Donor pre-treatment with everolimus or cyclosporine does not reduce ischaemia–reperfusion injury in a rat kidney transplant model

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Abstract

Background. Immunosuppressive agents have been investigated in renal ischaemia–reperfusion injury (IRI) and have frequently demonstrated a beneficial effect. Most studies focused on treatment of the recipient at the time of transplantation. Pre-treatment of these organs before injury (pharmacological pre-conditioning) may particularly protect these organs. This study aimed to investigate the possible protective effects of donor pre-treatment with cyclosporine (CsA) or the mTOR inhibitor everolimus or their combination against IRI during renal transplantation in a rat model.

Methods. Donors received vehicle, CsA (5 mg/kg), everolimus (0.5 mg/kg) or CsA+everolimus. Two oral doses were administered to the donors at 24 h and again at 6 h prior to donor kidney removal. Syngeneic rat kidneys were preserved in UW solution for 24 h prior to transplantation. After 24 h of reperfusion, blood and tissue samples were collected from recipients for further analysis.

Results. Renal functions as determined by creatinine and necrosis scores were not different between the experimental groups. Cleaved caspase-3, heat shock protein 70 (HSP70), tumor-necrosis factor-alpha (TNF-α) and nitrotyrosine protein levels were not statistically different between the four treatment groups at 24 h post-transplantation. Blood NMR analysis on metabolic markers for IRI reveals no beneficial effects of donor pre-treatment on the 24-h outcome in transplantation.

Conclusions. When given alone or as a combination to donors before organ recovery, cyclosporine or everolimus does not appear to ameliorate IRI.

Keywords: cyclosporine; everolimus; ischaemia/reperfusion injury; metabolomics; rat kidney transplantation

Introduction

Ischaemia–reperfusion injury (IRI) during renal transplantation remains a significant problem, especially in extended criteria donors. Aggravated IRI can contribute to the development of delayed graft function. There is also growing evidence that IRI may increase immunogenicity, possibly resulting in increased rejection rates. Several studies have demonstrated that commonly used immunosuppressive agents exhibit protective effects during IRI [1–4]. Each drug has a distinct spectrum of side effects associated mostly with long-term use of these immunosuppressants. The immunosuppressive protocols based on chronic cyclosporine (CsA) administration have reported serious nephrotoxic episodes that are characterized by a decrease in glomerular filtration rate (GFR), afferent arteriolopathy and striped tubulointerstitial fibrosis [5–7]. Other side effects include hyperlipidaemia, diabetes and neurotoxicity [7–10]. However, ultra-short-term use is unlikely to be associated with adverse effects. Furthermore, the combination of CsA with other immunosuppressive agents may either increase or decrease its toxicity. In the brain for example, studies have shown enhancement of cyclosporine toxicity when co-administered with the immunosuppressant sirolimus. Although not nephrotoxic alone, sirolimus has been shown to enhance the nephrotoxic effects of cyclosporine, while everolimus, a sirolimus derivative, does not necessarily increase the nephrotoxic potential of CsA [9,11].

There is controversy in the literature regarding the impact of immunosuppressive treatment on the outcome of IRI after renal transplantation. Some studies reported pharmacological pre-conditioning with CsA in the kidney [4,12], which has similarly been seen in hypoxic brain and ischaemic heart [2,3,13,14]. In these studies, pre-treatment with CsA led to improved renal functions and histology, increased heat shock protein 70, and decreased expression
of pro-inflammatory cytokines (decreased expression of interleukin-1 and tumor necrosis factor-alpha) as well as amelioration of oxidative stress in CsA-pre-treated rat kidney after IRI [4,12]. In contrast, other studies observed aggravated IRI in rat kidney by CsA [1,15–18] as detected by increased renal dysfunction, decreased GFR and delayed tubular regeneration. Similarly, some studies have reported negative effects of sirolimus on IRI (including renal dysfunctions, delayed tubular regeneration and increased expression of heme oxygenase-1) [17], while others observed no negative effect of sirolimus pre-treatment on renal outcome after IRI [18]. These existing controversies regarding the possible effects of CsA and sirolimus pre-treatment on IRI in the kidney can be partly explained by the various dose ranges used in the different studies. Recently, FTY720 (a new immunosuppressant that promotes lymphocyte sequestration into lymph node) was reported to reduce renal fibrosis as a result of IRI, both alone and in conjunction with CsA (CsA was found to be a negative factor for IRI in these studies) [1,15,19]. Pharmacological pre-conditioning of the organ donor has been less investigated, in part due to logistical concerns and fear of unwarranted side effects. However, the increasing disparity of available organs and patients on the waiting list for transplantation requires use of extended criteria organs. These organs are known to experience greater susceptibility to injury during the transplant process. Protection of these organs prior to any insult would seem to be the best strategy.

The aim of this pilot study was to use a rat renal transplantation model to assess the effect of donor pre-treatment with cyclosporine and everolimus on renal IRI during transplantation.

Materials and methods

Animals
All animal protocols were reviewed and approved by the University of California San Francisco Committee on Animal Research and were in agreement with the National Institutes of Health guidelines for ethical animal research (NIH publication No. 80-123, revised 1985). Kidney donors and recipients were inbred male Lewis rats (200–250 g; Charles River Laboratories, Wilmington, MA, USA). Syngeneic animals are frequently used in order to focus solely on IRI and exclude any injury due to immunogenecity. Animals were housed in individual cages in a temperature- and light-controlled environment under a 12-h light, 12-h dark cycle. Water and chow were available ad libitum.

Drug pre-treatment of donors
CsA and everolimus (both Novartis Pharmaceutical, Basel, Switzerland) were dissolved in milk and diluted to appropriate concentrations prior to administration via oral gavage.

Drug doses were determined based on studies performed by our group. In the donor treatment group, the animals received two doses of 5.0 mg/kg CsA, 0.5 mg/kg everolimus, or CsA+everolimus (5 mg/kg + 0.5 mg/kg, respectively) 24 and 6 h prior to renal harvest. Drug concentrations were determined 6 h after the last drug dose was administered. The control group received milk as vehicle at the highest volume. The timing was chosen based on clinically relevant time points during the organ recovery process. Additionally, previous studies have indicated that, for example, heat shock protein 70 up-regulation after CsA administration is most pronounced ~6 h after the last administration.

Animal surgery
All procedures were performed under anaesthesia with isoflurane. The animals were placed on a heating pad, and the temperature was continuously monitored with a rectal probe.

In the donor the left kidneys were procured, flushed with ViaSpan University of Wisconsin (UW) solution (Barr Laboratories, Pomona, NY, USA) and stored in UW solution at 4°C for 24 h. Insulin 40 IE (Ely Lilly, Indianapolis, IN, USA) and dexamethasone 16 mg (American Regent Laboratories, Shirley, NY, USA) were added to each liter of UW solution.

Recipients underwent bilateral native nephrectomies followed by heterotopic kidney transplantation using an established microsurgical technique [20]. Briefly, end-to-side anastomoses between the renal vessels and the recipient’s abdominal aorta and inferior vena cava were created using continuous 8-0 nylon sutures; an end-to-end uretero-bladder anastomosis was performed using interrupted 11-0 nylon suture. Warm ischaemia was defined as time to complete vascular anastomoses of the graft in the donor. After recovery from surgery and anaesthesia, recipients were transferred to the housing facility and followed up for 1 day. The degree of IRI and the possible effect of interventions can be easily monitored at this time point [21–23].

Graft function
Assessment of renal graft function was determined by measuring the concentration of creatinine in serum after 24 h of reperfusion. Samples were analyzed by a veterinary laboratory service (IDEXX Veterinary Services, Sacramento, CA, USA).

Renal morphology
Semi-quantitative assessment of histological damage was carried out as described previously [22,24]. Briefly, kidney grafts were cross-sectioned through their mid-section, fixed in buffered formalin, embedded in paraffin, and stained with haematoxylin and eosin. Sections were examined histologically for characteristic morphologic changes and tubular cell necrosis resulting from IRI: nuclear pyknosis, karyorrhexis and/or disruption of cell membranes. An analysis was performed on four different randomly selected high-power fields on each slide by a pathologist who was blinded to the experimental condition of the animals. Light microscopic examination was performed under standard conditions with a total magnification of ×40 and ×200. The histological assessment of renal necrosis following ischaemia–reperfusion injury was rated by applying the score ranging from 1 to 5, where 1 is <5% necrosis, 2 is 5–25% necrosis, 3 is 25–50% necrosis, 4 is 50–75% necrosis and 5 is >75% necrosis.

Western blot analysis
We examined expression levels of several proteins involved in mediating IRI, including cleaved caspase-3, heat shock protein 70 (HSP70), TNF-α and nitrotyrosine. Kidney samples (100 mg) were homogenized by a hand-held homogenizer in ice-cold tissue protein extraction buffer (Pierce Rockford, IL, USA) with 1 mM EDTA and 1:100 protease inhibitor (Sigma, St. Louis, MO, USA). The contents were transferred to QIAshredder (Qiagen, Valencia, CA, USA) and centrifuged at 14 000 rpm for 10 min at 4°C. The supernatant was then transferred to a new tube and centrifuged again. The resulting supernatant was stored at −80°C, and the protein concentration in all samples was determined using a standardized assay kit.

Fifty microgram of protein from kidney samples was mixed with 1× LDS buffer and reducing agent (both Invitrogen, Carlsbad, CA, USA) and heated at 65°C for 5 min. The mixture was then subjected to SDS–PAGE. Following electrophoretic separation, the protein was transferred to nitrocellulose membranes that were incubated for 1 h at room temperature in Tris-buffered saline–TWEEN (TBST) containing 5% dry milk. After blocking, the membranes were incubated overnight in TBST + 0.5% milk containing an antibody specific for TNF-α, HSP70, nitrotyrosine or actin (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) at concentrations of 1:500, 1:1000, 1:1000 and 1:2500, respectively. Cleaved caspase-3 was purchased from Cell Signaling Technology.
(Danvers, MA, USA) and used at a dilution of 1:500. The membranes were then washed and incubated for 1 h at room temperature with respective horseradish peroxidase-conjugated secondary antibodies. The membranes were developed using Super Signal West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA) and quantified using spot densitometry. The protein levels were compared by the ratio of measured protein band density and reference protein (actin) band density and normalized to vehicle control.

$^1$H-NMR-based metabolomics on rat blood

All blood samples were collected from recipients at the time of sacrifice 24 h after transplantation. Whole blood was collected in heparin-coated tubes, snap-frozen and then processed using dual chloroform/methanol extraction [23]. An extended metabolic blood profile by proton nuclear magnetic resonance ($^1$H-NMR) is an established method to observe metabolic protection during IRI in the kidney [20,23]. Each blood sample (1 mL) was carefully vortexed and processed with 2 mL of cold chloroform/methanol (1:1, vol/vol). After centrifugation, the supernatants were collected, then the pellets were re-suspended with 1 mL of chloroform/methanol and centrifuged, and the supernatants were collected. The supernatant was washed with 1 mL ice-cold water. The water phase was removed and added to the pellet. Two milliliters of water was added, then the pellet was centrifuged and the supernatant was lyophilized overnight (water-soluble extracts). Afterwards, the water-soluble part was dissolved in 0.45 mL of $D_2$O and analyzed by proton NMR. The lipids in the organic phase were evaporated to dryness in a speed-vacuum centrifuge. The lipid extracts were dissolved in 0.6 mL of deuterated chloroform/methanol (2:1, vol/vol) for NMR analysis.

All water-soluble and lipid blood extracts were analyzed using a 500-MHz high-resolution TopSpin software (Bruker Medical, Billerica, MA, USA). An inverse TXI 5-mm probe head was used for all experiments. In order to suppress water residue in extracts, a standard Bruker water pre-saturation sequence was used (‘zgpr’) (operating frequency for proton channel: 500.24 MHz; power level pl1 = 3 dB; power level for water suppression pl9 = 59 dB; power angle $\varphi_1 = 7.5$ μs (90° pulse); power angle for water suppression $\varphi_1 = 60$ μs; water suppression at Q1 = 4.76 ppm; relaxation delay $d_1 = 12.85$ s (5 × T1); delay for power switching $d_12 = 20$ μs; short delay $d_13 = 3$ μs; spectral width sw = 12 ppm; total number of scans $n = 40$). An external standard substance, trimethylsilyl propionic-2,2,3,3-$d_4$ acid (TSP, 20 and 50 mM in $D_2$O), was added into a thin glass capillary. The final TSP concentration (0.5 and 1.2 mmol/L for water-soluble and lipid extracts, respectively) in the capillary was calculated prior to NMR experiments on study extracts using a standard amino acid solution. The TSP capillary was placed into the NMR tube during the experiment and served as an external standard which allowed for absolute metabolite quantification in each study extract. $^1$H chemical shifts were referred to TSP signal at 0 ppm. After performing Fourier transformation and making phase and baseline corrections, each $^1$H peak was integrated using Bruker 1D WinNMR program. The absolute concentrations of single metabolites were then referred to the TSP integral and calculated according to equation (1):

$$C_x = \frac{I_x \times N_x \times C}{I_9} \times V : M,$$

where $C_x$ is the metabolite concentration, $I_x$ is the integral of metabolite $^1$H peak, $N_x$ is the number of protons in metabolite $^1$H peak (from CH, CH$_2$, CH$_3$, etc.), $C$ is the TSP concentration, $I_9$ is the integral of TSP $^1$H peak at 0 ppm (-9 since TSP has 9 protons), $V$ is the volume of the extract and $M$ is the volume of blood sample.

Statistical analysis

All groups were compared using ANOVA. Treatment groups were compared with control with Dunnett’s test. All data are displayed as mean ± SD. P <0.05 was considered statistically significant. All statistical tests were performed using SPSS 10.0 for Windows (SPSS, Inc., Chicago, IL, USA).

Results

Drug concentrations 6 h after administration of the last drug dose

Animals treated with CsA alone had average drug concentrations of 197 ± 58 ng/mL. Mono-treatment with everolimus resulted in drug concentrations of 4.05 ± 0.4 ng/mL. Combined treatment of CsA and everolimus resulted in significantly higher drug concentrations for both drugs, 369 ± 159 and 9.5 ± 2.3 ng/mL, respectively ($n = 5$ all groups, P < 0.05).

Ischaemia time

Cold ischaemia time for all organs was 24.1 ± 0.5 h. Warm ischaemia time was 25.7 ± 4.6 min. There were no differences in cold or warm ischaemia times among the treatment groups. Two animals, which died briefly after surgery due to technical problems, were replaced, and their data were not reported. Otherwise, all animals survived the observation period.

Graft function

There were no differences in serum creatinine levels in recipients prior to transplantation (Figure 1). At 24 h post-transplantation, creatinine levels for the vehicle-, CsA-, everolimus- and combination-pre-treated groups were 4.0 ± 1.6, 3.7 ± 1.3, 4.6 ± 0.3 and 4.6 ± 0.5 mg/dL, respectively (Figure 1). There were no significant differences between treatment groups.

Renal morphology

At 24 h post-transplantation, renal necrosis for the vehicle-, CsA-, everolimus- and combination-pre-treated groups were scored as follows: 2.00 ± 1.15, 2.20 ± 0.84, 1.60 ± 0.55 and 2.00 ± 0.00, respectively (Figure 2). There were no significant differences between donor treatment groups.

Western blot analysis

We chose HSP70, TNF-α, nitrotyrosine and cleaved caspase-3 expression for several reasons. HSP70 is part of a highly conserved family of essential proteins that are believed to be mediators of ischaemic tolerance and can confer cellular protection against various forms of cellular injury. CsA has been previously shown to up-regulate HSP70. At 24 h post-transplantation, relative HSP70 levels for the vehicle-, CsA-, everolimus- and combination-pre-treated groups were 1.0 ± 0.84, 0.84 ± 0.84, 1.10 ± 0.93 and 0.71 ± 0.90, respectively (n.s., Figure 3A).

TNF-α, also modulated by CsA, is a cytokine known to mediate both inflammation and the extrinsic pathway of apoptotic cell death. At 24 h post-transplantation, relative TNF-α levels for the vehicle-, cyclosporine-, everolimus- and CsA+everolimus-pre-treated groups were 1.0 ± 0.37, 1.02 ± 0.08, 0.95 ± 0.10 and 1.20 ± 0.43, respectively (n.s., Figure 3B).
Nitrotyrosine expression serves as an indicator of cell damage and inflammation, as well as of the production of nitric oxide (NO). The concentration of nitrotyrosine is used as a surrogate marker for oxidative damage caused by NO. At 24 h post-transplantation, relative nitrotyrosine levels for the vehicle-, CsA-, everolimus- and combination-pre-treated groups were $1.0 \pm 0.37$, $0.92 \pm 0.45$, $1.16 \pm 0.45$ and $1.37 \pm 0.56$, respectively (n.s., Figure 3C).

Caspase-3 is a mediator of renal tubular cell death in IRI. The degree of apoptosis was evaluated by relative levels of the activated cleaved form of caspase-3. At 24 h post-transplantation, relative cleaved caspase-3 levels for the vehicle-, cyclosporine-, everolimus- and CsA+everolimus-pre-treated groups were $1.1 \pm 0.19$, $1.94 \pm 0.93$, $1.63 \pm 1.24$ and $2.12 \pm 1.30$, respectively (n.s., Figure 3D).

**Quantitative metabolomics for IRI biomarkers**

Our group has recently demonstrated that targeted quantitative metabolomics is well suited to detect immunosuppressive agent-induced metabolic changes or injury-induced metabolic alterations such oxidative stress, either in the blood or tissue. We have also shown that metabolic changes in the blood are of more clinical relevance than urinary markers [26]. Specifically, increased levels of allantoin, the end product of uric acid and therefore xanthine metabolism, have been shown to be a sensitive marker of oxidative stress in rats, which correlates with increased uric acid levels in transplanted patients [23,26].

38 endogenous metabolites were quantified from each blood sample using 1H-NMR-selective metabolites, and their levels among study groups are presented in Table 1. Pretreatment of kidney donors with CsA for 24 h demonstrated biochemical modifications (most of them directly associated with nephrotoxic effects): increases in glucose, lactate, hydroxybutyrate, acetate and triacylglycerol were seen in recipients 24 h after transplantation. In addition, an increase in total glutathione in CsA-pre-treated group was indicative of increased oxidative stress. The everolimus-pre-treated group showed no significant changes (except for increased glutamine blood levels, Table 1). Finally, when combined with CsA, everolimus partly ameliorated CsA-induced disturbances in blood biochemical homeostasis. Glucose, TAG and hydroxybutyrate levels returned to normal. However, increased glutathione, glutamate and lactate levels were still present. There were no differences between groups neither for trimethylamine-N-oxide (TMAO, an abundant renal osmolyte and a marker of medullary injury) nor for allantoin.

**Discussion**

The current experimental rat study demonstrated that pre-treatment of kidneys in the donor before transplantation with either therapeutic CsA or everolimus drug concentrations or a combination of both does not protect the kidney from subsequent IRI during transplantation. Moreover, CsA pre-treatment led to increased circulating levels of glucose, lactate, hydroxybutyrate, acetate and glutathione (typical biochemical changes for CsA-induced nephrotoxicity) [20,25]. Although combination with everolimus partly ameliorated these negative toxic effects, total glutathione was still increased in the combination group despite higher CsA exposure.

Based on histology and creatinine, our results suggested that at therapeutic immunosuppressive doses, neither cyclo-
Sporine nor everolimus nor the combination of both appear to be protective during IRI in a rat kidney transplant model. After IR, none of the experimental treatments resulted in modulation of HSP70 or TNF-\(\alpha\). The role of HSP70 in renal IRI is documented. Up-regulation of HSP70 before injury is considered protective, and over-expression after injury often considered a marker of the severity of injury. Several reports have demonstrated that CsA can modulate HSP70 and thus confer protective effects. However, most studies examined warm ischaemia as opposed to transplantation ischaemia, which also includes cold ischaemia. Similarly, TNF-\(\alpha\) expression was not affected by any treatment. TNF-\(\alpha\) is released from the kidney in response to renal ischaemia–reperfusion, and has been implicated in the pathogenesis of renal ischaemia–reperfusion injury. TNF-\(\alpha\) is a potent pro-inflammatory cytokine and is an important mediator of inflammatory tissue damage.

Proton NMR spectroscopic analysis allows for the simultaneous detection, identification and quantification of hundreds of low-molecular-weight (maximum 20 kDa) metabolites within a biological matrix. Several publications have demonstrated the feasibility of NMR for establishing various end points for different clinical scenarios in transplantation. For example, allantoin is a marker for oxidative stress via the xanthine oxidase pathway [22,23]. Increased levels of glucose, lactate, total glutathione, ketone

**Fig. 2.** Necrosis scores and representative light microscopy images of donor-treated and recipient-treated animals. The severity of tubular damage was graded on a scale from 1 to 5. The different grades were defined as follows: 1 is <5% necrosis, 2 is 5–25% necrosis, 3 is 25–50% necrosis, 4 is 50–75% necrosis and 5 is >75% necrosis. \(n = 7, 5, 5, 5\) for milk-, cyclosporine-, everolimus- and CsA+everolimus-treated groups, respectively. There were no significant differences between groups.
bodies and TAG were reflective for CsA-induced renal dysfunctions, and TMAO was a kidney injury marker associated with both IRI and acute and chronic rejection [20]. Confirming our HSP70 and TNF-α results, we found no differences in biochemical markers for IRI among the groups: allantoin and TMAO levels remained statistically unchanged among the groups. Surprisingly, the only differences among study groups were related to increased blood markers of CsA-related nephrotoxicity. Even 24 h of donor pre-treatment led to a significant increase in glucose, lactate, ketone bodies, TAG and total glutathione. Everolimus alone had almost no effect on biochemical homeostasis and partly ameliorated CsA-induced nephrotoxicity in the combination group despite higher CsA concentrations.

In summary metabolic, we did not see any pre-conditioning effects or protection against IRI by CsA, everolimus or their combination. In our previous study with prolonged kidney ischaemia times (up to 42 h), blood as well as kidney allantoin levels were significantly increased 24 h after reperfusion [23]; this effect was partly amended by donor pre-treatment with N-acetylcysteine [22]. In this study, 24 h after cold storage followed by transplantation, allantoin blood levels were not different between treatment groups and control animals. In the presence of oxidative stress, highly reactive nitric oxide species are formed, including nitrotyrosine.

Nitrotyrosine is generated when superoxide anions react with nitric oxide to create peroxynitrate, which in turn modifies tyrosine residues in signaling proteins. Consist-

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**Fig. 3.** Normalized levels of HSP70 (A), TNF-α (B), nitrotyrosine (C) and cleaved caspase-3 (D) in the transplanted kidney 24 h after transplantation. The values are given for the following study groups: vehicle (n = 5), CsA (n = 6), everolimus (n = 5–6) and combination (CsA+everolimus) (n = 5–6) donor pre-treatment. There were no significant differences between groups.
ent with our allantoin findings, we were unable to identify differences between experimental groups. Our results are in contrast to findings recently published. Shihab et al. demonstrated that a single dose of calcineurin inhibitor treatment in the donor 24 h or 7 days before organ recovery improved renal function after 2 h of cold ischaemia followed by transplantation [27]. However, the cold ischaemia time in this study was significantly shorter (2 h vs. 24 h), and the CsA dose was twice as high (10 mg/kg vs. 5 mg/kg) and administered at different time points. Two hours of cold ischaemia is minimal and is mainly seen in living donation kidney transplantation. We chose 24 h of cold ischaemia since this better represents the clinical reality when using organs from deceased donors. These organs are likely to benefit the most from protective intervention. Furthermore, 10 mg/kg of CsA may transiently result in high drug levels that are likely to be in the toxic range. Thus, the ability to compare the results of these two studies is limited. Nevertheless, it does imply that further investigations in pharmacological pre-treatment are warranted. Specifically, the presence of metabolomic markers of CsA nephrotoxicities in the present study, however, suggests that the cause is much more complex. Thus, a different study using variable cold ischaemia time frames and drug dosages should be conducted in the future to explore further the effect of pre-conditioning. In summary, our data demonstrate that donor pre-treatment with CsA or everolimus does not reduce IRI during transplantation when organ donors are pre-treated twice within 24 h before organ recovery; in contrary, CsA-induced nephrotoxicity...
should be taken into account while conducting future studies even with short-term exposure to CsA.

**Acknowledgements.** We would like to thank Vivian Tan, MD, Assistant Professor of Pathology, Department of Pathology, University of California San Francisco, for reviewing the histologic specimens. This study was in part financially supported by a research grant from Novartis Pharmaceuticals Corporation, Basel, Switzerland.

**Conflict of interest statement.** None declared.

**References**


**Table 1. Selective metabolites and their blood concentrations as determined by NMR**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Control</th>
<th>CsA</th>
<th>Everolimus</th>
<th>CsA+Everolimus</th>
<th>ANOVA P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>0.07 ± 0.02</td>
<td>0.13 ± 0.04</td>
<td>0.11 ± 0.03</td>
<td>0.11 ± 0.05</td>
<td>0.01</td>
</tr>
<tr>
<td>CH3 Acetyl</td>
<td>1.0 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.3 ± 0.1(5)*</td>
<td>0.006</td>
</tr>
<tr>
<td>Choline</td>
<td>1.2 ± 0.2</td>
<td>1.4 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.2 ± 0.1(5)*</td>
<td>0.048</td>
</tr>
<tr>
<td>Glu Taur Arg</td>
<td>1.4 ± 0.3</td>
<td>2.3 ± 0.8</td>
<td>2.1 ± 0.7</td>
<td>2.0 ± 0.5(5)</td>
<td>0.03</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.4 ± 0.4</td>
<td>2.4 ± 0.6</td>
<td>1.5 ± 0.6</td>
<td>1.7 ± 0.2</td>
<td>0.009</td>
</tr>
<tr>
<td>Glutamate</td>
<td>0.33 ± 0.06</td>
<td>0.46 ± 0.06</td>
<td>0.38 ± 0.11</td>
<td>0.48 ± 0.08(5)*</td>
<td>0.0006</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.40 ± 0.08</td>
<td>0.50 ± 0.07</td>
<td>0.62 ± 0.09</td>
<td>0.52 ± 0.13(5)</td>
<td>0.004</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.6 ± 0.1</td>
<td>2.0 ± 0.2</td>
<td>1.6 ± 0.2</td>
<td>1.8 ± 0.1</td>
<td>0.0008</td>
</tr>
<tr>
<td>Lysine + Arg</td>
<td>0.29 ± 0.03</td>
<td>0.29 ± 0.02</td>
<td>0.31 ± 0.04</td>
<td>0.34 ± 0.03(5)*</td>
<td>0.04</td>
</tr>
<tr>
<td>OH Butyrate</td>
<td>0.21 ± 0.04</td>
<td>0.41 ± 0.10</td>
<td>0.20 ± 0.04</td>
<td>0.20 ± 0.09(5)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TAG</td>
<td>0.60 ± 0.15</td>
<td>1.04 ± 0.29</td>
<td>0.87 ± 0.37</td>
<td>0.85 ± 0.35(5)*</td>
<td>0.02</td>
</tr>
<tr>
<td>Total glutathione</td>
<td>0.40 ± 0.08</td>
<td>0.54 ± 0.04</td>
<td>0.45 ± 0.07</td>
<td>0.52 ± 0.06(5)*</td>
<td>0.004</td>
</tr>
<tr>
<td>Val Leu ile</td>
<td>0.94 ± 0.10</td>
<td>1.06 ± 0.15</td>
<td>1.05 ± 0.17</td>
<td>1.19 ± 0.16(5)*</td>
<td>0.04</td>
</tr>
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Data are mean ± SD.

*Significantly different from control (Dunnett’s test).