholic; GCDCA, glycochenodeoxycholic acid. At day 15 cyclosporine A determines a significant decrease in GCDCA output compared with tacrolimus ($P < 0.04$). This difference tends to normalize thereafter, with no statistical significance at days 30 and 90.

Table 3. Biliary concentration of individual bile acid (sum of tauro + glycoconjugate) at different time points, according to type of immunosuppression.

	Cholic acid (mM)		Chenodeoxycholic acid (mM)		Deoxycholic acid (mM)	
Day	CyA	TCR	CyA	TCR	CyA	TCR
1	1.2 ± 1.3	1.6 ± 1.5	1.1 ± 1	1.1 ± 1.3	n.a.	n.a.
3	2.5 ± 2.6	2.5 ± 2.7	0.9 ± 0.6	0.8 ± 0.7	n.a.	n.a.
7	3.1 ± 2.1	5.1 ± 5.7	1.4 ± 2.1	1.5 ± 1.5	n.a.	n.a.
15	9.5 ± 7.4	11.7 ± 7.3	2.5 ± 2.4	4.8 ± 4.4	n.a.	n.a.
30	9.5 ± 7.7	8 ± 3.1	6.1 ± 6.5	11.6 ± 7	0.7 ± 0.9	1.3 ± 2
90	7.1 ± 3.6	6.2 ± 4.6	7.3 ± 4.9	7.5 ± 5.2	2.5 ± 2.1	1 ± 1.7

Data are mean ± SD. Differences between groups were not statistically significant.
n.a., not assessable; CyA, cyclosporine A; TCR, tacrolimus.

(ii) animal *in vivo* studies [28,29]; and (iii) liver-transplanted children, employing stable isotopic dilution technique [30]. Interestingly, CyA-associated BA repression seems preferential for the CDCA synthetic pathway rather than for the CA one [8]. Few reports focused on the comparative effects of CyA or TCR on bile lipid composition [17,18]. McCashland et al. [17] showed that in the early phase after OLT a specific inhibition of CDCA synthesis was present with CyA but not with TCR administration. This finding was not confirmed in a later study of OLT recipients, reporting similar BA-dependent bile flow and CDCA bile concentrations with either CyA or TCR immunosuppression [18]. Both these studies, however, are limited by the fact that patients were receiving multiple

immunosuppressive therapies (steroids, azathioprine), and bile analysis was restricted to day 10 after transplant. In addition, in the McCashland *et al.*'s [17] study the TCR group was composed of only five patients. A comparative study of bile lipid secretion between different dosages of CyA or TCR was also conducted in rodents [19]. This report confirmed a CyA-induced direct inhibition of CDCA synthesis, but was present only at the highest CyA dosage; however, the drugs were administered through an unusual i.m. route and liver transplantation was not part of the experimental design. In the present study, we readdressed this issue in a prospective human study, where patients were randomized to receive CyA or TCR monotherapy from the first day after transplant. Our results support the finding that after liver transplantation CyA and TCR have similar effects on biliary lipid concentration, including total BA, cholesterol, and total phospholipids. In addition, the extension of the study until the third month after grafting allows to conclude that differences on total bile lipid content between CyA and TCR are unlikely in the long-term follow up. With regard to the reported direct CyA inhibition of CDCA synthesis by McCashland *et al.* [17] and not confirmed by other authors, our data suggest that this phenomenon: (i) may be restricted to GCDCA only, (ii) is transient, and (iii) tends to fade leaving no differences between CyA and TCR. These findings help clarify some of the disagreements between different clinical reports. In fact we demonstrated that a correct assessment of CyA inhibition of CDCA production after transplant, requires an extension of bile analysis until day 15 (time point at which a complete recovery of bile lipid composition is approached), and that this evaluation has to include the extent of conjugation with either taurine or glycine of each single BA. A possible limitation in the comparison between our data and previous studies are that we did not express bile lipids in terms of secretion. This was due to the observation that the T-tube, in our experience, is an unreliable tool to achieve bile flow data. In fact this device does not recover the variable amount of bile that is driven into the intestine trough its lower portion [31]. In the absence of a correct measure of bile flow we considered appropriate to assess biliary lipid concentration as the best surrogate parameter for bile lipid secretion. Other authors followed different approaches to obtain a correct measure of bile flow in humans, such as the triple lumen duodenal catheter or the Fogarty balloon device [32,33]. However, these methods were considered inapplicable in our setting because of ethical consideration. The possible implications of CyA inhibition on GCDCA biliary concentration after OLT are not completely clear at this stage. However, speculations are possible on the basis of GCDCA physical and chemical characteristics. In fact differences in BA chemical structure and conjugation with either taurine or glycine are responsible for their hydrophilic/hydrophobic balance [34,35]. The BA in this study, according to their detergent capacity, are ordered as follows: GCDCA > glycocholic acid > taurochenodeoxycholic acid > taurocholic acid. A greater detergency determines an increased capacity to solubilize cholesterol, thus preventing cholesterol meta-stability and potential cholesterol crystal formation [36]. Theoretically one may conclude that CyA impairment of GCDCA biliary concentration should expose transplanted patients to a higher risk of choledocholithiasis in the short term. However, as after grafting bile is very diluted, and several variables (genetic, hormonal, diet-related, etc.) influence the complex process of biliary cholesterol solubility [37–40], this mild impairment in GCDCA production is not likely to induce a relevant lithogenic effect. In addition, as changes in GCDCA concentration are limited in time, it seems reasonable to conclude that this phenomenon should not be of major importance on other biochemical or clinical events including the reported BA-induced bile duct injury after OLT [41]. This latter condition is mainly determined by a high BA/phospholipid ratio, when BA detergency is not counteracted by adequate phospholipid biliary concentrations. However, this parameter was not different between CyA and TCR in our study (Table 2). In conclusion, CyA or TCR have comparable effects on total bile lipid composition after liver transplant. However, CyA determines a specific reduction of GCDCA biliary concentration, which is evident at day 15 after surgery. This alteration is progressively corrected and disappears at 3 months.

Acknowledgement

The authors express gratitude to Paola Piccolo (MD), for helpful discussions and critical review of the text.

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