行政院國家科學委員會專題研究計畫 成果報告

以系統生物學模式探討併用黃酮與免疫抑制劑對免疫系統之影響(第 2 年)
研究成果報告(完整版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 95-2320-B-028-001-MY2
執 行 期 間 ：96 年 08 月 01 日至 97 年 07 月 31 日
執 行 單 位 ：國立臺灣體育大學（臺中）競技運動系

計 畫 主 持 人：方世華
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報 告 附 件 ：出席國際會議研究心得報告及發表論文

公 開 資 訊 ：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 97 年 10 月 05 日

附件一

行政院國家科學委員會補助專題研究計畫 ■ 成 果 報 告 □期中進度報告

（計畫名稱）

以系統生物學模式探討併用黃酮與免疫抑制劑對免疫系統之影響

計畫類別：■個別型計畫　□ 整合型計畫
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計畫參與人員： 史佩玉；賴慶忠

成果報告類型(依經費核定清單規定繳交)：□精簡報告 ■完整報告

本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
■出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
■涉及專利或其他智慧財產權，□一年■二年後可公開查詢

執行單位：國立台灣體育大學(臺中)

中 華 民 國 97 年 10 月 1 日

前言

Cyclosporine (CsA) is an effective immunosuppressive agent widely used to prevent allograft rejection and chronic inflammation in clinical practice. CsA exerts it immunosuppressive action by interfering the activation and proliferation of T cells. There is growing evidence that regular exercise can increase immune function. Patients taking CsA may be benefit from regular exercise in improving cardiovascular and musculoskeletal functions. However, the effects of exercise on patients' immune functions those who are taking CsA are unclear.

研究目的

The aim of this study was to investigate the interactive effect of CsA administration and regular exercise on the functions of immune cells in mice.

文獻探討

Cyclosporin (CsA), a lipophilic cyclic peptide isolated from the fungus *Hypocladium inflatum gams* [(1)], is a potent immunosuppressant extensively prescribed in the treatment of autoimmune diseases and allograft transplantation [(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, nuclear factor of activated T cells (NF-AT) 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)].

It has been suggested that exercise can affect various components of the immune system (Nieman 1998; Hoffman-Goetz & Pedersen 1994). Many immune functions are stimulated by moderate physical activity and following long-term regular training. It's reported that regular exercise play an important role in the rehabilitation of patients with autoimmune rheumatic disease (Nordemar R 1981; Minor MA 1989). On the other hand, more vigorous effort and periods of heavy training exhibit to suppress immune responses (Nehlsen-Cannarella 1998). It's important to know that what level of exercise is clinically beneficial or deleterious for the individual. However, the mechanisms of these beneficial effects are poorly understood.

Since CsA is necessary for chronic inflammation patients, the health risk associated with exercise for those taking CsA routinely is an important clinical issue. We hypothesized that CsA may have clinically relevant interaction with exercise in the immune cells functions. The present study aimed to investigate the interactions of exercise with CsA through measuring the related immune responses in mice.

研究方法

Mice

Female 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-h 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).

Drugs

Cyclosporine (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, U.S.A.). 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, U.S.A.).

Animals and drug administration

Mice were randomly divided into 6 groups: 0-Ex (no CSA, no exercise), 0+Ex (no CSA, exercise), 10-Ex (10 mg/kg/d CSA, no exercise), 10+Ex (10 mg/kg/d CSA, exercise), 20-Ex (20 mg/kg/d CSA, no exercise), and 20+Ex (20 mg/kg/d CSA, exercise), with eight mice in each group. The 3 exercise groups were trained 3 times a week at approximately 75% VO2max for 8 weeks.

Cell culture

Animals were sacrificed by cervical spine dislocation. The spleen was removed and crushed into a single cell suspension, and red blood cells were lysed with Tris-buffered ammonium chloride before washing three times with HBSS. The cell numbers were determined with a hemocytometer, and viabilities were assessed by trypan blue dye exclusion. Cells were seeded at a density of 2 x 10^6 cells/ml and incubated at 37 ºC in humidified 5% CO_2/95% air. All culture materials were disposable and free of endotoxin.

Cell proliferation assay

Mitochondrial respiration-dependent MTT assay was employed to determine the cell viability. MTT in PBS (0.1 mg) was added into each well and then incubated at 37ºC for 4 h. The formazan crystals were dissolved by addition of acid-isopropanol and mixed at room temperature. After 20 min, the optical density (OD) was measured with a microplate reader (BIO-RAD, model 3550, U.S.A.) at 570 nm ($OD_{570-620}$). The mean value of the four wells was used to assess the cell proliferation.

Determination of nitric oxide (NO)

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

Cytokine assay

Spleen cells, $5x10^6$/ml, were incubated with and without 5 μg/ml ConA in 24-well plates for 48 h. The culture supernatants were collected and stored at –80ºC prior to being analyzed by enzyme-linked immunosorbent assay (ELISA) (PharMingen, San Diego, CA) as described earlier [28]. Briefly, 96-well plates were coated with monoclonal antibody with specificity for IFN-γ or IL-4 and incubated overnight at 4ºC, washed with 0.05% Tween 20 in PBS and blocked by RPMI 1640 supplement with 10% FCS for 1 h at room temperature (RT). Serially diluted culture supernatants and standards prepared from recombinant mouse IFN-γ or IL-4 separately (PharMingen, San Diego, CA) were added for 2 h at RT. The wells of the plates were washed and biotin-conjugated rat anti-mouse IFN-γ or IL-4 was added for another 1 h at RT. After proper washing, avidin-horseradish peroxidase was added and incubated for 1 h 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, U.S.A.) at 450 nm. The detection sensitivities of IFN-γ and IL-4 were 31.3 pg/ml and 7.8 pg/ml, respectively.

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 * $p< 0.05$; ** $p<0.01$.

結果與討論

In order to investigate the interaction between the effects of exercise and CsA on immune activities *in vivo*, we used the optimal dose of CsA 10 mg/kg as described in our previous study [31]. We monitored the growth of mice in each group by their weight, food consumption, and mobile activities everyday. The 0+Ex, 10+Ex, and 20+Ex groups were performed at approximately 75% VO2max exercise 3 times a week for 8 weeks. The changes of weight showed that the exercise groups had lower but insignificant weight than non-exercise groups (Fig.1). Food consumption and mobile activities were similar among all groups.

After 6 weeks, the function of peritoneal macrophages was determined by monitoring the levels of nitric oxide (NO) from peritoneal macrophages under LPS/IFN-γ stimulation. The result demonstrated that NO production ability by mitogen-stimulated macrophages were higher in 10+Ex and 20+Ex groups comparing to 10-Ex and 20-Ex group, respectively (Fig. 2).

Spleen is one of the major secondary immune organs and CsA is known to inhibit T cell activation. Therefore, we investigate whether the influence of CsA on the functions of spleen cells would be altered by exercise. The mitogenic activity of T cells was monitored by stimulating spleen cells with 5 μg/ml ConA for three days and cell proliferation analysis was performed by the MTT method. Although mitogen-stimulated cell proliferation is suppressed by CsA but exercise slightly enhances the T cell proliferation (Fig. 3). Further comparison of cytokine levels secreted by ConA-stimulated spleen cells indicated that T helper type 1 (Th1) cytokine, IFN-γ but not Th2 cytokine, IL-4, was markedly decreased after treatment with CsA alone. We found that exercise resulted in a significant increase of IFN-γ (Fig. 4A), however, no significant difference for IL-4 (Fig. 4B) among these groups.

To our knowledge, this is the first study to investigate the effect of exercise on the CsA-induced immunosuppression *in vivo*. Previous studies have revealed that the negative side effects of CsA include nephrotoxicity, hepertension, hepatotoxicity and decreased exercise capacity (Mercier JG 1995; William TJ 1997). Our results showed that 10 mg/kg CsA administration everyday did not affect the 0+Ex, 10+Ex, and 20+Ex groups to perform at approximately 75% VO2max exercise 3 times a week for 8 weeks. Meanwhile, they showed similar in food consumption and mobile activities among these six groups. Although the exercise groups were observed to get lower weight than non-exercise groups but there is no significant differences. This phenomenon is also supported by the regular exercise would cause more energy loss in healthy people.

Previous report indicated that CsA reduced the levels of inducible nitric oxide synthase (iNOS), NO, and cyclooxygenase-2 produced by LPS-activated RAW264.7 cells (Attur *et al.*, 2000). Previous clinical study indicated that a well-organized exercise program resulted in significant improvement in the functional capacity of heart transplant patients (Karapolat H 2007). In addition to the significant increase in VO2, improved immune function may contribute to the better quality of life of these patients. Our results showed that exercise significantly enhanced NO production by LPS/IFN-γ activated macrophages also support this concept.

It's well known that CsA is a drug with a wide immunosuppressant action because of its ability to inhibit the calcium/calmodulin dependent phosphatase, calcineurin, and consequently the NF-AT-induced transcription, finally inhibiting the T lymphocytes proliferation (Matsuda S 2000; Annamaria DL 2005). 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) (Sauma, D. 2003)[(38)]. Moreover, CsA reduced co-stimulatory molecule expression and further altered the antigen presenting function of DCs for T cell activation (Lee, J. I., 1999). However, calcium acts as a second messenger in skeletal muscle (Koulmann and Bisgard 2006) during prolonged exercise. And through calcineurin signaling, exercise would activate many target genes expressions. It's well known that calcineurin about 10-fold higher concentrations in neuronal and muscle cells than other cell types (Olson EN 2000). Whether exercise also activates the Ca2+-dependent regulatory pathway in T lymphocytes needs further studies. The intensity of exercise also plays an important role in the activation pathway. In addition, it's still unclear that the combined effects of regular exercise and CsA on the activity of calcineurin.

It's well known that the immunological and hormonal systems have close inter-regulatory links (Besedovsky H 1989). In healthy individuals, exercise leads to a rise in cytokines, such as interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF). IL-1β interacts with the endocrine system to cause corticotrophin to release hormone (Besedovsky H 1987). In addition, glucocorticoids levels rise during moderate or sever exercise. Glucocorticoids are anti-inflammatory hormones and have a clinically therapeutic effect on the treatment of autoimmune rheumatic disease (Saldanha C 1986). There is accumulating evidence suggests that exercise of a high intensity or long duration can cause immunosuppression and increase susceptibility to infection (Health GW 1992; Chung HY 2005). In this study, we found that exercise resulted in a significant increase of T helper type 1 (Th1) cytokine, IFN-γ, that is the

major cytokine in innate and acquired immunity. And mitogen-stimulated cell proliferation suppressed by CsA also slightly enhanced by exercise. These results suggest that regular exercise may enhance CsA-induced immunosuppression.

Whether exercise indirectly decreased CsA absorption or increased CsA clearance due to metabolizing enzymes induction, increased p-glycoprotein activity, changed protein binding, or induction of extrahepatic metabolism to result in decrease of CsA bioavailability needs further studies. In conclusion, exercise significantly decreased the CsA-induced immunosuppression and resulted in the higher macrophage and Th1 type activities than giving CsA alone in mice. Therefore, in clinical, patients who need to take CsA everyday to control their chronic inflammation are encouraged to undergo regular and modulate exercise to prevent infection caused by CsA administration.

Acknowledgements

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Fig. 1 The weight of each group during the eight weeks. The 0+Ex, 10+Ex, and 20+Ex groups were performed at approximately 75% VO2max exercise 3 times a week for 8 weeks. The weight of mice was monitored every 2 weeks. The data were expressed as the mean.

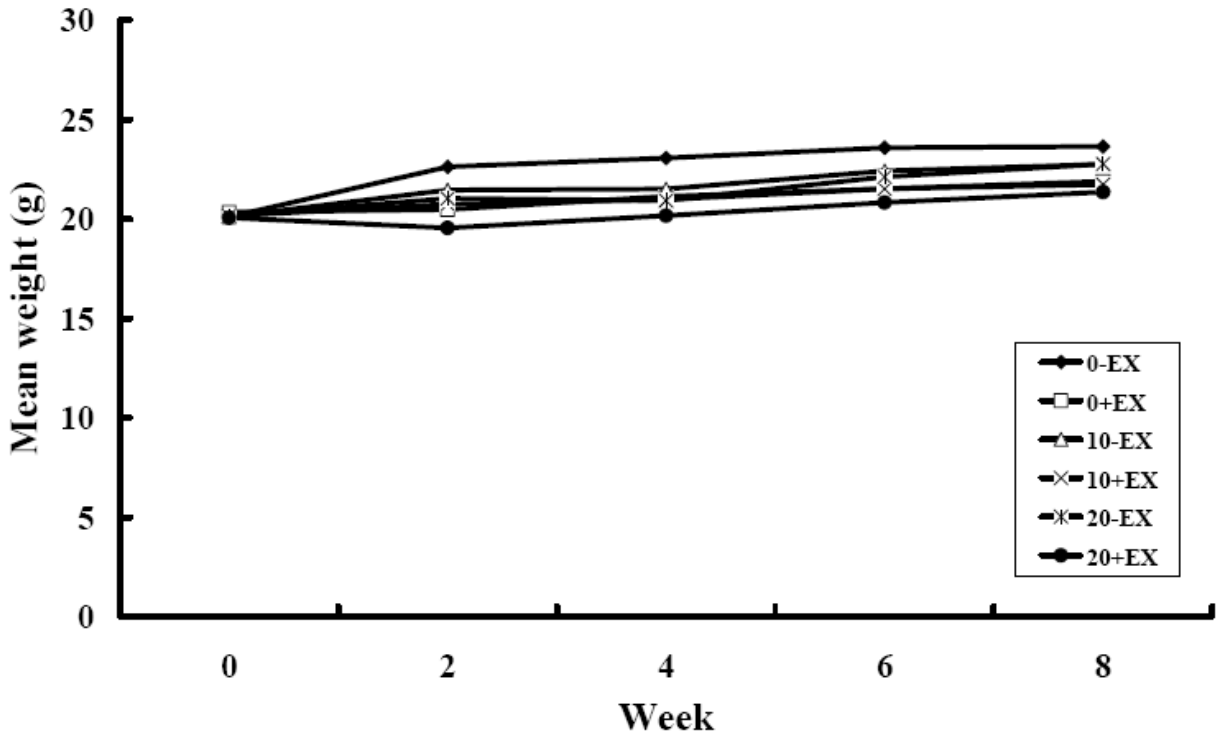


Fig. 2 Effect of different combinations of CsA and exercise on nitric oxide production from macrophages. Murine peritoneal macrophages were added with LPS (2 μg/ml) plus IFN-γ (10 U/ml) and supernatants were collected 48 h after the initiation of the cultures to determine the amount of NO by the method of Griess. The data were expressed as the mean ± SD.

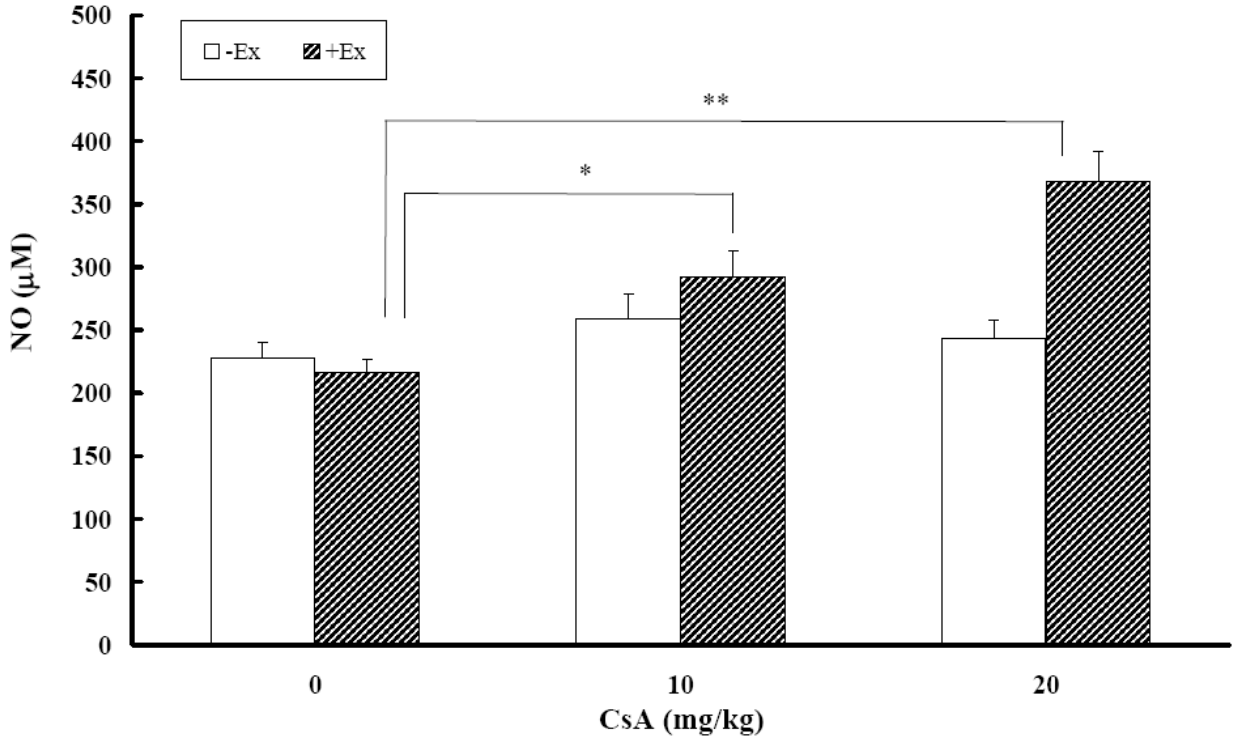


Fig. 3 The cell proliferation of ConA-stimulated spleen cells. $5x10^6$ spleen cells were incubated with and without 5 μg/ml ConA in 24-well plates for 72 h. The culture supernatants were collected and analyzed using a sandwich-ELISA as described in Methods. The data were expressed as mean ± SD.

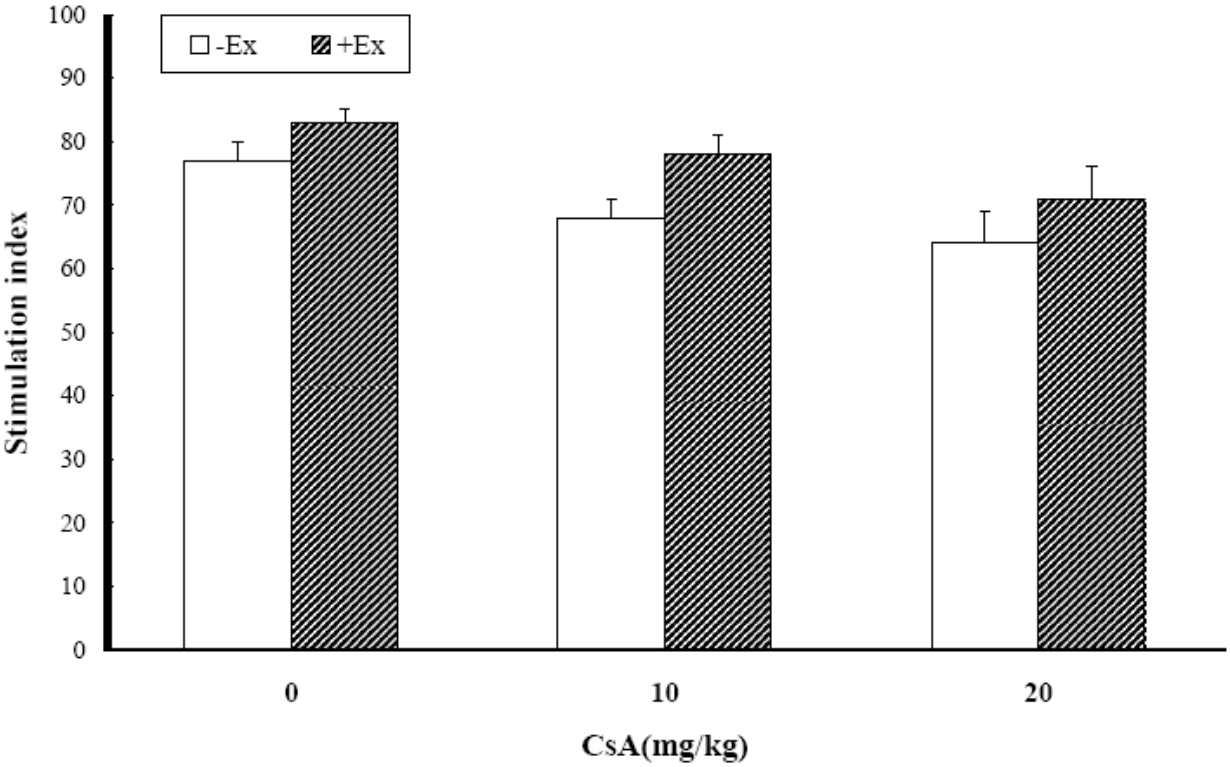
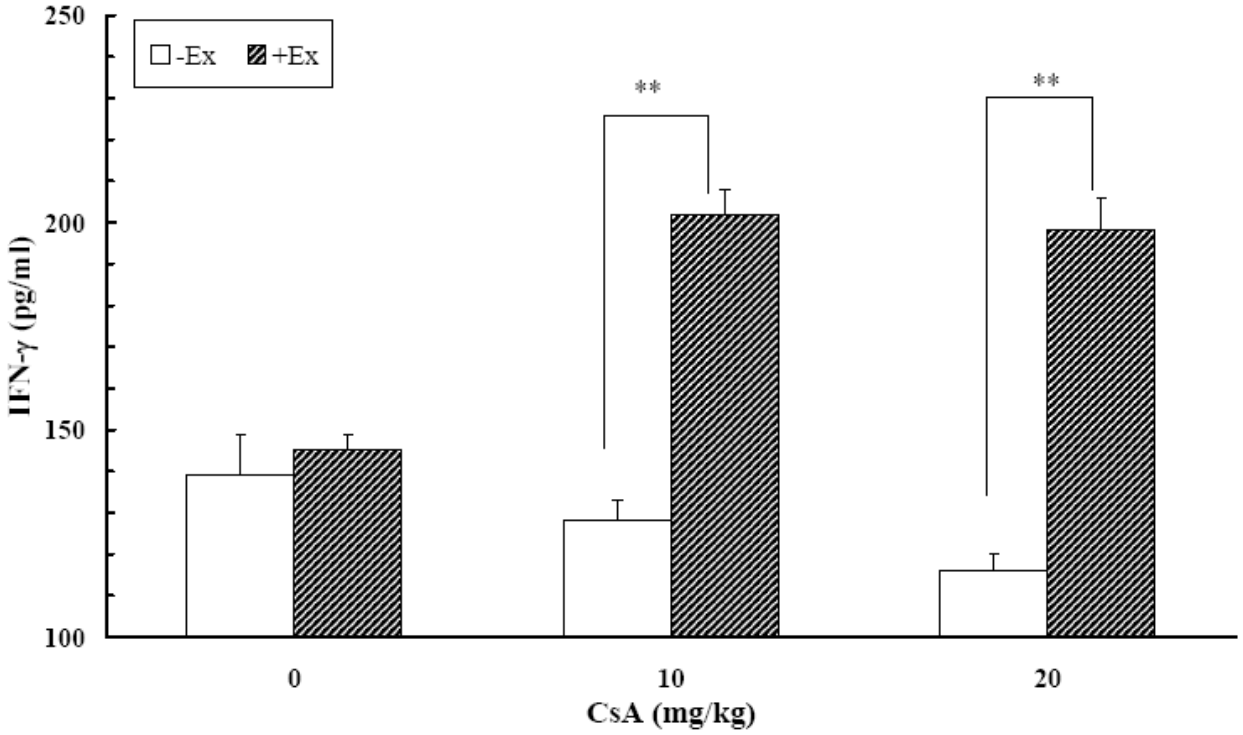
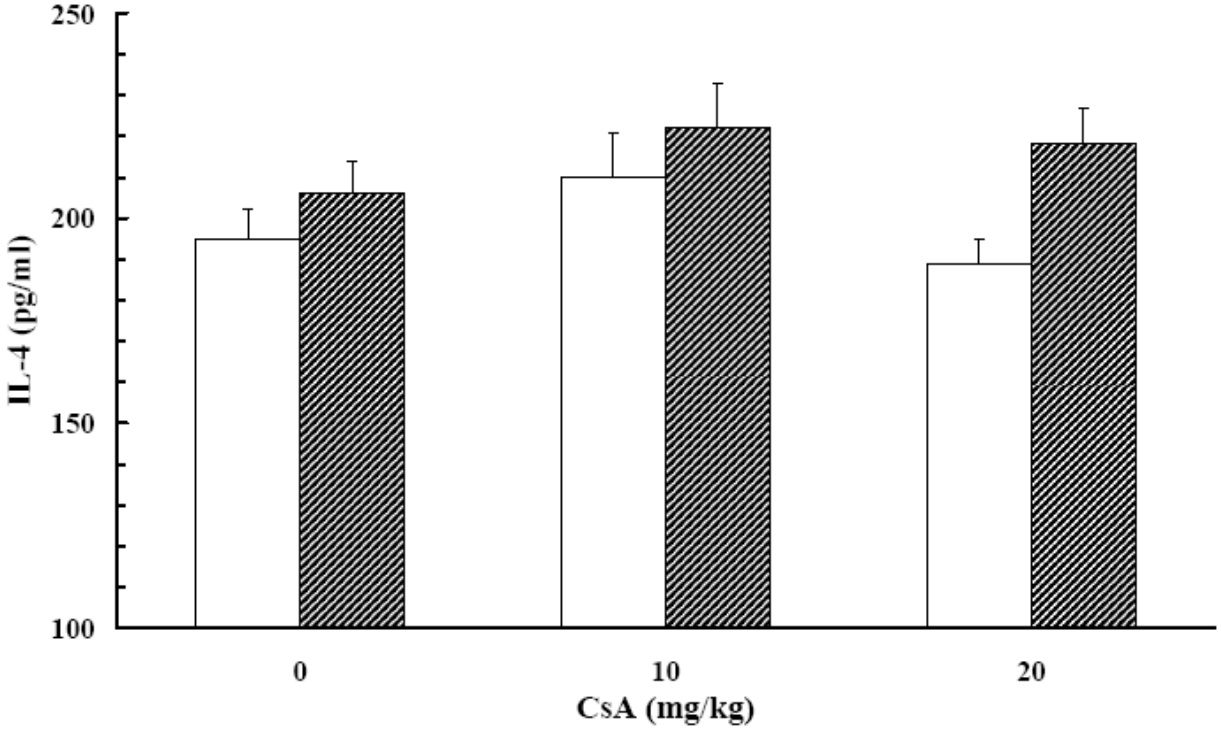


Fig. 4 The levels of IFN-γ (A) and IL-4 (B) cytokines secreted from ConA-stimulated spleen cells. $5x10^6$ spleen cells were incubated with and without 5 μg/ml ConA in 24-well plates for 48 h. The culture supernatants were collected and analyzed using a sandwich-ELISA as described in Methods. The data were expressed as mean ± SD.





計畫成果自評部份：

目前這一部分的研究成果已完成投稿模式，將先投稿至國際學術期刊。其他結果有部份仍在繼續做更深入的實驗探討中。

出席國際學術會議心得報告

計畫編號	NSC 95－2320－B －028 －001－MY2
計畫名稱	以系統生物學模式探討併用黃酮與免疫抑制劑對免疫系統之影響
出國人員姓名 服務機關及職稱	方世華 國立台灣體育大學(台中)競技運動學系教授
會議時間地點	上海 97 年 5 月 11 日至 97 年 5 月 13 日止
會議名稱	「第二界亞太平洋藥物研討會（2nd Asian Pacific Regional Meeting）」
發表論文題目	Effect of rutin on acute renal failure and the relative bioavailability between oral intake of rutin and Sophorae Flos decoction in rats

一、參加會議經過

5/10 抵達上海後立即前往會議中心報到，開會期間大會每天早上先安排 keynote presentation，主題包括目前國際學術上最重要的發展方向：評估藥物作用的分子標的；藥物的輸送管道對藥物進出細胞的角色與重要性；非藉由病毒基因的細胞傳遞機轉；系統生物學與代謝質體學再藥物開發上的應用等等。另外大會安排各個領域的研究成果口頭論文發表與壁報論文發表，三天密集的呈現超過一千篇的論文，研究資訊暨豐富又新穎，獲益良多。

二、與會心得

感謝國科會的補助得以有機會到上海參與藥學界年度國際盛會，各國藥學專家齊聚一堂，了解國際上研究之趨勢，尤其更深入了解發炎與免疫調節藥物發展的方向，對本身的研究領域專長有相當大的啟發，同時也藉由壁報論文的發表，與相關研究人員交換經驗，有助於提高台灣在國際藥學研究上學術知名度。