Upregulation of IL-2, IL-4 and IL-10 on mouse immune responses by aqueous-ethanol extract of Rhodiola crenulata in vitro

Chuan-Cheng Wu¹  Ching-Yi Chu²

¹Hospital of KangTein
²Department of Nursing, Hungkuang University

Received 7 December 2010; accepted 10 February 2011

Abstract

Rhodiola, used in Chinese medicine, has a major biological factor in resisting hypoxia, eliminating fatigue, preventing high altitude sickness, nervous system diseases, and tumors, as well as being cardiovascular-protective. In this study, we investigated the influence of the aqueous-ethanol extract of Rhodiola crenulata (RCAE) on antibody productions, cytokine patterns and proliferations of mouse spleen cells and MPC-11 B cell line. Results showed that 5-10μg/ml RCAE could enhance about 20% of the cytotoxicity of MPC-11 cells (for the IgG secretion) and increase about 10% of the viability of mouse spleen cells by MTT assay, but no significantly difference below 2.5μg/ml of RCAE-treated in both. Data demonstrated that RCAE promoted the IgG productions of MPC-11 and PWM-stimulated mouse spleen cells by ELISA assay. More then, there has been evidenced that RCAE induced the mRNA and protein expressions of IL-2, IL-4 and IL-10 on PWM-stimulated mouse spleen cells by RT-PCR and ELISA assay. In conclusion, RCAE was indicated to stimulate mouse spleen lymphocytes for enhance of IgG response and through the regulation of IL-2, IL-4 and IL-10.

Key words: Rhodiola crenulata, IgG, IL-2, IL-4, IL-10
Introduction

Rhodiola is widely distributed at high altitudes (3000-5400m) in mountainous regions all over the world. There are over 200 different species of Rhodiola and about 20 of them are used in Asian traditional medicine, such as *Rhodiola rosea*, *Rhodiola crenulata* and *Rhodiola sacra* (Brown et al., 2002). It is commonly used to promote adaptogenic activity, enhance work and exercise performance, eliminate fatigue and prevent altitude sickness (Brown et al., 2002; Abidov et al., 2003). Beside these traditional functions, newly discovered properties have received a great deal of attention recently including: stimulation of nervous system (Perfumi et al., 2007), memory enhancement (Chen et al., 2008), anticancer properties (Tu et al., 2008), antioxidant properties (De Sanctis et al., 2004), regulation of blood sugar and antihypertensive actions (Kwon et al., 2006), cariotonic effect (Wang et al., 2006), and immune tonic properties (Shen et al., 2003; Mishra et al., 2006).

An effective immune response for preventing cancer diseases and infections has been correlated with the activities of antibody via the complement activation and ADCC pathway (Correa-Oliveira et al., 1989), macrophage (Ottesen EA, 1979) and T-cell (Buchanan et al., 1973). The differential role of CD4+ T-cell sub-populations is referred to as $T_{H1}$ and $T_{H2}$ (Mosmann et al., 1986; Mosmann et al., 1989). It is now believed that the $T_{H1}$ subset, which is IL-2 and INF-γ secreted, can promote the activation of $T_C$ and NK cells, and acts mainly in helping cell-mediated responses, including cytotoxicity to tumors and virus-infected cells (Young HA, 1996). IL-2 plays an important role in promoting T-cells proliferation, cytokine production, and the function properties of B cells, macrophages and NK cells (Blachere et al., 2006). On the other hand, the secretion of IL-4 and IL-10 from $T_{H2}$ activation can enhance the proliferation and antibody production of B cells, and helped humoral responses against infections (Lafreniere et al., 2006; Beissert et al., 2006). Redundancy-regulation has been noted between IL-2 and IL-4 on the class switch of B cells to produced IgG2a (Kindt et al., 2007).

Previous studies had shown that oral administration of *Rhodiola rosea* extract for superficial bladder carcinoma patients could improve the parameters of leukocyte integrins and T-cell immunity (Bocharova et al., 1997). *Rhodiola crenulata* could enhance the formation of antibodies and increase the moving index of white cells in heavy exercise training rats (Shi et al., 1999). The aqueous extract of *Rhodiola rhizome* stimulated IL-6, p-IkappaB, and NF-kB activation in PBMC and RAW264.7 macrophage cell line (Mishra et al., 2006). Salidroside of *Rhodiola algida* induced increase in IL-1, IL-2, IL-4, IL-8, IL-10 and TNF-α secretion and those gene expressions in human peripheral blood lymphocytes (Li et al., 2009). In this study, we evaluated the immunomodulatory activity of an aqueous-ethanol extract of *Rhodiola crenulata* (RCAE) on MPC-11 (mouse B cell line and secreted for IgG) and mouse spleen cells. Therefore, RCAE will exanimate to induce the
mRNA and protein production of IL-2, IL-4 and IL-10 on PWM-stimulated mouse spleen cells, and also activate the secretion of IgG on those spleen and MPC-11 cells. The major objectives of this investigation were determining: whether RCAE could regulate mouse immune responses.

**Materials and Methods**

*Aqueous-Ethanol Extraction of Rhodiola crenulata*

The herb of *Rhodiola crenulata* H. ohba was obtained from Tibet (China) by TCM BIOTECH INTERNATIONAL CORP. (Taipei, Taiwan), on October 2006. It has been confirmed by TCM BIOTECH INTERNATIONAL CORP. and kept in our lab numbered 920216. Two g of dried *Rhodiola crenulata* powder was extracted with 200 ml water in an 80°C heated water bath for 6 hours. This cold extract was precipitated by 4 folds of 95% Ethanol at 4°C overnight, and collected the precipitant by centrifugation and lyophilized. A crude brownish sample was weighted and resolved in PBS (the recovery was about 68.76~71.22%). After centrifugation at 10,000xg for 30 min, the resolute was filtrated through a Millipore filter (0.2μm) and stored at 4°C for the assay.

**Cell line and Mouse spleen cells**

MPC-11 cells secreted IgG were cultured in a complete medium containing RPMI-1640 (Gibco BRL Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Hyclone, Utah), 4 mM L-glutamine (Hyclone, Utah), 100 units/ml penicillin and 100 μg/ml streptomycin (Hyclone, Utah). These exponential growth cells were used for the following assay.

Spleen cells, separated from BALB/c male mice (age for 8-10 weeks), were washed in a complete medium as described above. Then, the spleen cells were mixed with 5 ml of cold ACK lying buffer for 5 min and added 20 ml of RPMI medium. After washed 3 times, these cells incubated 10% CO₂ 37°C for 3 hours. The suspended cells main contained B and T lymphocytes were used for the following assay.

**MTT assay**

Samples (1x10⁵ cells/ml) of MPC-11 and PWM-stimulated mouse spleen cells, which were added to a suboptimal dose of pokeweed mitogen (a final concentration as 2.5μg/1x10⁶ cells/ml) (PWM;Sigma, MO. USA), were treated with RCAE ranging from 0, 0.3125, 0.625…to 20μg/μl and analyzed by MTT assay (Chu et al., 2002). The viability was expressed as the ratio of OD₅₉₀ value of RCAE-induced cells to that of control cells.

**Determination of IgG production by ELISA assay**

Samples (1x10⁶ cells/ml) of the MPC-11 cells and 2.5μg/ml of PWM-stimulated mouse spleen cells were treated with RCAE in a dose dependent manner (0, 30, 60 and 120μg/ml) and harvested daily over a five-day period. The supernatants were added to a 96-well ELISA plate which was coated with Goat-anti-mouse IgG (Zymed, SSF. USA), stored at 4°C overnight, and then blocked with 1.0% Gelatin/PBS (Sigma, MO. USA) at 37°C for
1 hour. After 2 hours reaction at 37°C, each well of the plate was washed 3 times with 0.05% tween 20 / PBS (Sigma, MO. USA), and added HRP-Goat-anti-mouse IgG (Zymed, SSF. USA) at 37°C over 1 hour. All of the wells were washed with 0.05% tween 20 / PBS 3 times, and added 100μl of tetramethylbenzidine (TMB ; Nalgene, MA. USA) for each well and stood in a dark room over 20 min at room temperature. Then, we dropped 50μl of 2N H₂SO₄ to each well, after which allowed to determined the levels of OD₄₅₀ and OD₅₇₀ using a microplate reader (VERSAmax, MDC, USA). A standard curve of mouse IgG (Calbiochem, USA) was also detected as described.

**Determination of IL-2, IL-4 and IL-10 production by ELISA Kits**

Samples (1x10⁶ cells/ml) of mouse spleen cells received 2.5μg/ml of PWM and RCAE ranging from 0, 30, 60, to 120μg/ml, then harvested daily over 3 days. The production of IL-2, IL-4 and IL-10 of the culture medium taken from those cells were measured by Mouse Cytokine ELISA Kits (R&D, DY. USA). The assays were run using the general ELISA protocol with a sandwich design.

**RCAE-induced IL-2, IL-4 and IL-10 mRNA expression of PWM-stimulated spleen cells by RT-PCR assay**

**Isolation of RNA**

Samples (2x10⁷ cells/ml) of spleen cells were treated with 60μg/ml of RCAE and 2.5μg/1x10⁶ cells/ml of PWM for 12, 24, and 36 hours. After the harvested cells were washed twice with 1 x RNase-free PBS, the RNA isolation of these cells was performed as Chu and Tseng (2002). A 2 μl aliquot was used for RNA quantification according to GeneQuant II (Pharmacia, USA).

**Synthesis of cDNA**

The synthesis of cDNA from each mRNA sample was performed as Chu and Tseng (2002) and subsequently used as the PCR template.

**Primers**

The primers (Invitrogen, SSF, USA) used for PCR amplification were as follows: IL-2 sense sequence 5'-GACACTTGTGCTCCTTGTTCA-3’ and antisense sequence 5’-TCAATTCTGTGGCCTGCTTG-3’; IL-4 sense sequence 5’-TGCCTCCAAGAACACAACTG-3’ and antisense sequence 5’-AACGTACTCTGGTTGGCTTC-3’; IL-10 sense sequence 5’-ATGCAGGACTTTAAGGGTTACTTG-3’ and antisense primer: 5’-TAGACACCTTGGTCTGGAGCTTA-3’; β2 microglobulin sense sequence 5’-TGACCGGCTTGTATGCTATC-3’ and antisense sequence 5’-CAGTGAGCCAGGATATAG-3’ (Chopra et al.,1997; Ryan et al.,1998).

**PCR amplification**

The conditions for amplification were denaturizing at 94°C for 50 sec (5 min for the first cycle), IL-2, IL-4, IL-10 and β₂m annealing at 60°C for 45 sec, and extension at 72 °C for 45 sec (5 min for the last cycle) (Chopra et al.,1997; Ryan et al.,1998). The PCR products were subjected to
gel electrophoresis, staining by ethidium bromide, and an immediate density measurement for each band using a Multilmage™ system (Alpha Innotech, USA).

**Statistical analysis**

All data were expressed as mean ± SD, and the differences between groups were assessed with the student’s t-test. A value of p < 0.05 was considered statistically significant.

**Results**

**Effect of RCAE to the viabilities of MPC-11 mouse lymphoma cells and normal spleen cells**

In this study, we found that 5-10μg/ml of RCAE could increase about 10% of the viabilities of mouse spleen cells and decrease about 20% for MPC-11 (1x10⁵) by MTT assay, but without the effectiveness for the growth and viability of these cells below 2.5μg/ml of RCAE treatment (Fig.1A).

**RCAE induced the IgG production in MPC-11 and normal spleen cells**

Data showed that 60μg/ml of RCAE promoted the IgG production of MPC-11 cells (1x10⁶) from the second to fifth day of RCAE treatment (Fig.1B). On the other hand, the IgG secretions of PWM-stimulated mouse spleen cells in 30-120μg/ml of RCAE-added were enhanced significantly compared with the control groups from the third to fifth day treatment (Fig.1C). Furthermore, the RCAE effect to the cytokine regulation of mouse spleen cells could prove interesting and is therefore indicated in the following analysis.

**RCAE increase the mRNA and protein expressions of IL-2, IL-4 and IL-10 on mouse spleen cells**

To investigate the involvement of IL-2, IL-4 and IL-10, the protein expression for RCAE-treated mouse spleen cells was assayed by ELISA, and the level of mRNA expression in these cells was estimated using RT-PCR. In 60-120μg/ml of RCAE, the IL-2 and IL-4 secretions of PWM-stimulated mouse spleen cells were significant increase compared as those of the control groups (Fig.2A, Fig.2B). On the same condition, the IL-10 secretion of those cells by RCAE treatment showed little increase as compared with the untreated cells (Fig.2C). More then, there has been demonstrated that 60μg/ml of RCAE induced the mRNA expression of IL-2, IL-4 and IL-10 on PWM-stimulated mouse spleen cells as compared with the controls (Fig.2D). Results for the IL-2, IL-4 and IL-10 mRNA production of PWM-stimulated spleen cells with RCAE treatment gave virtually correlated results to the protein expression data as described above.

Taken together, this study demonstrated that the effect of RCAE in enhance of IgG response was via IL-2, IL-4 and IL-10 regulation pathway.

**Discussion**

In this paper, we clearly found that the viability of mouse spleen cells was increased after RCAE-treatment. More then, our findings indicated that RCAE could induce the IgG secretion of
MPC-11 and mouse spleen cells. Data supported that RCAE could enhance immune responses and showed no cytotoxic properties in the spleen cells of mouse. Beside the immunomodulatory activity, high doses of RCAE offered a cytotoxic effect on MPC-11 lymphoma cells indicated that RCAE probably help the anti-lymphoma function.

On the other hand, the mRNA and protein expressions of IL-2, IL-4 and IL-10 from T cell activation in PWM-stimulated mouse spleen cells of RCAE-treated were tested. Results demonstrated that the IL-2 and IL-4 mRNA and protein productions of spleen cells with 60-120 µg/ml RCAE-addition were dominantly expressed, and the IL-10 expressions of those cells were lightly increased. Together with the enhanced productions of IL-2, IL-4 and IL-10 on the spleen cells by RCAE-treatment, could increase the activation of B cells, T cells, NK cells, macrophages, and then promote the apoptotic ability to anti-cancer, infection-defense and other diseases. Therefore, RCAE may be considered a complementary alternative drug and an immunostimulatory potentiator. This strategy would likely have no toxic side-effects.

Diverse components from *Rhodiola crenulata* with different preparation act their different abilities as preventive and therapeutic agents. For example, Polysaccharides extracted from *Rhodiola sachalinensis* increased the activities of natural killer cells and proliferation in the spleen of Coxsackie B-3 virus-infected mice (Sun et al., 2001). Rhodiola Salidrosides A. Bor Gadoloside played mitogen-like effect to spleen lymphocytes and enhanced the cytotoxicity of macrophages in mice (Xie et al., 2003). Polysaccharide of Rhodiola increased the proliferation of antibody secretion cells and the Delayed-type hypersensitivity, and decreased the activity of IL-2 and T<sub>β</sub>/T<sub>β</sub> ratio in mice (Yuan et al., 2007). Additionally, Salidroside of *Rhodiola algida* enhanced the IL-1, IL-2, IL-4, IL-8, IL-10 and TNF-α secretion on human lymphocytes (Li et al., 2009). Therefore, further study on the relationship between the compounds of RCAE and their immune enhance activities are interesting and important.

**Acknowledgments**

This work was kindly supported by a grant from the Hungkuang University, Hospital of KangTein, and TCM Biotech International Corp., Taiwan (HK-KTOH-97-07).

**References**


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transcription factor nuclear factor-kappaB.

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Fig. 1A

Viability (%)/1x10^5 cells

RCAE (μg/ml)

MPC-11 cells
Mouse Spleen cells

Fig. 1B

IgG (μg/ml)/1x10^6 MPC-11 cells

RCAE 0 μg/ml
RCAE 15 μg/ml
RCAE 30 μg/ml
RCAE 60 μg/ml

Time (days)

*
Fig. 1C

Fig. 1: RCAE promoted the IgG productions of MPC-11 and mouse spleen cells, and no affect to the viabilities of those cells in the lower doses (A) The viabilities of MPC-11 and mouse spleen cells were shown in RCAE-treatment. (B, C) RCAE induced the IgG secretion of MPC-11 cells (B) and PWM-stimulated mouse spleen cells (C). Data are the means ± SD of eight similar experiments. * p < 0.05 indicated a significant difference from the untreated cells.
Fig. 2A

Fig. 2B
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Fig. 2: RCAE induced the IL-2, IL-4 and IL-10 mRNA and protein expressions in mouse spleen cells. (A) RCAE enhanced the IL-2 production on the PWM-stimulated mouse spleen cells. (B, C) RCAE increased the IL-4 (B) and IL-10 (C) secretions in the same cells. Data are the means ± SD of eight similar experiments. * p < 0.05 indicated a significant difference from the untreated cells. (D) Time-dependent gene expressions of IL-2, IL-4 and IL-10 in the PWM-stimulated mouse spleen cells were increased with RCAE-treatment. Experiments were repeated three times and yielded similar results.
大花紅景天乙醇萃取水溶液
調節老鼠IL-2, IL-4和IL-10
免疫功能之評估

吳全成1 朱敬儀2

1光田綜合醫院內科
2弘光科技大學護理系(所)

摘要

約20種紅景天(Rhodiola)植物在臨床上有治療心血管疾病、神經系統疾病與抗惡性腫瘤等雙向
調節活性功能。本實驗主要評估大花紅景天(Rhodiola crenulata)水-乙醇萃取液(簡稱RCAE)調節老鼠
體液性免疫功能之影響，結果發現：低濃度RCAE (≦2.5 µg/ml)對老鼠B淋巴癌細胞株MPC-11(IgG
分泌型)及老鼠脾臟淋巴細胞(1x10^5)的存活率並無顯著影響；而高濃度RCAE (5-20 µg/ml)對MPC-
11具有約20%的胞殺作用；卻對老鼠脾臟淋巴細胞增加約10%的存活率。同時，以酵素免疫分析
法(ELISA)檢測RCAE對MPC-11及老鼠脾臟淋巴細胞的IgG分泌活性，數據顯示60 µg/ml RCAE會增
加MPC-11(1x10^6)細胞IgG分泌量；而30-120 µg/ml RCAE亦可增加老鼠脾臟淋巴細胞(1x10^6)細胞IgG分泌
量。進一步利用RT-PCR及ELISA方法分析60 µg/ml RCAE對老鼠脾臟淋巴細胞分泌第二介白質(IL-
2)、第四介白質(IL-4)及第十介白質(IL-10)等細胞激素的變化，結果印證RCAE能活化老鼠脾臟淋巴
細胞，增強IL-2、IL-4及IL-10之mRNA表現量及蛋白質分泌量。綜合上述，本研究證實RCAE不僅
能促進老鼠B淋巴球活化，增強製造IgG；並可藉由激活T淋巴細胞分泌IL-2、IL-4及IL-10等細胞激
素來調節促進免疫反應。

關鍵詞：大花紅景天、IgG、第二介白質、第四介白質及第十介白質