Journal of Food and Drug Analysis, Vol. 14, No. 2, 2006, Pages 166-173 藥物食品分析 第十四卷 第二期

Effects of *Citrus grandis* Peels on Cyclosporin Concentration and Immune Responses in Mice

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(Received: October 21, 2005; Accepted: January 18, 2006)

ABSTRACT

A previous study indicated that the coadministration of decoction of Citri Grandis Pericarpium (CGP), the peels of *Citrus grandis* (Rutaceae), significantly increased the blood level of cyclosporin (CsA) and resulted in acute intoxication of pigs. In this study, we aimed to measure the effects of CGP on the distribution of CsA in both lymphoid and non-lymphoid tissues as well as on the immune cell function in mice. Our results showed that coadministration of 3 g/kg CGP with CsA (Neoral®, 10 mg/kg) significantly decreased the concentrations of CsA in blood, liver, kidney and spleen by 37 %, 45 %, 46 % and 51 %, respectively, after a two week treatment. The decrease in CsA level correlated positively with the CGP dose. Pharmadynamically, coadministration of CGP restored the phagocytosis activity in blood decreased by CsA. Moreover, the nitric oxide production of activated macrophages and helper T cell type 1 (Th1) cytokines production suppressed by CsA were also reversed by the coadministration of CGP. In conclusion, coadministration of CGP significantly decreased the systemic exposure of CsA and resulted in higher macrophage and Th1 type activities than giving CsA alone in mice. Therefore, the combined use of CGP with CsA requires close monitoring clinically.

Key words: *Citrus grandis*, cyclosporin, immune response, Th1/Th2 cytokines

INTRODUCTION

Cyclosporin (CsA), a lipophilic cyclic peptide isolated from the fungus *Hypocladium inflatum* gams[(1)], is a potent immunosuppressant that is widely used for the treatment of autoimmune diseases and allograft rejection[(2,3)]. It is well known that CsA binds to cyclophilin to form an active complex which inhibits the enzyme calcineurin phosphatase[(4)]. Without the dephosphorylation by calcineurin, NFAT (nuclear factor of activated T cells) family members are unable to translocate into the nucleus to activate cytokine genes in T cells to result in the suppression of immune responses[(5,6)]. In addition, CsA has been recently found to block the p38 and JNK signaling pathways triggered by antigen recognition in T cells[(5)].

The therapeutic window of CsA is very narrow[(7)]. CsA and its metabolites have been reported to exert many side effects, such as nephrotoxicity, hepatotoxicity, neurotoxicity, and systemic hypertension[(8,9)]. Therefore, therapeutic drug monitoring of CsA is essential for clinical application. In addition, CsA is a substrate for both CYP3A4 and P-glycoprotein (P-gp), a drug efflux transporter. Numerous drugs such as diltiazem[(10)], ketoconazole[(11)] and nefazodone[(12)] have been reported to interact with CsA by altering the CYP3A4 metabolic pathway or affecting P-gp function in both liver and intestine mucosa[(12-14)].

In recent decades, many immunosuppressive drugs have been prescribed to prevent allograft rejection and their mechanisms were found to inhibit the expression of IL-2, to suppress the immune responses mediated by B lymphocytes, to exert anti-inflammatory effect, to induce the apoptosis of activated T lymphocytes as well as to block the p38 and JNK signaling pathways[(15)]. On the other hand, natural flavonoids were recently reported to exhibit a variety of beneficial pharmacological properties, including antioxidation, antiviral, and anti-inflammatory activities[(16)]. Therefore, people are encouraged to ingest flavonoid-rich diet. Flavonoids are the most abundant polyphenols present in human diet such as fruits, vegetables, tea and red wine. However, flavonoids were reported to modulate CYP 3A4 and P-gp[(17-19)]. Since CsA is a substrate for both CYP3A4 and P-gp, the health risk associated with high intake of flavonoid for those taking CsA routinely is an important clinical issue.

Previous studies indicated that grapefruit juice interacted with CsA pharmacokinetically, led to increased CsA blood levels[(20-22)] and resulted in serious clinical consequences. Citri Grandis Pericarpium (CGP), the peels of *Citrus grandis* (Rutaceae), is a Chinese herb containing flavonoid constituents such as naringin and naringenin like grapefruit[(23)]. We hypothesized that CsA was subject to clinically relevant interaction with CGP decoction as with grapefruit juice. An earlier study indicated that the coadministration of decoction of Citri Grandis Pericarpium (CGP), the peels of *Citrus grandis* (Rutaceae), significantly increased the blood level of cyclosporin (CsA)

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and resulted in acute intoxication of pigs[24]. Furthermore, because CGP contains antioxidant flavonoids which may exert pharmacological activities on immune systems, the present study aimed to investigate the pharmacokinetic and pharmacodynamic interactions of CGP with CsA through measuring CsA tissue distribution and related immune responses in mice.

MATERIALS AND METHODS

I. *Mice*

Balb/c mice were purchased from National Laboratory Animal Center (Taipei, Taiwan) and maintained in the Animal Center of China Medical University. The animal room was at a 12-hr light and dark cycle with constant temperature and humidity. All mice used were 8 weeks old. All procedures were performed according to the Guide for the Care and Use of Laboratory Animals (NRC, USA).

II. *Drugs*

Cyclosporin (Neoral®, 100 mg/mL) was provided by Novartis (Taiwan) Co. Ltd. Lipopolysaccharide (LPS), Concanavalin A (ConA) and Phosphate buffered saline (PBS) were purchased from Sigma Chemical (St. Louis, MO, USA). Recombinant interferon-γ (IFN-γ) was purchased from PeproTech (Margravine, London, England). The spleen cells and peritoneal excluded macrophages were maintained in the RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 1% penicillin, 1% streptomycin, and 200 mM L-glutamine (Gibco BRL, Grand Island, NY, USA).

III. *CGP Decoction Preparation and Quantitation of Flavonoids*

The crude drug of CGP was purchased from an herbal store in Taichung. Four liters of water were added to 200 g of sample and heating was carried out on a gas stove. After boiling, gentle heating was continued for about 2 hr until the volume reduced to less than half of the original volume. The mixture was filtered while hot and sufficient hot water was added to make 2 L, which was frozen at -20°C for later use. For the quantitation of flavonoids, CGP decoction (300 μL) was added with 700 μL of methanol. After centrifugation, the supernatant was added with equal volume of methanol containing 6,7-dimethoxycourmarin as the internal standard and then subjected to HPLC analysis. The HPLC apparatus included two pumps (LC-10AL, Shimadzu, Japan) and an UV spectrophotometric detector (SPD-10A, Shimadzu, Japan). The RP-18 column (LiChrospher®, 4.0 × 250 mm) was equipped with a prefilter. The mobile phase was acetonitrile and 0.1% *ortho*-phosphoric acid (22:78) and the flow rate was 1.0 mL·min^{-1} with the detection wavelength set at 288 nm. Quantitation results showed that the dissolved amounts of naringin and naringenin into the decoction from CGP crude drug were 1.7 ± 0.1 mg·g^{-1} and 20.5 ± 0.3 μg·g^{-1}, respectively.

IV. *Animals and Drug Administration*

Mice were randomly divided into A~H groups. Each group included eight mice. Group A was the control and was given equivalent amount of water. Groups B~E were given a dose of 10 mg/kg cyclosporin (Neoral®, diluted with olive oil) with CGP at daily doses of 0, 1, 2 and 3 g/kg, respectively. CGP was given right before CsA. The drugs were administered via gastric gavage. Groups F~H were given CGP alone at 1, 2 and 3 g/kg, respectively.

V. *Determination of Cyclosporin Concentration in Blood, Liver, Kidney, and Spleen*

After dosing different combinations of CsA and CGP to the mice for two weeks, the concentrations of CsA in blood and various tissues were measured[25]. Mice were anesthetized with intramuscular injection of ketamine (20 mg/kg) and then blood sample (100 μL) was withdrawn from the retro-orbital venous plexus. Spleen cells (1×10^7) and 100 mg of liver or kidney were individually homogenized in 100 μL of PBS. The CsA concentration was measured by employing a specific monoclonal fluorescence polarization immunoassay[26] using a CsA kit (Abbott, Abbott Park, IL, USA). The assay was calibrated for concentrations ranging from 25.0 to 1500.0 ng/mL. The LLOQ (lower limit of quantitation) of this assay is 25.0 ng/mL. The accuracy of this method was further assessed with recovery studies by spiking CsA into homogenates of spleen, liver, kidney and water in triplicates to afford 100.0, 200.0 and 500.0 ng/mL. Afterwards, the concentrations obtained in various tissues with the corresponding ones in water were compared.

VI. *Quantification of Phagocyte Activity*

Blood sample was withdrawn from the retro-orbital venous plexus after dosing CsA and CGP to mice for two weeks. The quantitative analysis of phagocyte activity was performed using a Phagotest® kit (ORPEGEN Pharma, Heidelberg, FRG) following the manufacturer's instruction[27]. Briefly, heparinized whole blood (100 μL) was incubated with the fluorescein isothiocyanate (FITC)-labelled *E. coli* (50 μL) at 37°C and a negative control sample remained on ice. The phagocytosis is stopped by placing the sample on ice and adding quenching solution to quench the FITC fluorescence of surface bound bacteria, leaving the fluorescence of internalized particles unaltered. After washing, the percentages of phagocytes that had ingested bacteria and the activities (the number of bacteria per cell) were monitored using a FACscan flow cytometer (Becton Dickinson). Data analysis was performed using

the Cell Quest Software (Becton Dickinson, San Diego, CA) and calculated by (the percentage of phagocytes which have ingested FITC-labeled *E. coli* at 37°C) minus (the percentage of phagocytes which have ingested FITC-labeled *E. coli* bacteria on ice).

VII. *Determination of Cell Populations in Splenocytes*

Surface staining was performed as described above[28]. Spleen cells (1×10^6) were incubated with fluorescein isothiocyanate [FITC]-conjugated anti-CD3 (Leinco Technologies, Inc., St. Louis, MO, USA), and phycoerythrin [PE]-conjugated anti-CD4, or [FITC]-anti-CD3 and [PE]-anti-CD8, or [FITC]-anti-CD3 and [PE]-anti-CD19 (PharMingen, San Diego, CA, USA), respectively, at 4°C for 30 min. The cells were washed twice, suspended in 0.5 mL of PBS and subjected to FACScan analysis. A total of 10,000 cells were counted and the frequency of each cell surface marker was determined using appropriate software. The flow cytometry was regularly calibrated with CaliBRITE beads (Becton Dickinson, Mountain View, CA, USA).

VIII. *Determination of Nitric Oxide*

Peritoneal excluded macrophages were obtained from mice[29] and incubated in RPMI 1640 medium in the presence and absence of LPS (2 μg/mL) plus IFN-γ (10 U/mL), respectively. NO was determined by measuring the accumulation of nitrite, a stable end product, in the culture supernatant according to the Griess reaction[30]. Equal volumes of culture supernatant or serum were mixed with Griess reagent and left over for 10 min at room temperature. The optical density (OD) was measured with a microplate reader (BIO-RAD, model 3550, USA) at 540 nm and the nitrite concentration in medium was calculated using sodium nitrite as a standard.

IX. *Cytokine Assay*

Spleen cells (5×10^6) were incubated with and without 5 μg/mL ConA in 24-well plates for 48 hr. The culture supernatants were collected and stored at -80°C prior to the analysis of enzyme-linked immunosorbent assay (ELISA) (PharMingen, San Diego, CA, USA) as described earlier[28]. Briefly, 96-well plates were coated with monoclonal antibody with specificity for IL-2, IFN-γ, IL-4, or IL-10 and incubated overnight at 4°C, washed with 0.05% Tween 20 in PBS and blocked by RPMI 1640 supplement with 10% FCS for 1 hr at room temperature (RT). Serially diluted culture supernatants and standards prepared from recombinant mouse IL-2, IFN-γ, IL-4, and IL-10 separately (PharMingen, San Diego, CA, USA) were added for 2 hr at RT. The wells of the plates were washed and biotin-conjugated rat anti-mouse IL-2, IFN-γ, IL-4, or IL-10 was added for another 1 hr at RT. After proper washing, avidin-horseradish peroxidase was added and incubated for 1 hr at RT. After aspirating and washing, substrate (tetramethylbenzidine and hydrogen peroxide) was added for 30 min at RT in the dark. The optical density (OD) was measured with a microplate reader (BIO-RAD, model 3550, USA) at 450 nm. The detection sensitivities of IL-2, IFN-γ, IL-4, and IL-10 were 3.13 pg/mL, 31.3 pg/mL, 7.8 pg/mL, and 31.3 pg/mL, respectively.

X. *Statistical Analysis*

All data are expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Dunnetts *post-hoc* test, and the significant difference was set at $*p < 0.05$; $**p < 0.01$.

RESULTS

I. *Effect of CGP Coadministration on the Tissue Distribution of CsA*

In order to investigate the *in vivo* interaction of CGP and CsA, we used the optimal dose of CsA 10 mg/kg as described in our previous study[31]. The recoveries of CsA from liver, kidney and spleen were 104.8 ± 3.21, 90.7 ± 2.16 and 103.7 ± 1.28% for the concentrations of 100.0, 200.0 and 500.0 ng/mL, respectively. After giving CsA with different doses of CGP to mice, the concentrations of CsA in blood, liver, kidney and spleen indicated that CGP dose dependently decreased the levels of CsA in blood, liver, kidney and spleen (Figure 1, Groups B~E). At the dose of 3 g/kg CGP (Group E), the most profound reduction of CsA concentration was found in spleen (51%), and the least reduction in blood (37%), and the moderate decrease in liver and kidney (about 45%).

II. *Effect of CGP Coadministration on the Phagocytic Activity in Blood*

To further measure that the effect of CGP coadministration on the phagocytic activity, blood samples were obtained from rats in Groups A~H and analyzed by a Phagotest® kit. The phagocytic activities in the peripheral blood were found comparable among groups of mice before experiment. After two weeks of treatment, Group B receiving CsA alone showed significantly lower phagocyte activities in the peripheral blood than Group A, a control group (Figure 2), whereas Groups C~E coadministered CGP with CsA showed significantly higher phagocytic activity than Group B. In addition, Groups F~H receiving CGP only showed comparable phagocytic activity as Group A.

III. *Effect of CGP Coadministration on the Function of Peritoneal Macrophages*

The function of peritoneal macrophages by monitoring the secreted levels of nitric oxide (NO) under

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Table 1. The percentage of immune cells in spleen after various treatment

Group		CD3	CD4	CD8	CD19
A	Negative control	53.28 ± 1.15	34.13 ± 2.25	18.22 ± 1.25	43.33 ± 2.35
B	CsA alone	47.24 ± 2.63#	30.39 ± 1.35#	14.57 ± 1.56#	49.16 ± 3.53#
C	CsA + 1 g/kg CGP	52.32 ± 1.36*	36.61 ± 1.05*	15.61 ± 1.17	44.83 ± 3.36*
D	CsA + 2 g/kg CGP	52.18 ± 2.73*	36.97 ± 2.22*	16.76 ± 2.42	45.96 ± 2.78*
E	CsA + 3 g/kg CGP	52.51 ± 2.25*	36.74 ± 2.36*	16.27 ± 2.24	45.16 ± 3.05*
F	1 g/kg CGP alone	54.36 ± 3.62	36.84 ± 2.46	18.32 ± 1.66	43.78 ± 3.27
G	2 g/kg CGP alone	54.78 ± 3.44	36.07 ± 2.27	18.82 ± 2.62	41.96 ± 3.56
H	3 g/kg CGP alone	54.62 ± 3.64	36.36 ± 2.47	18.86 ± 2.24	40.16 ± 2.85

represents $p < 0.05$ compared to group A.
* represents $p < 0.05$ compared to group B.

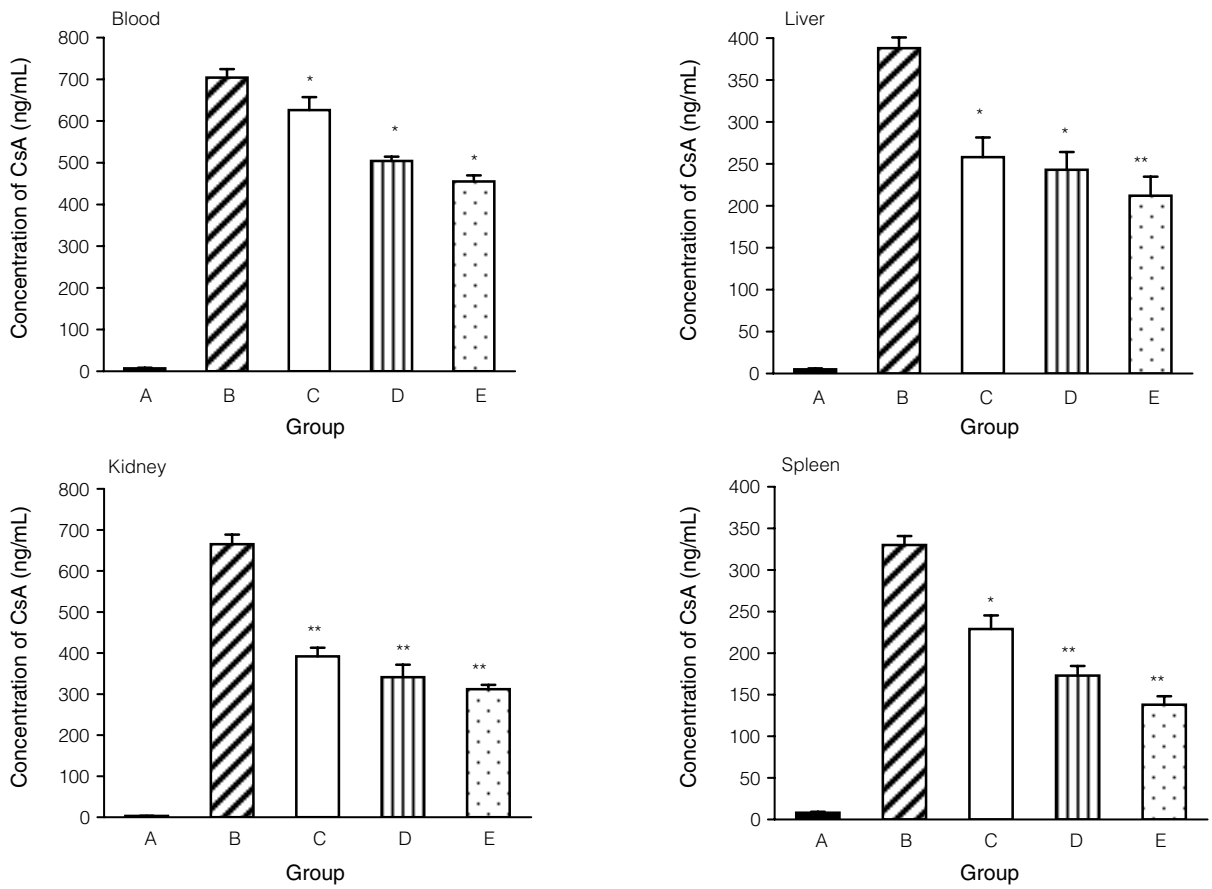

Figure 1. The concentrations of CsA in blood and various tissues. One hundred micro liter of blood, 1×10^7 spleen cells, 100 mg of liver or kidney were homogenized in 100 μL of PBS after dosing different combinations of CsA and CGP to mice for two weeks. The CsA concentration was measured by using a CsA kit (Abbott, Abbott Park, IL, USA) and expressed as ng/mL. Statistically compared with Group B: *$p < 0.05$; **$p < 0.01$.

LPS/IFN-γ stimulation demonstrated that treatment with CsA alone (Figure 3, Group B) significantly decreased NO production. However, coadministration of various doses of CGP with CsA significantly restored the decreased NO production in a dose-dependent manner (Figure 3, Groups C~E). In addition, treatments with various doses of CGP alone (Groups F~H) showed no significant effects compared to Group A.

IV. *Effect of CGP Coadministration on the Population and Function of Immune Cells in Spleen*

The populations of total T cells ($CD3^+$ cells), including $CD4^+$ helper T cells, $CD8^+$ cytotoxic T cells, and $CD19^+$ B cells in spleen are shown in Table 1, indicating that CsA significantly decreased the percentage of total T cells in spleen ($p < 0.05$), especially $CD8^+$ T cells to the greatest extent, with a concomitant increase of $CD19^+$ B

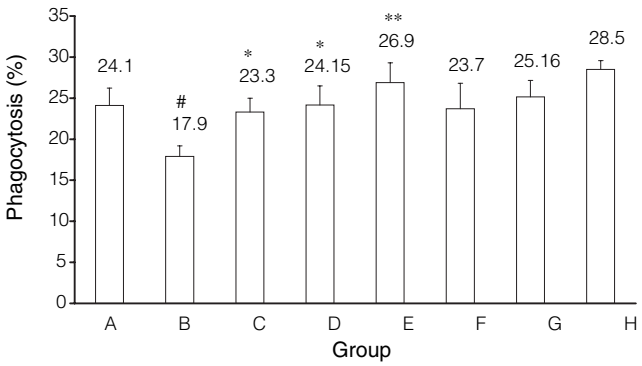

Figure 2. Effect of different combinations of CsA and CGP on phagocyte activity of macrophages *in vivo*. The percentage of phagocytes which have ingested bacteria was monitored by using a FACscan flow cytometer (Becton Dickinson), and calculated by the percentage of phagocytes which have ingested FITC-labeled *E. coli* bacteria at 37°C minus the percentage of phagocytes which have ingested FITC-labeled *E. coli* bacteria on ice. The data were expressed as mean ± SD. Each group contains 8 mice. Statistically compared with Group B: *$p < 0.05$; **$p < 0.01$ and compared with Group A: #$p < 0.05$.

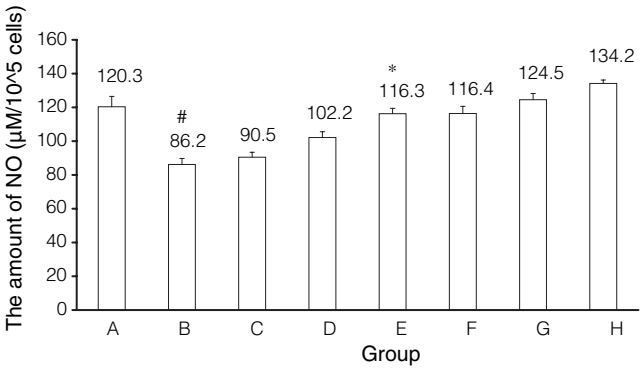

Figure 3. The level of nitric oxide produced by peritoneal macrophages. Murine peritoneal macrophages were added with LPS (2 μg/mL) plus IFN-γ (10 U/mL) and supernatants were collected 48 hr after the initiation of the cultures to determine the amount of NO by the method of Griess. Groups F~H were given CGP alone at 1, 2 and 3 g/kg, respectively. The data were expressed as the mean ± SD. Statistically compared with Group B: *$p < 0.05$ and compared with Group A: #$p < 0.05$.

cells ($p < 0.05$). Coadministration of CGP (Groups C~E) significantly restored the percentage of $CD4^+$ T cells ($p < 0.05$). In contrast, administration of CGP alone resulted in no significant change on the percentages of total T cells and B cells (Table 1, Groups F~H). Further comparison of cytokine levels secreted by ConA-stimulated spleen cells indicated that T helper type 1 (Th1) cytokines, IL-2 and IFN-γ were markedly decreased after treatment with CsA alone. However, coadministration with various doses of CGP resulted in significant increase of Th1 cytokines (Figure 4, Groups C~E compared to Group B, $p < 0.05$). In addition, no significant difference was observed between Group A and each group receiving CGP alone (Groups F~H), indicating CGP treatment alone exerted no effect on Th1 response. The results indicated that coadministration with CGP reversed the effect of CsA on Th1 cytokine production. However, no significant difference for Th2 type cytokines, IL-4 and IL-10 was found among eight groups.

DISCUSSION

This first report of the effect of CGP coadministration on the concentrations of CsA in blood, kidney, liver and spleen indicated that CGP significantly decreased the *in vivo* concentrations of CsA in mice. However, a previous pig study reported that the blood CsA level after coadministration of single dose of CGP decoction was higher than that after giving single dose of CsA alone[24]. This discrepancy might be due to species difference between pigs and mice or different dosage regimen. Nevertheless, the influences in both species were significant.

Previous studies have reported that, both in human and mice, CsA and calcineurin inhibition peaked at 1 hr after dosing and had higher concentration in spleen and kidney than in blood[25]. The mechanisms of CsA toxicity had been reviewed[32] and include elements such as vasoconstriction and interstitial fibrosis. The high concentration of CsA in kidney and liver might probably account for the serious nephrotoxic and hepatotoxic side effects. Our results showed that CsA levels in blood, spleen, liver and kidney were decreased by the coadministration of CGP dose-dependently (Figure 1). This indicated that CGP significantly decreased the bioavailability of CsA, implying that CGP might decrease the efficacy of CsA.

Traditional therapeutic monitoring is based on the blood level of CsA. In the case of sirolimus, a novel immunosuppressive drug, previous studies had shown that the coadministration of sirolimus with CsA resulted in an increase of CsA levels by 2-3 folds[33,34] and increased the inhibition of lymphocyte function[35]. In addition, many studies indicated that St. John's wort interacted with CsA pharmacokinetically, leading significant decrease of CsA exposure and resulted in acute rejection of transplant organs[20-22,36]. From pharmacokinetic point of view, the CGP-CsA interaction might result in graft rejection as St. John's wort.-CsA interaction for organ transplant patients.

Previous report indicated that CsA reduced the levels of NO and cyclooxygenase-2 produced by LPS-activated RAW264.7 cells[37]. Our results showed that coadministration of CGP significantly restored the NO production suppressed by CsA. In addition to the reduced activity of activated macrophages, we demonstrated that CsA also decreased the percentage of $CD4^+$ and $CD8^+$ T cells with a concomitant increase of $CD19^+$ B cells (Table 1). Meanwhile, CsA was known to cause significant decrease of Th1 type cytokines, IL-2 and IL-12, as well as to inhibit IFN-γ from dendritic cells (DCs)[38]. Moreover,

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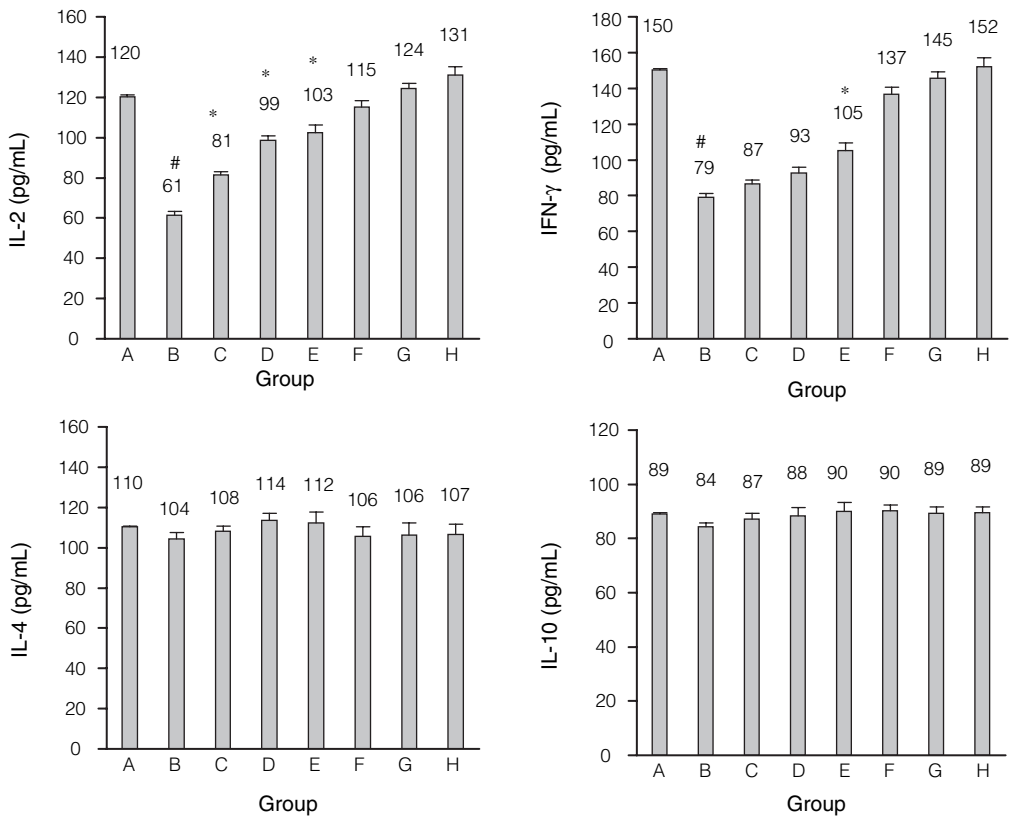

Figure 4. The levels of cytokines secreted from ConA-stimulated spleen cells. Spleen cells (5×10^6) were incubated with and without 5 μg/mL ConA in 24-well plates for 48 hr. The culture supernatants were collected and analyzed using a sandwich-ELISA as described in Methods. The data were expressed as mean ± SD. Statistically compared with Group B: *$p < 0.05$ and compared with Group A: # $p < 0.05$.

CsA reduced co-stimulatory molecule expression and further altered the antigen presenting function of DCs for T cell activation[39]. In our study, IL-2 and IFN-γ were markedly inhibited by CsA. However, when 3 g/kg CGP was coadministered with CsA, the production of Th1 type cytokines was significantly enhanced in ConA-stimulated spleen cells. These pharmacodynamic effects should be resulted from the CGP-CsA pharmacokinetic interaction which showed a marked decrease of *in vivo* CsA concentration.

Previous studies suggested that grapefruit juice inhibited the intestine CYP3A4, but naringin, narirutin and naringenin were not the major agents responsible for the grapefruit juice-CsA interactions[40,41]. A recent report indicated that inhibition of P-gp was proposed to be a more important mechanism for enhanced CsA absorption than for inhibition of CYP 3A4[42]. Whether CGP elevated CYP 3A4 or/and P-gp activity or increased the elimination of CsA to result in significant decrease of CsA exposure in mice awaits further studies and the causative agents in CGP decoction remain to be clarified.

In conclusion, coadministration of CGP significantly decreased the systemic exposure of CsA and resulted in higher macrophage and Th1 type activities than giving CsA alone in mice. The pharmacodynamic effect of CsA was diminished by the pharmacokinetic interaction caused by coadministration of CGP. Therefore, the combined use of CGP with CsA requires close monitoring clinically.

ACKNOWLEDGEMENTS

This study was supported by NSC 93-2320-B-039-048 granted by the National Science Council, ROC, and CMU 94-104 by the China Medical University, ROC.

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