

# 行政院國家科學委員會補助專題研究計畫成果報告

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※ 薑黃素於移植醫學之應用--II

※ (1) 引發移植鼠心長期存活之機轉

※ (2) 預防慢性異種移植排斥及治療急性排斥

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計畫主持人： 關士傑

共同主持人： 賴明坤

計畫參與人員： 鄧方晟、劉穎昇

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## 一、中文摘要

1980年代以來環孢靈 (cyclosporine; CsA) 及各種新藥相繼問世，使得器官移植的預後大幅提昇。使得移植後的急性排斥發生率有下降的趨勢。但是，即使將其血中濃度調整到最適合的治療劑量，臨床上還是會有部份的病例發生排斥，顯示免疫系統的活化與排斥的發生，單靠環孢靈阻斷細胞素-2 (interleukin-2; IL-2) 基因活化的前置步驟 (即 calcineurin)，並不足以竟全功。很明顯地，一定有其它的訊息傳遞步驟可以不經由 calcineurin 而活化 IL-2 的產生，這其中最重要的是與 CD28 有關的訊息傳導。最近有實驗顯示薑黃素 (curcumin; CCM)，不僅有防止缺血再灌流損傷的功效，而且在生體外，對環孢靈無法抑制的抗-CD28 抗體及 PMA 所引發的淋巴球增生也有明顯的抑制功效。目前經文獻搜尋，並沒有進一步有關這方面動物實驗，或根本機轉的研究。

我們以首先利用大鼠異位心臟移植之模式了解薑黃素與環孢靈併用是否對預防或延緩急性排斥的發生有加成或相乘的治療作用。另外，我們以生體外實驗之混合淋巴球培養 (mixed lymphocyte culture; MLC)，來探討 CCM 與 CsA 對特異種系之淋巴球增生的抑制作用。

在異位心臟移植研究結果中，我們於文獻中次首證明 CCM 併用低濃度之 CsA 能有效延長移植心臟之存活時間，甚至能造成其長期存活 (long-term survival)，此結果指出 CCM 與 CsA 在生體內的確可能有相加或相乘之免疫抑制效果，並值得進一步探討其機轉。

在淋巴球交配反應中，我們評估了組織不

相容品系鼠:WKY (RT1<sup>b</sup>) x BN (RT1<sup>a</sup>) 在一系列遞增濃度的 CsA 與/或 CCM 的存在下，我們看到以嚴格之 combination index 來評估，CCM 與 CsA 在 MLR 反應中確實有相乘效果 (synergistic effect)，這對於免疫活化的基本了解中認為 CsA 阻斷與 Calcineurin 有關之 NFAT 活化，而 CCM 阻斷 second signal (CD28) 下游之活化機轉的推論是相吻合的。

關鍵詞：薑黃素，器官移植，排斥，環孢靈

## 二、英文摘要

A major contribution to recent significant progress in the field of organ transplantation comes from rapid developments of immunosuppressive medication. Cyclosporine (CsA) made a marked improvement of graft survival. The advent of FK-506 slightly decreased the incidence of acute rejection further. Yet, even with the most well monitored blood concentration of CsA or FK-506 therapy, the occurrence of acute allograft rejection is still not an infrequent event in clinical practice. This suggests an existence of alternate signal transduction pathways, bypassing the calcineurin step that was blocked by either CsA or FK-506, to initiate interleukin-2 (IL-2) expression and to activate effector limb of the immune response. The most important one of these alternate ways is

the CD28 co-stimulation signal. Previous studies in the CD28/ B-7 pathway were focused on the use of CTLA4-Ig, which yielded initial promising but later controversial results.

Recent studies showed that curcumin (diferuloyl methane; CCM) possesses not only anti-oxidant and anti-inflammatory actions, but also the ability to block CsA-resistant CD28 co-stimulatory pathway of nonspecific human T-cell proliferation in vitro. Up to now, there is no further publications for *in vivo* evaluation of CCM as an immunosuppressant in animal models, and in depth studies for its possible mechanism to prevent immune activation. We have studied the combination effects of CCM and subtherapeutic dose of CsA with heterotopic cardiac transplantation models in rats. We also examined the effect of CCM in mixed lymphocyte reaction.

For the first time, our results on rat heart allograft models demonstrated that CCM (dose 100mg/day, po, x14d or x28d), when used with low dose CsA can prolong MST of the allografts, and even more, achieve long-term survival, we speculated possible synergistic or additive effect occurred in this model

In mixed lymphocyte reaction, two MHC-incompatible rat strains (BNxWKY) in the presence of increasing concentrations of Curcumin and/or CsA. These two drugs showed synergistic effect when examined with Combination index analysis (CI=0.22~0.81, <1). This result supports previous understanding of CsA to block NFAT activation of Calcineurin pathway while CCM to block downstream activation of the CD28

pathway.

### 三、研究目的

近年來器官移植醫學的長足進步有一大部份原因來自於抗排斥藥物的快速發展。1980 年代以來環孢靈 (cyclosporine) 的問世，使得器官移植的預後大幅提昇。爾來 FK-506 等新藥的發展，使得移植後的急性排斥發生率有稍下降的趨勢。但是，不管是以環孢靈或 FK-506 治療，即使將其血中濃度調整到最適合的治療劑量，臨床上還是會有部份的病例發生排斥，顯示免疫系統的活化與排斥的發生，單靠環孢靈或 FK-506 阻斷細胞素-2 (interleukin-2; IL-2) 基因活化的前置步驟 (即 calcineurin)，並不足以竟全功。很明顯地，一定有其它的訊息傳遞步驟可以不經由 calcineurin 而活化 IL-2 的產生，這其中最重要的是與 CD28 有關的訊息傳導。以往針對阻斷 CD28 路徑的研究著重於 CTLA4Ig 的使用，雖然動物實驗的結果不錯，但是目前臨床上的成效上仍不明確。

最近有實驗顯示薑黃素 (curcumin)，一個從咖哩中分離出的化合物，不僅有防止缺血再灌流損傷的功效，而且在生體外具有抑制離體人類 T 淋巴球增生的功能，對環孢靈無法抑制的抗-CD28 抗體及 PMA 所引發的淋巴球增生也有明顯的抑制功效。目前經文獻搜尋，並沒有進一步有關抗排斥這方面動物實驗，或根本機轉的研究。我們對於薑黃素的初步研究也顯示了不錯的結果，給予我們一個具有潛力的研究方向。

### 四、結果討論

(1) 環孢靈與薑黃素使用於大鼠心臟移植模式

材料與方法:

薑黃素之免疫抑制效果首先以大鼠異位心

RT1<sup>b</sup>) 鼠心移植至 WKY (RT1<sup>a</sup>)接受鼠或 Buffalo (BUF, RT1<sup>b</sup>)鼠心移植至 Wistar-Furth(WF, RT1<sup>a</sup>) 接受鼠。薑黃素單獨使用或合併使用環孢靈給藥，移植存活天數由心跳之存在為評估依據。

結果:

在 BN x WKY 模式，薑黃素單獨使用時，有意義地延長平均存活天數(MST $\pm$ SD)至 20.5 $\pm$ 7.8 (100 mg/d x 14 d 組) 附圖一，及 24.5 $\pm$ 6.3 (100 mg/d x 28d 組) 附圖二，相較於不給藥控制組的 9.1 $\pm$  1.9 日。合併投與薑黃素及次低劑量環孢靈(10 mg/kg/d x 7d)更使存活天數之延長比兩藥單獨使用(28.5 $\pm$ 5.1 及 35.6 $\pm$ 5.9 天)效果還佳，(P< 0.05)。在 BUF x WF 模式，薑黃素單獨使用並未延長平均存活日，但當次劑量環孢靈合併投與，則接近三分之二移植鼠可存活 60 天以上，達長期存活 (P< 0.05) 附圖三。

## (2) 環孢靈與薑黃素使用於淋巴球交配反應

材料與方法:

以單向淋巴球交配反應(one-way MLC) 評估薑黃素之免疫抑制效果，從手術取下新鮮脾臟，尼龍網篩出組織細胞液，以密度分層離心法分離淋巴球，以 mitomycin-C 去活化之刺激淋巴球 (1\*10<sup>5</sup>/孔密度) Brown Norway (BN, RT1<sup>b</sup>) 與接受者淋巴球(1\*10<sup>5</sup>/孔密度) WKY (RT1<sup>a</sup>) 共同培養於一系列遞增濃度下之環孢靈(0 to 1.0\*10<sup>3</sup> ng/ml) 與薑黃素(0 to 50  $\mu$ M) 之 RPMI1640/10% FCS 96 孔培養盤 37°C; 5 % CO<sub>2</sub> 中 144 小時後，加入 1 $\mu$ Ci [<sup>3</sup>H] thymidine，再 6 小時後收取淋巴球，以閃爍記數儀測量 cpm 作為細胞增值指標。實驗重複三次以上以平均值/標準差表示。

結果:

薑黃素可抑制此兩種組織不相容品系鼠 MLR 反應，並呈現濃度依賴性。

(ED<sub>50</sub>=1.44 $\mu$ M)，此外薑黃素各濃度下之環孢靈並存於培養液時有相乘效果 (synergistic suppressions)。例如，MLC 反應中 [<sup>3</sup>H] thymidine 結合之閃爍記數值於 10  $\mu$ M 薑黃素及 50ng/ml 環孢靈抑制至 (5415 $\pm$ 521 cpm) 相較於任一藥劑單獨使用; 10  $\mu$ M 薑黃素(8297 $\pm$ 2247cpm); 50ng/ml 環孢靈(12982 $\pm$ 1877cpm)。附圖四、五。在合併用藥指標(CI, combination index) 薑黃素及環孢靈在 MLR 反應中確實有相乘效果 (synergistic effect, CI=0.22~0.81, <1) 附圖六。這對於免疫活化的基本了解中認為 CsA 阻斷與 Calcineurin 有關之 NFAT 活化，而 CCM 阻斷 second signal(CD28)下游之活化機轉的推論是相吻合的。

## 五、計劃自評

我們在完成本年度研究之後，我們已建立有系統的大鼠異位心臟移植模式，有助於以後急性與慢性排斥模式下的各種藥物作用分析。

在第一部分的異位心臟移植研究結果中，我們於文獻中次首證明 CCM 併用低濃度之 CsA 能有效延長移植心臟之存活時間，甚至於造成其長期存活(long-term survival)，此結果指出 CCM 與 CsA 在生體內的確可能有相加或相乘之免疫抑制效果，並值得進一步探討其機轉。

在淋巴球交配反應中，我們評估了組織不相容品系鼠:WKY(RT1<sup>a</sup>) x BN (RT1<sup>b</sup>) 在一系列遞增濃度的 CsA 與/或 CCM 的存在下，我們看到以嚴格之 combination index 來評估，CCM 與 CsA 在 MLR 反應中確實有相乘效果(synergistic effect)，這對於免疫活化的基本了解中認為 CsA 阻斷與 Calcineurin 有關之 NFAT 活化，而 CCM 阻斷 second signal(CD28)下游之活化機轉的推論是相吻合的。

進一步以淋巴球在藥物調控下受激活後之細胞素(cytokine)分泌研究更可証實此

模式之正確性，此部分屬於本計劃第三年實驗，在我們初步採自移植鼠心臟組織樣本，並抽取RNA進行RT-PCR實驗中，由於技術上的問題目前尚未得到可靠的結論，因此我們計劃先採取組織不相容品系鼠淋巴交配反應的淋巴球細胞作為生體外RNA來源(ref 36.)，以期在較易控制的條件下使實驗結果更穩定。

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### Survival Functions (BN-WKY)

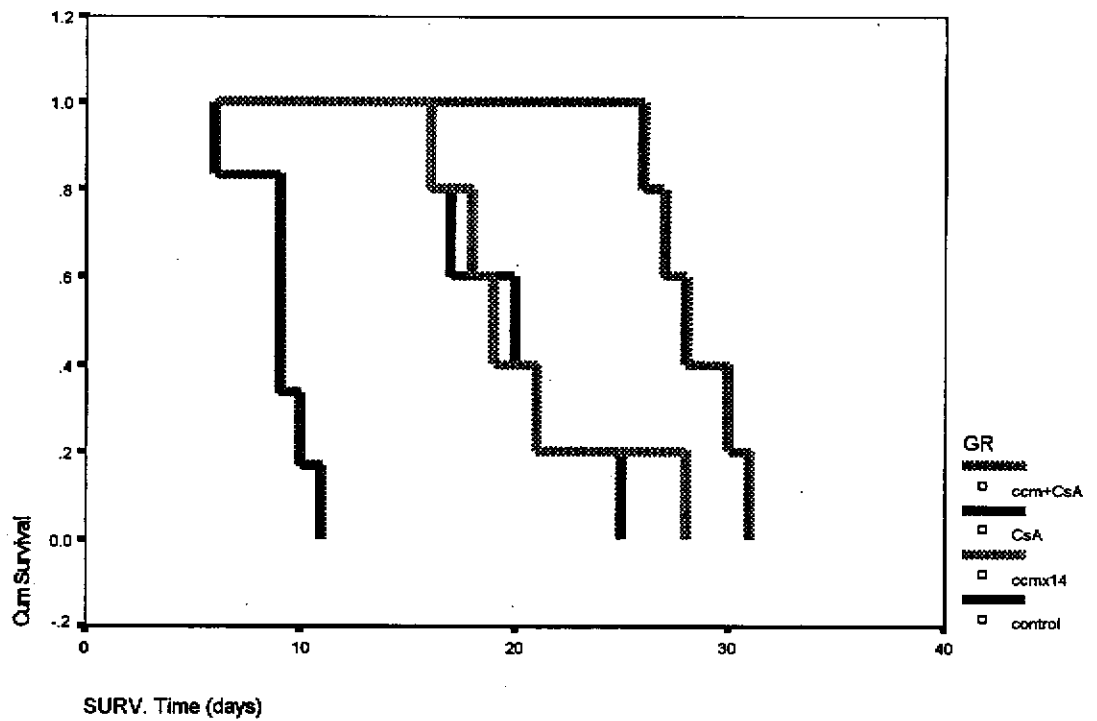


Figure 1: In BN to WKY model, CCM alone significantly increased the mean survival time (MST)  $\pm$  SD to  $20.5 \pm 7.8$  (100 mg/d x 14 d) as compared to  $9.1 \pm 1.9$  d of non-treated controls.

### Survival Functions (BN-WKY)

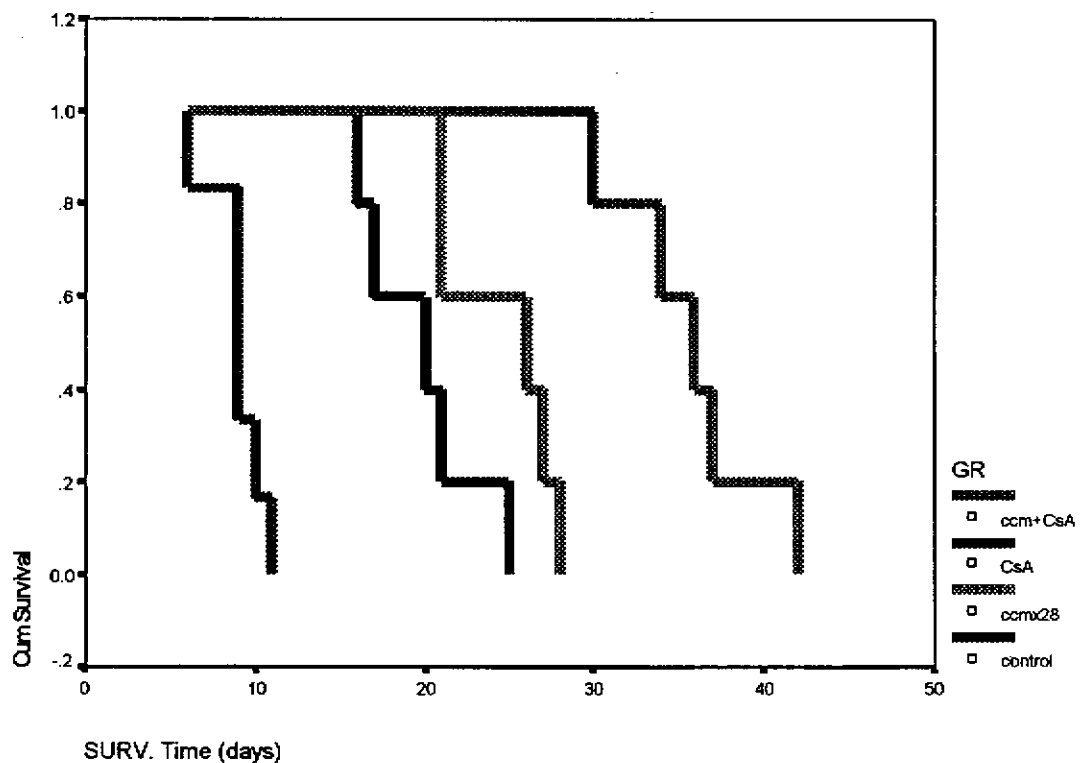
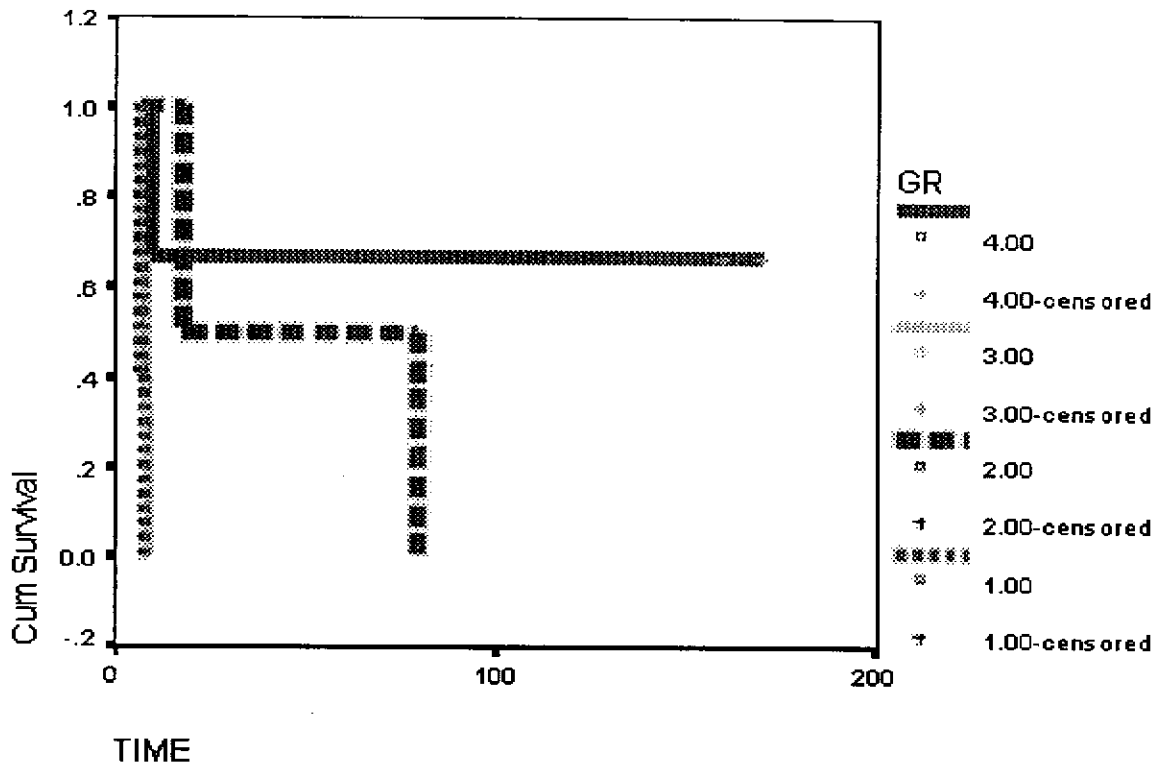


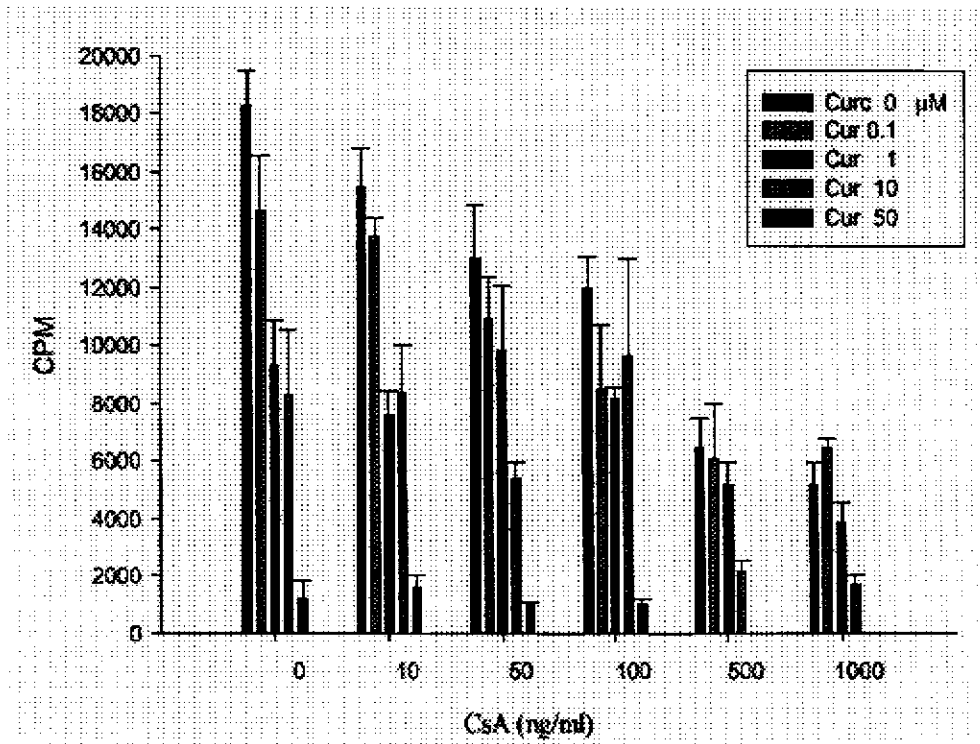
Figure 2: In BN to WKY model, CCM alone significantly increased the mean survival time (MST)  $\pm$  SD to  $24.5 \pm 6.3$  (100 mg/d x 28d) days, as compared to  $9.1 \pm 1.9$  d of non-treated controls.



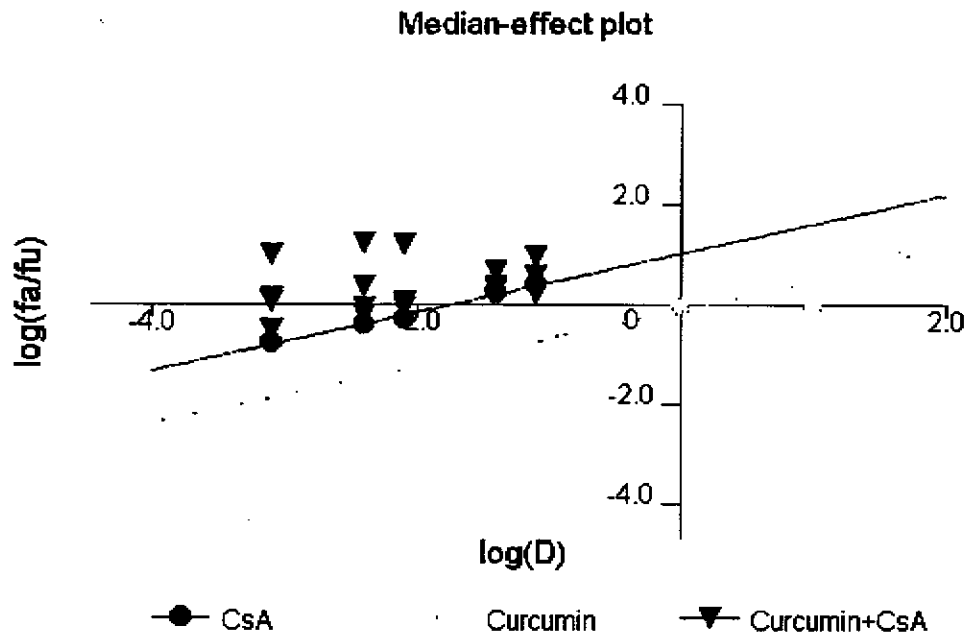
## Survival Functions



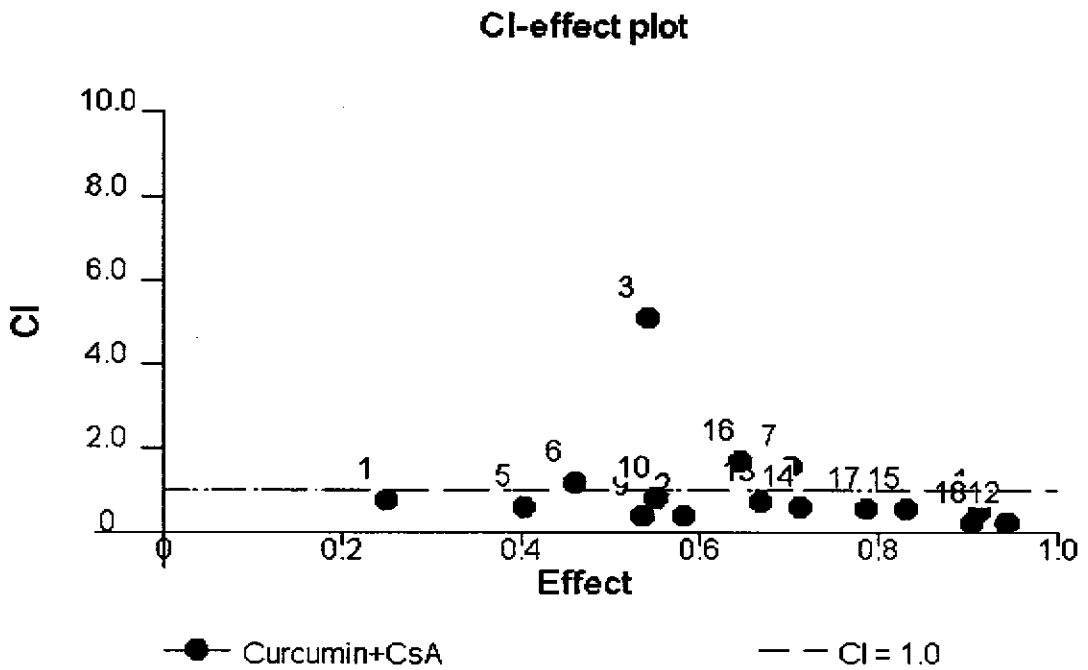
图三：Combination of CCM and subtherapeutic doses of CsA (10 mg/kg/d x 7d) produced further prolongation of MSTs to 28.5±5.1 and 35.6±5.9 days, better than that of CCM or CsA alone (P<0.05).



图四：The effect of Curcumin and CsA in increasing concentrations described on text and figure, on the primary one-way mixed lymphocyte culture of BNx WKY experiment .



圖五：The Median effect plot of Curcumin and CsA in increasing concentrations described on text and figure, in the BNx WKY MLC experiment .



圖六：The Combination index plot of Curcumin and CsA in increasing concentrations described on text and figure, in the BNx WKY MLC experiment.