The synergistic effects of C. Sinensis with CsA in preventing allograft rejection

Chenguang Ding[1, 2], Puxun Tian[1], Lining Jia[1, 2], Yang Li[1], Xiaoming Ding[1], Heli Xiang[1], Wujun Xue[1], Yong Zhao[2]

[1]*Department of Renal Transplantation, the First Affiliated Hospital, Medical college of Xi'an Jiaotong University, Xi'an 710061, China, [2]Transplantation Biology Research Division, State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Beijing, 100101, China*

TABLE OF CONTENTS

1. ABSTRACT

The severe side-effects of Cyclosporin A (CsA) limited its long term clinical application in allograft recipients. In the present study, we investigated the potential synergistic effects of cordyceps sinensis (Cs) with CsA in an allograft kidney transplant rat model. Cs alone or a low dose of CsA treatment did not prolong graft survival. However, the combined therapy of a low dose of CsA with Cs significantly prolonged graft survival in rats. The allografts showed significantly improved function in a combined therapy in rats as determined by urine volume and serum creatinine levels. Furthermore, significantly less mononuclear cell infiltration in kidney grafts, lower levels of CD4+ T cells in peripheral blood, and less serum IL-2 and IFN-γ production was observed in recipients treated with combined therapy, as compared with recipients treated with Cs alone or a low dose of CsA. These data indicate that Cs and CsA have synergistic effects to block allogeneic graft rejection, which may be applied in transplant recipients to decrease the dose of CsA and avoid CsA associated side-effects.

2. INTRODUCTION

In the past decades, with the development of efficient immunosuppressive drugs, tissue and organ preservation, tissue typing and matching and surgical techniques, 1-year kidney allograft survival rates have exceeded 90 %(1-3). Common therapies to avoid acute rejection usually employ strong immunosuppression such as combinations of several agents, including corticosteroids, antilymphocyte antibodies, azathioprine or mycophenolate mofetil (MMF), and calcineurin inhibitors (CNI) (4, 5). CNI, such as cyclosporin A (CsA) or tacrolimus (FK506) are usually indispensable for the suppression T cell responses in allograft recipients. However, their severe toxicities greatly limit their use in clinical immunosuppressive therapy (4-6). In order to reduce the dose and side effects of CNI, numerous studies of combined therapy of other drugs with CNI have been carried out in both experimental models and clinical recipients (4-7). Antibody therapy with antithymocyte globulin (ATG) or anti-CD3 antibody (OKT3) has been employed for induction therapy or for rescuing acute

rejection. However, these antibodies deplete lymphocytes in a non-specific manner, resulting in side effects such as infections and malignancies, which may diminish its overall benefits for long-term graft survival.

Cordyceps sinensis (Cs), one of the popular traditional Chinese medicine, has been widely used for the treatment of multiple diseases in China (8). Recent studies have identified multiple pharmacological actions of Cs (9-11). The experimental findings on the effects of Cs in immune system are rather controversial (12-14). It is reported that Cs monotherapy prolonged transplant survival though the effect was limited (14). Cs in combined with other drugs was also able to ablate allograft vasculopathy (15). However, the influences of Cs on kidney transplantation were unclear so far. In the present study, the effects of Cs on graft survival and immune responses in recipients with allogenetic kidney grafts were investigated in which Cs was used as an adjuvant therapy with CsA.

3. MATERIALS AND METHODS

3.1. Animals

Male Lewis (weight 230–250g) and Wistar rats (weight 200–230g) were purchased from Vitalriver Inc. (Beijing, China). Lewis rats served as recipients of kidney allografts from Wistar rats. All animals were housed in plastic cages with controlled light/dark cycles and provided with food and water ad libitum. All experiments were performed according to approved animal care protocols.

3.2. Surgical Technique

Orthotopic kidney transplantation was performed with end-to-end anastomosis of the left renal vessels and ureter by use of a 10-0 Ethilon suture using the modified microsurgical technique published by Hölzen JP (16). Grafts were preserved transiently (5 min) in 4°C saline before transplantation. The mean ischemic time was 25 mins. Right sided native nephrectomy was performed following engraftment.

3.3. Experimental groups and drug administration

Rat kidney allograft recipients were randomly divided into 4 groups and orally administrated Cs or CsA according different protocols. Group 1: Cs (50mg/kg/day) (Huadong Medicine Group Co., Ltd**,** Hangzhou, China) alone; Group 2: a low dose CsA(2mg/kg/day) (Novartis, China) alone; Group 3: a high dose CsA alone(5mg/kg/day); Group 4: a combined therapy of Cs (50mg/kg/day) with a low dose CsA (2mg/kg/day). Every recipient was administered daily from the day of transplantation to day 37 post transplantation or death.

3.4. Assessment of graft survival

Graft loss was defined as death of recipients duo to acute rejection, which was confirmed histologically by autopsy. After the transplantation, animals having no urination or no temporal decrease in the serum creatinine and no histological findings of acute rejection in autopsy were considered to have operational technical failure and excluded from this study.

3.5. Allograft function and histological analysis

Serum creatinine was monitored after transplantation. Animals were housed in metabolic cages. Peripheral blood was collected from vena caudalis and analyzed for serum creatinine (enzymatic assay, Creatinine-Pap, Roche Diagnostics, Mannheim, Germany). The urine volume in 24 h was also recorded.

Graft tissues were fixed in 10% formalin and embedded in paraffin. 5 μm sections were cut and stained with Harris' Haematoxylin and 0.5% Eosin (H & E) and assessed for histological changes.

3.6. Cytokine detection by enzyme-linked immunosorbent assay (ELISA)

IL-2, IL-10 and IFN-γ in the recipient serum were quantified by commercially available ELISA kits (R&D Systems, Minneapolis MN, USA) according to the manufacturer's instructions.

3.7. Flow cytometry (FCM) analysis of the peripheral blood lymphocytes (PBLC)

PBLCs were incubated with optimal concentrations of phycoerythrin (PE)-conjugated anti-rat-CD4 (OX-35; IgG2a) and fluorescein isothiocyanate (FITC)-conjugated anti-rat-CD8 (OX-8; IgG1) antibodies (both from BD Biosciences PharMingen San Diego, CA, USA) for 30 min at 4 °C in the dark. Cells were washed three times and resuspended with FCM buffer (PBS with 0.1% BSA and 0.1% NaN$_3$). At least ten thousand cells were counted by a FASCalibur flow cytometer (Becton Dickinson, CA), and data were analyzed with CellQuest software. Propidium iodide (PI) staining was used to gate out non-viable cells. The percentage of positive cells was determined by subtracting the percentage of cells stained nonspecifically with the negative control mAbs from that of cells stained with specific anti-rat mAbs.

3.8. Statistics

Statistical analysis was performed using SPSS11.0. Data are presented as mean ± standard deviation. Graft survival was evaluated by the Kaplan-Meier test. Student's unpaired t test was used to analyze the differences of mean graft survivals between groups. A level of significance of P < 0.05 was considered as sufficient in all experimental groups.

4. RESULTS

4.1. Cs, in combination with low dose CsA, significantly prolonged the recipients' survival and protected allograft function

First of all, the effect of Cs on the graft survival was determined in a rat allo-kidney transplant model. As shown in Figure 1, Cs alone or a low dose of CsA showed little potential to block acute kidney rejection, resulting in a mean graft survival of no more than 9 days. In contrast, the combination of Cs with a low dose of CsA significantly delayed the occurrence of allo-kidney graft rejection as efficiently as a high dose of CsA alone did. At 37 day after grafting, 60% kidney-grafted mice treated with Cs and a low dose of CsA survived, while rats treated with a high dose of CsA showed only 30% survival.
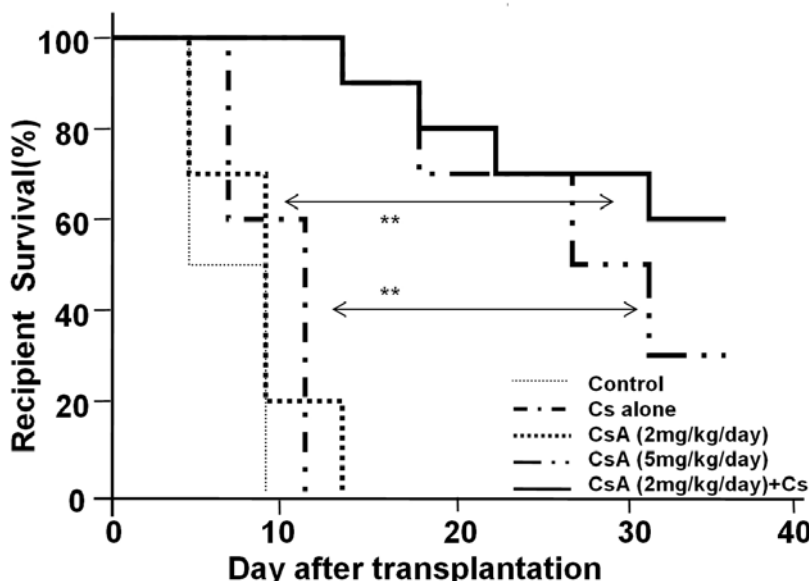
Figure 1. Survival time of recipient rats with allogeneic kidney grafts. Rats were treated with Cs and/or different doses of CsA as mentioned in materials and methods. K-M survival curve was done. Ten rats in each group were performed. ** P<0.01 compared among the indicated groups.

Meanwhile, allo-kidney graft function was assessed by urine volume and serum creatinine levels. The urine volume of kidney-grafted rats treated with Cs alone or with a low dose of CsA was decreased significantly on day 5 compared with that on day 1 (p < 0.01, Figure 2A). All allo-kidney recipients treated with a combined therapy of Cs with a low dose of CsA maintained a normal urine volume without hematuria for more than 37 days, while those treated with a high dose of CsA maintained a normal urine volume only for 31 days. The serum creatinine levels were inversely correlated well with the urine volume (Figure 2A and 2B). The serum creatinine in rats treated with Cs alone or a low dose of CsA began to rise by day 5 and increased progressively thereafter until death. In contrast, serum creatinine in recipients treated by combined therapy of Cs with a low dose of CsA remained normal until day 37 after grafting, similarly as rats treated with a high dose of CsA alone.

4.2. A combined therapy of Cs with CsA significantly prevented kidney allograft rejection

For elucidating the effect of Cs on allograft rejection, the histological changes of allo-kidney grafts were determined by H-E staining. At day 5 post transplantation, allo-kidney grafts in rat recipients treated with Cs alone or a low dose of CsA showed massive infiltration of interstitial mononuclear cells, widespread tubulitis and patchy necrosis accompanied with hemorrhage, severe glomerulitis with extensive capillary occlusion caused by endothelial swelling, and intimal arteritis in the cortex (Figure 3A and B). In contrast, allo-kidney grafts in rat recipients treated with a high dose of CsA or a combination of Cs with a low dose of CsA displayed a nearly normal glomerular and tubule structure (Figure 3C and D). At day 37 post transplantation, allo-kidney grafts in rat recipients treated with a high dose of

CsA displayed thickening of arterial intima, interstitial fibrosis, and tubular atrophy along with the deterioration of graft function (Figure 3E). Impressively, allo-kidney grafts in rat recipients treated with Cs and a low dose of CsA showed significantly decreased infiltration of mononuclear cells in the tubular interstitium, reduced tubular atrophy and delayed interstitial fibrosis (Figure 3F).

4.3. The significantly decreased T cell proportion and the ratio of CD4+/CD8+ T cells in peripheral blood of allo-kidney grafted rats treated with Cs and CsA

Proportions of T cell subsets in the periphery blood were assayed by flow cytometry. As shown in Figure 4, allo-kidney grafted rats treated with a combined therapy of Cs with a low dose of CsA or with a high dose of CsA showed significantly decreased percentages of CD4+ and CD8+ cells as compared with allo-kidney grafted rats treated with Cs or a low dose of CsA alone at day 5 after grafting (P<0.05 or P<0.01, Figure 4A and B). The ratio of peripheral CD4+/CD8+ T cells in rats treated with a combined therapy of Cs with a low dose of CsA was significantly lower than other groups at day 5 after grafting, though it recovered to normal level by day 37 after grafting (Figure 4C).

4.4. The significantly decreased levels of Th1 cytokines in sera of allo-kidney grafted rats treated with Cs and a low dose of CsA

The immune responses were also determined by the cytokine production in sera of grafted rats. At day 5 post transplantation, two Th1 cytokines, IL-2 and IFN-γ, in sera of rats treated with either a high dose of CsA or a combination of Cs with a low dose of CsA were significantly lower than those in rats treated with Cs alone or a low dose of CsA. (p<0.05, or p<0.01, Figure 5 A and B). In contrast, serum IL-10, a Th2 cytokine, did not show
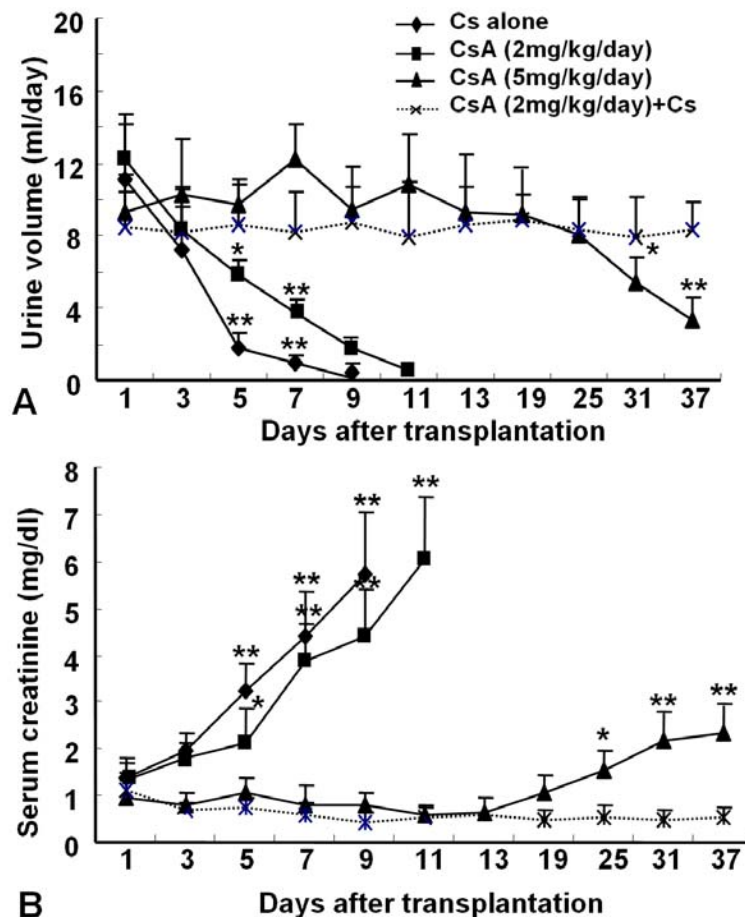
Figure 2. Allokidney graft function in recipient rats treated with different protocols. Urinary volume (A) and serum creatinine (B) were detected as described in materials and methods. Ten rats in each group were performed. *P<0.05, **P<0.01 compared with day 1. Data are presented as Mean $\pm$ SD.

significant difference among these groups (P>0.05, Figure 5C).

At day 37 post transplantation, serum IL-2 and IFN-γ were identical in allo-kidney grafted rats treated with either a high dose of CsA or a combination of Cs with a low dose of CsA (P>0.05). However, serum IL-10 level in allo-kidney grafted rats treated with a combination of Cs with a low dose of CsA was significantly higher than that of rats treated with a high dose of CsA (P<0.05).

5. DISCUSSION

Cs was demonstrated to have mild immunosuppressive effects when used as a monotherapy in different transplant animal models and clinical organ transplantation (14-15, 17-19). In the present study, we were particularly interested in its effect on allograft rejection when used as an adjuvant therapy with CsA. Therefore, we investigated the effects of Cs alone, or Cs in combination with a low dose of CsA, or a high dose of CsA on MHC incompatible renal allograft models in inbred rats. The poor immunosuppressive efficacy of Cs monotherapy in this model is not unexpected, which is identical to the data reported by Jordan (15). A combination of Cs with a low dose of CsA, however, significantly prolonged allo-kidney graft survival, suggesting synergistic effects of these agents in clinical transplant patients. The usage of Cs could significantly decrease the doses of CsA treatment so that it may potentially decrease the side effects caused by high doses of CsA.

The treatment of Cs combined with a low dose of CsA significantly decreased the percentages of CD4+ T cells and CD8+ T cells, as well as the CD4+/CD8+ ratio in peripheral blood as compared with Cs alone or a low doses of CsA in a kidney transplant model. The decreased T cell proportions in Cs and CsA-treated rats may be caused by inhibition on T cell proliferation and/or cell death. The significantly reduced ratio of CD4+/CD8+ cells in allo-kidney grafted rats treated with a combined therapy of Cs with a low dose of CsA indicates that this combination treatment down regulated more CD4+T cells than CD8+T cells in this model.
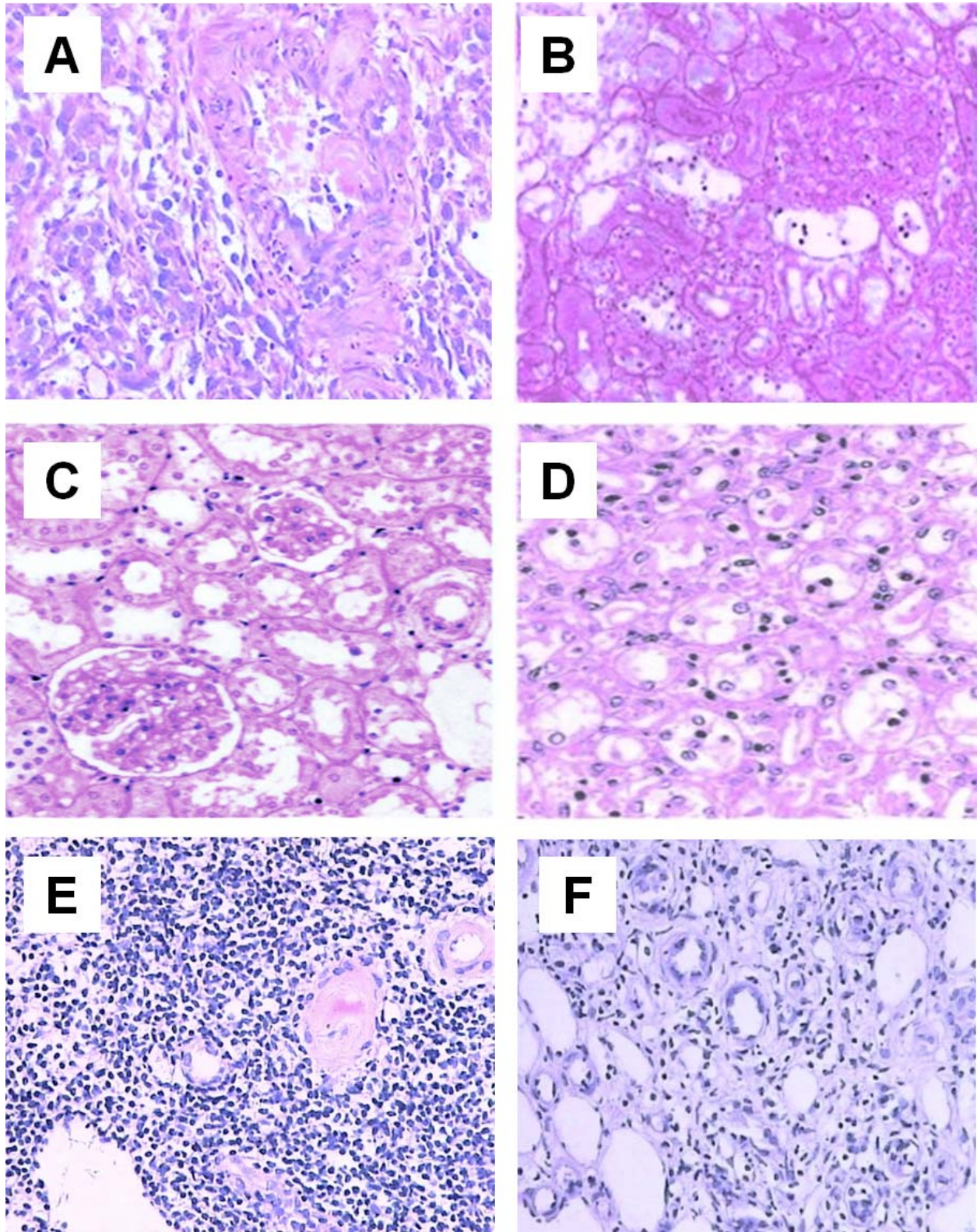
Figure 3. Cs as adjuvant therapy significantly prevented kidney allograft rejection. At day 5 (A, B, C, D) and day 37 (E, F) after transplantation, H&E staining of 5 μm sections of allo-kidney grafts were performed as described in materials and methods. (A) Cs alone(50 mg/kg); (B) A low dose of CsA (2 mg/kg/d); (C, E) A high dose of CsA (5 mg/kg); (D, F) A combination of Cs with a low dose of CsA; Five rats in each group were performed.
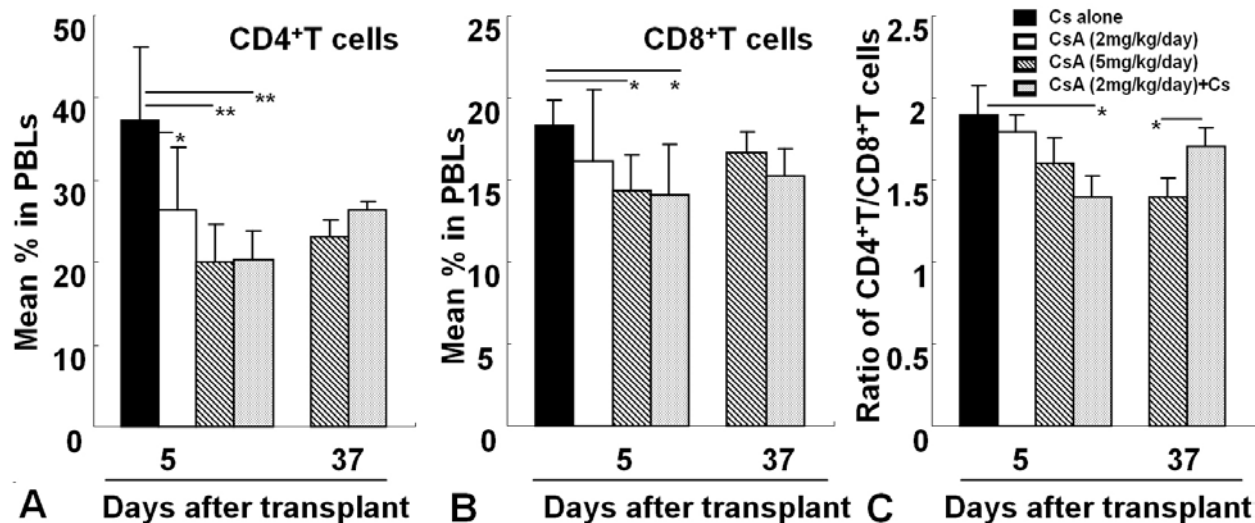
Figure 4. Significantly decreased percentages of T cells in the periphery blood of allo-kindey grafted rats treated with Cs and a low dose of CsA. The T cell subsets in the periphery blood of rats were assayed by flow cytometry. Allo-kidney grafted rats treated with a combination of Cs and a low dose of CsA or with a high dose of CsA showed significantly decreased percentages of CD4+ and CD8+ cells as compared with allo-kidney grafted rats treated with Cs or a low dose of CsA alone at day 5 after grafting (A and B). The ratio of peripheral CD4+/CD8+ T cells in rats treated with combined therapy of Cs and a low dose of CsA was significantly lower than that in other groups at day 5 after grafting, though it recovered to normal level by day 37 after grafting (C). Ten rats in each group were performed. *P<0.05, **P<0.01 with the indicated group. Data are shown as Mean ± SD.
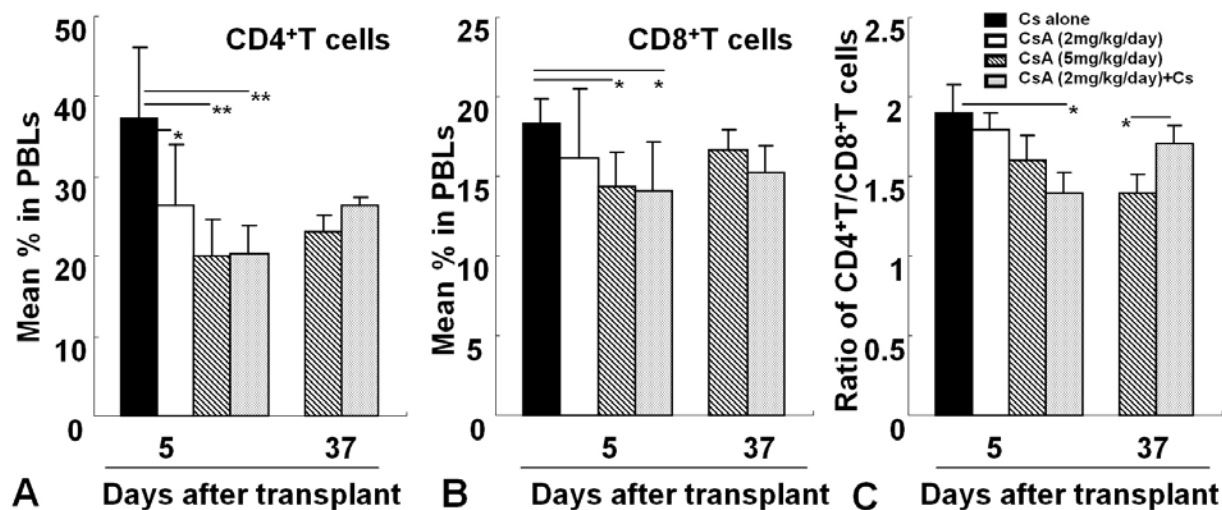

Figure 5. Significantly decreased Th1 cytokines in the peripheral blood of allo-kidney grafted rats treated with combined therapy of Cs and a low dose of CsA. The cytokines in sera of allo-kidney grafted rats were assayed by ELISA as described in materials and methods. At day 5 post transplantation,serum IL-2 and IFN-γ in rats treated with either a high dose of CsA or both Cs and a low dose of CsA were significantly lower than those in rats treated with Cs alone or a low dose of CsA (A and B). IL-10 did not show significant difference among these groups (C). At day 37 post transplantation, serum IL-2 and IFN-γ did not show significant differences between the rats treated with a high dose of CsA and those treated with a combination of Cs and a low dose of CsA (A and B). However, IL-10 was significantly higher in rats treated with a combination of Cs and a low dose of CsA than that in rats treated with a high dose of CsA. Ten rats in each group were performed. *P<0.05, **P<0.01 compared with the indicated group. Data are presented as Mean ± SD.

Furthermore, the synergistic effects of Cs and CsA were also reflected by the inhibition on Th1 cytokine production. The treatment of Cs in combination with a sub-therapeutic dose of CsA significantly inhibited the expression of Th1 cytokines, IL-2 and IFN-γ as compared with rats treated with Cs alone or a low dose of CsA. It is interesting that IL-10, one of the Th2 cytokines, was significantly enhanced in rats treated with a combined therapy of Cs with a low dose of CsA at day 37 after grafting, as compared with rats treated with a high dose of CsA. Whether the combination of Cs with a low dose of CsA would be favorable to transplant tolerance induction via IL-10 needs to be addressed in the future.

In conclusion, our data indicate that Cs has a synergistic role with CsA to significantly reduce T cell proportions, inhibit Th1 cytokine (IL-2 and IFN-γ) productions, and enhance IL-10 production in an allo-kidney grafted rat model. Thus, the treatment of Cs in combination with a low dose of CsA remarkably prolonged allogeneic kidney graft survival in rats. The present study may have potential impacts for clinical application of Cs in transplant patients.

6. ACKNOWLEDGEMENTS

7. REFERENCES

1. Tang IY, Meier-Kriesche HU, Kaplan B. Immunosuppressive strategies to improve outcomes of kidney transplantation. *Seminars in Nephrology*, 27, 377-92 (2007)

2. Vincenti F. A decade of progress in kidney transplantation. *Transplantation*, 77, S52–S61 (2004)

3. Nashan B. Maximizing the clinical outcome with mTOR inhibitors in the renal transplant recipient: defining the role of calcineurin inhibitors. *Transplant int*, 17, 279-85 (2004)

4. Knight SR, Morris PJ. Does the evidence support the use of mycophenolate mofetil therapeutic drug monitoring in clinical practice? A systematic review. *Transplantation*, 85, 1675-85 (2008)

5. Cantarovich D, Vistoli F, Soulillou JP. Immunosuppression minimization in kidney transplantation. *Frontiers in Bioscience*, 13, 1413-32 (2008)

6. Williams D, Haragsim L. Calcineurin nephrotoxicity. *Advance in Chronic Kidney Disease*, 13, 47-55 (2006)

7. Jiang WL, Jiang JG, Xu DS, Wang X, Qi SJ, Ouyang J, Ma AL, Vu MD, Bilolo KK, Chen HF. Synergistic effect of vincristine with tacrolimus or sirolimus in prevention of acute heart allograft rejection in the rat. *Microsurgery*, 23, 117-22 (2003)

8. Paterson RRM. Cordyceps - A traditional Chinese medicine and another fungal therapeutic biofactory? *Phytochemistry*, 69, 1469-95 (2008)

9. Wu HY, Zhang QX, Leung PH. Inhibitory effects of ethyl acetate extract of Cordyeeps sinensis mycelium on various cancer cells in culture and B16 melanoma in C57BL/6 mice. *Phytomedicine*, 14, 43-49 (2007)

10. Lee SKW, Wong CK, Kong SK, Leung KN, Lam CWK. Immunomodulatory activities of HERBSnSENSESTM Cordyceps - in vitro and in vivo studies. *Immunopharmacology and Immunotoxicology*, 28, 341-60 (2006)

11. Hsu CC, Tsai SJ, Huang YL, Huang BM. Regulatory mechanism of Cordyceps sinensis mycelium on mouse Leydig cell steroidogenesis. *FEBS Letters*, 543, 140-3 (2003)

12.Xiaoxia Zhou, Claudius U Meyer, Peter Schmidtke, Fred Zepp. Effect of cordycepin on interleukin-10 production of human peripheral blood mononuclear cells. *European Journal of Pharmacology*. 453, 309-17 (2002)

13. Yu L, Zhao J, Zhua Q, Li S.P. Macrophage biospecific extraction and high performance liquid chromatography for hypothesis of immunological active components in Cordyceps sinensis. *J. Pharmaceutical and Biomedical Analysis*. 44, 439–43 (2007)

14. Zhang Z, Xia SS. Cordyceps sinensis-I as an immunosuppressant in heterotopic heart allograft model in rats. *J Tongji Medical University*. 10, 100–3 (1990)

15. J.L. Jordan, G.M. Hirsch, T.D.G. Lee. C. sinensis ablates allograft vasculopathy when used as an adjuvant therapy with cyclosporin A. *Transplant Immunology*, 19, 159-66 (2008)

16. Holzen JP, Palmes D, Langer M, Spiegel HL. Microsurgical training curriculum for learning kidney and liver transplantation in the rat. *Microsurgery*, 25, 614-23 (2005)

17. Zhao X, Li L. Cordyceps sinensis in protection of the kidney from cyclosporine A nephrotoxicity. *Zhong hua Yi Xue Za Zhi*, 73,410–2, 447 (1993)

18. Zhu XY, Yu HY. Immunosuppressive effect of cultured Cordyceps sinensis on cellular immune response. *Zhong Xi Yi Jie He Za Zhi,* 10, 485–7, 454 (1990)

19. Sun Ming, Yang Yu-ru, Lu Yi-Ping, Gao Rui, Wang Li, Wang Jia, Tang Keshi. Clinical study on application of bailing capsule after renal transplantation. *Zhongguo Zhong Xi Yi Jie He Za Zhi,* 24, 808-10 (2004)

Abbreviations: IFN-γ:Interferon-γ, IL-2:Interleukin-2, IL-10:Interleukin-10, CsA: Cyclosporin A, Cs.: Cordyceps sinensis, ELISA:Enzyme-linked immunosorbent assay, FCM: Flow cytometry, PI:Propidium iodide, ATG: Antithymocyte globulin, OKT3:Anti-CD3 antibody, CNI: Calcineurin inhibitors

Key Words: Kidney Transplantation, Cordyceps sinensis, Cyclosporin A, Rejection

Send correspondence to: Puxun Tian, Department of Renal Transplantation; the First Affiliated Hospital; Medical college of Xi'an Jiaotong University; Xi'an 710061; China, Tel: 86-29-85323958, Fax: 86-29-85323958, E-mail: yuantian@mail.xjtu.edu.cn