# Immunotoxicity of Hydrocortisone on Th1/Th2-Related Cytokine Production Is Associated with *Yang*-Deficient State in Traditional Chinese Medicine

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Steroid hormone serving as an immunosuppressor often induces immunotoxicity when administered in highest dosage or accumulated in long-term usage. The stage of high concentration of steroid hormone leading to a wide range of symptoms is associated to the *yang*-deficient state, which is the part of *yin-yang* imbalance involved in processes of many diseases in traditional Chinese medicine. Here we intend to investigate the profile of Th1/Th2-related cytokine transcriptions under *yang*-deficient conditions in a *yang*-deficient animal model by intramuscular injection of hydrocortisone (a kind of steroid hormone). The *yang*-deficient symptoms were estimated by detecting activity, appetite, body weight and so on. T cell proliferation and cytokine transcriptions were analyzed. The results showed that *yang*-deficient mice were established successfully since typical *yang*-deficient symptoms were observed in this model with decreased activities, appetite, body weight and temperature. More interestingly, the transcriptions of IFN- $\gamma$ , IL-2, IL-4 and IL-10 in this model were markedly suppressed and the proliferation of lymphocytes significantly decreased as well. The results suggested that *yang*-deficient symptoms were related to the steroid-induced reduction of cytokine transcription and impairment of lymphocyte proliferation. Therefore, novel strategies through regulating cytokine production might be considered as potent approach to patients with *yang*-deficiency symptoms. *Cellular & Molecular Immunology*. 2007;4(5):383-388.

Key Words: hydrocortisone, *yin-yang* balance, traditional Chinese medicine, cytokine

# Introduction

Homeostasis refers to the bodys maintenance of a consistent internal environment, which is maintained by numerous parameters including immune factors and hormone. Homeostatic mechanisms are necessary to control a vast number of parameters when challenged with stressor and invaders in the continuation of life (1, 2). Especially, a pair of counteractive events can regulate the local equilibrium by negative and positive regulation. In immune system, cytokines are involved in the lymphoid homeostasis as these pleiotropic

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factors can modulate cell survival, growth, differentiation and apoptosis (3). In particular, the T-cell cytokines play crucial roles in immune homeostasis by counteraction of Th1 and Th2 (4). IFN- $\gamma$  and IL-2 (Th1) are responsible for cellular immune responses, while IL-4 and IL-10 being ascribed to type II cytokines are thought to accelerate the humoral immune responses (5). In normal condition, these cytokines interact with each other to balance the ability of generating a vigorous response to provide defense against invaders and the body tissues from being damaged in responses (6). This counterbalance of homeostasis can be disturbed by elevated steroid hormone due to suffering from chronic diseases or owing to therapies with high dose of glucocorticoid (GC). especially in acute alteration of GC. Under this condition, patients with various disorders often suffer from symptoms. Evidence was emerging of the involvement of IFN function in the development of the common symptoms in many diseases (7). Since symptoms can provide earliest information for patients, the traditional Chinese medicine (TCM) emphasizes the detections and treatments of disease-related symptoms to decrease the chance of developing the complication of diseases. The therapeutic methods depend upon the theory of TCM derived from hundreds of histories of clinical practices.

*Yin-yang* theory is an essential part of TCM in diagnosis and treatment based upon overall analysis of symptoms and

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phenotypes of the body (8). Yin-yang balance is the optimal state of life owing to the equilibrium of yin-yang counteraction within a narrow range of variation, caused by the interplay, interdependence, transformation and suppression of each other between the counterparts of *yin-yang*. Yin-yang imbalance is the status of maladjustment of *vin-vang* overrun that means one is in dominant state and the other is in inferior position. Yang-deficient involved in the yin-yang imbalance points at the functional lowlife showing various symptoms such as lassitude and impotence, cold-blooded and cold-feared, lumbago and fatigue. In most case, people accepted the clinical validity of *vin-vang* usage, but sometimes doubted its concepts and theories in science due to its lacking experimental evidences. In fact, cumulated clinical observations demonstrated vang-deficient patients diagnosed on their symptoms and complaints showed hypothalamic-pituitary-adrenal axis (HPA axis) disorganization with increased GC concentration (9, 10). Furthermore, the yang-deficient model mice established by hydrocortisone (HC) injection showed symptoms similar to clinical phenotypes of *yang*-deficient patients. Until now, little is known about the immunological mechanisms underlying the symptoms in *yang*-deficient condition. It remains unclear whether the mechanisms drive the common symptoms in different diseases. As an end effector of HPA axis, HC can inhibit the expression of Th1/Th2-related cytokines (11, 12). In the present study, we wanted to further know about the expression of Th1/Th2-related cytokine IFN-y, IL-2, IL-4 and IL-10 in HC-induced yang-deficient mice with standard vang-deficient symptoms.

## **Materials and Methods**

#### Mice

Inbred female BALB/c mice (7-8 weeks of age) were used in the experiments and kept in animal breeding unit with conventional conditions (23°C, 50%-60% humidity and pathogen-free condition) provided by School of Pharmacy, Shandong University, China. Mice were maintained six per cage with free access to food and water under a 12-h light/dark cycle. All murine treatments were accorded with the criterion of Ethic Committee of Shandong University, China.

#### Hydrocortisone treatment

Varying doses (5 mg/kg, 10 mg/kg and 20 mg/kg *b.w.*) of HC (Shanghai Pharmaceutical Co., China) dissolved in 500  $\mu$ l PBS were injected intramuscularly (*i.m.*) once per day for 7 days and a single dose of 20 mg/kg once per day for three time courses (3 days, 5 days and 7 days), respectively, to determine the feasible dosage to establish the *yang*-deficient model mice. Matched control mice were treated with corresponding volume of PBS. Six mice were used in each group of all experiments.

## Behavioral analysis of yang-deficient mice

The typical symptoms of yang-deficient mice were estimated

by murine activity (judged by the duration of forced swim of each mouse), appetite (the weight of consummation for 24 h of each mouse), temperature (in Celsius), and body weight conducted as following description.

## Judgment of activity by forced swim of mice

After treated with HC, the mice carrying a spring (weighing 2 g) around its tail were dropped individually into stainless steel cylinder (height 30 cm, diameter 30 cm) containing 20 cm depth of water that was maintained 21-22°C. A mouse was judged to be immobile when it floated in an upright position to keep its head above water. The duration of immobility of swimming mice regarded as activity, were measured before murine head sink into water (13, 14).

#### Detection of appetite, temperature and body weight

At the endpoint of treatment with HC for 7 days, mice were detected the appetite by examining the weight of consummation for 24 h. The rectal temperature of each mouse was measured with a digital thermometer in Celsius (T-50). The depth of rectal probe was 1 cm and the probe was maintained in the rectum until the temperature was stabilized.

## Splenocyte preparation

All mice were sacrificed after injection with different concentration of HC. Splenocytes were extracted and disrupted in ice-cold PBS. Single cell suspension was filtered through nylon mesh and isolated by Ficoll solution. After being washed twice with PBS, the splenocytes from differently treated mice were immediately stored at  $-80^{\circ}$ C for IFN- $\gamma$ , IL-2, IL-4 and IL-10 mRNA analysis by RT-PCR.

## Cytokine analysis by RT-PCR

RNA was extracted with Trizol (SIGMA) according to the manufacturer's protocol. The optical density of 260 nm and 280 nm determined the concentrations of RNA. First strand cDNA was synthesized from 2 µg of total RNA using M-MLV reverse transcriptase kit (Shanghai Shenggong, China) under recommended procedure. PCR reaction was conducted in a final volume of 50 µl containing 10 µl of cDNA mixture, 5 µl of 10× PCR buffer, 2 µl of 10 mM dNTP, 5 U Tag polymerase, and 25 nM a pair of specific primers. Sequences of primer were described previously (15). 28 cycles were conducted in 95°C, 55~58°C and 72 °C. The products of RT-PCR were electrophoresed in 2% agarose gels stained with EB. Alpha Innotech software was used to quantitatively analyze the intensity of cytokine expression according to the electrophoresis size and optical density of specific segments of each cytokine product (16).

## Cell culture

Splenocytes isolated from normal mice were suspended in ice-cold PBS. After being centrifuged in Ficoll-Hypaque gradient, the single cells were washed by PBS twice and suspended in completed RPMI 1640 (GIBCO) to an end concentration of  $5 \times 10^6$  cells/ml, which supplemented with penicillin 100 IU/ml, streptomycin 100 IU/ml and 10% fetal bovine serum. Viable cell counting was conducted by trypan

Table 1. Typical symptom in yang-deficient mice

Group	Physical Signs			
	Activity (min)	Appetite (g/d)	Temperature (°C)	Body weight (g)
Yang-deficient	$6 \pm 1.6^{*}$	$3.0 \pm 0.1^{*}$	$35.3 \pm 0.3^*$	$18.2 \pm 0.7^*$
Control	53 ± 11	$3.3 \pm 0.1$	$36.8 \pm 0.7$	$22.2 \pm 2.1$

The mice were treated with HC (20 mg/kg for 7 days, *i.m.*) and each group contains 6 mice. \*, compared with control mice p < 0.01.

blue exclusion. About  $1 \times 10^7$  cells were cultured per well in total volume of 2 ml in 24-well plates. The cells were cultured in different final concentration of HC ( $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$  and  $10^{-7}$ g/L) or  $10^{-5}$  g/L of HC with 5 µg/ml of Con A at 37°C with 5% CO<sub>2</sub>. As a matched control, the same volume of vehicle was added. After cultured for 4 h, the cells were harvested and stored at -80°C for cytokine transcription analysis by RT-PCR.

#### Splenocyte proliferation in HC-treated mice in vitro

Splenocytes separated from HC-treated mice were used to analyze the proliferation activity in response to mitogen *in vitro* by MTT (17). Splenocytes washed by PBS twice were resuspended in RPMI 1640 and adjusted to an end concentration of  $5 \times 10^6$  cells/ml as described above. The cells were cultured with 5 µg/ml concanavalin A (Con A, SIGMA) and incubated for 24 h or 48 h at 37°C in 5% CO<sub>2</sub>. For the last 4 h of incubation, 100 µl supernatant was discarded, and 10 µl of 5 mg/ml MTT were added to each well of 96-well plate. After 4 h additional incubation at 37°C with 5% CO<sub>2</sub>, 100 µl of 0.04 N HCl in isopropanolol was added to each well to dissolve the colored precipitated MTT. Absorbance was read using a dual wavelength of 570 nm and 630 nm by an automated reader.

#### Statistical analysis

Statistical analysis of data was conducted by SPSS software and all experiments were repeated at least three times. The p value less than 0.05 was regarded as statistically significant.

### Results

#### Typical symptoms in yang-deficient mice

It was reported that increased GC level could lead to behavioral and peripheral changes in *yang*-deficient patients. We were interested in whether mice with increased GC level could show the typical signs of *yang*-deficient symptoms characterized by decreased activity. Female BALB/c mice were used to establish the *yang*-deficient model by intramuscular injection with three dosages of hydrocortisone (5 mg/kg, 10 mg/kg and 20 mg/kg *b.w.* in 500  $\mu$ l PBS once per day for 7 days) and a single dose of 20 mg/kg once per day for three time courses (3 days, 5 days and 7 days after injection), respectively. After different treatments we found there was significant effect on the duration of immobility of swimming mice treated with 20 mg/kg of HC for 7 day. This group of mice had the typical symptoms of *yang*-deficiency such as decreased activity (duration of immobility), appetite, temperature and body weight (Table 1).

#### HC-induced inhibition of cytokine transcriptions in vitro

As the *yang*-deficient mice suffered from typical *yang*deficient phenomenon after treated with the high dosage of HC, we detected the effects of HC on IFN- $\gamma$ , IL-2, IL-4 and IL-10 transcriptions by RT-PCR *in vitro*. Splenocytes from normal mice were used to detect the dose-dependent suppression of HC on cytokine transcriptions. After cultured with different concentrations of HC for 4 hours, IFN- $\gamma$ , IL-2, IL-4 and IL-10 mRNA expressions were seriously inhibited in a dose-dependent manner (Figure 1A). To better





Figure 1. HC-induced inhibition of cytokine transcription *in vitro*. Splenocytes from normal mice were cultured with different concentrations of HC ( $10^{-7}$ ~ $10^{-3}$  g/L) for 4 h (A) or  $10^{-5}$  g/L of HC with Con A (5 µg/ml) stimulation (B). Total RNA was isolated from cultured splenocytes. RT-PCR was used to analyze the transcriptions of IFN- $\gamma$ , IL-2, IL-4 and IL-10 as described in Materials and Methods.



**Figure 2.** HC-induced inhibition of cytokine transcription in *yang*-deficient mice. Mice were intramuscularly injected with different doses of HC (5 mg/kg, 10 mg/kg or 20 mg/kg) for 7 days (A), or 20 mg/kg for 3, 5 and 7 days (B). Data were shown as electrophoresis image of RT-PCR bands and statistic results of relative intensity that were shown as mean  $\pm$  SD from six samples of each group. \*, p < 0.05, compared with matched control mice.

understand the cytokine production under active state, we also measured the cytokine mRNA levels in the presence of Con A stimulation *in vitro*. As shown in Figure 1B, IFN- $\gamma$ , IL-2, IL-4 and IL-10 transcriptions were seriously inhibited by HC (10<sup>-5</sup> g/L). IL-10 transcription showed more sensitivity to HC-induced reduction of cytokine in Con A stimulation.

## Inhibition of cytokine transcriptions in HC-induced yangdeficient mice

As an inductor of *yang*-deficient mice, HC showed inhibited effects on cytokine transcriptions *in vitro* both in absence and presence of Con A stimulation. To confirm the phenomenon of HC-induced suppression on cytokine mRNA *in vivo*, mice were intramuscularly injected with HC with different concentrations or a single dose for different time courses (3, 5 and 7 days). The results of RT-PCR exhibited that HC dramatically decreased IFN- $\gamma$ , IL-2, IL-4 and IL-10 mRNA transcriptions especially in high dose level for a long duration of HC injection (Figure 2).

## *Reduced proliferation of splenocytes from HC-induced yangdeficient mice*

Since mRNA transcriptions of IFN- $\gamma$ , IL-2, IL-4, and IL-10 were down-regulated in HC-treated mice, we further examined the proliferation of splenocytes from HC-treated

mice. We found that proliferation of splenocytes from HC-induced *yang*-deficient mice was dramatically inhibited in response to Con A stimulation *in vitro* (Figure 3).



Figure 3. Reduced proliferation of splenocytes in HC-treated *yang*-deficient mice. Splenocytes were isolated from HC-treated mice (0, 5, 10 and 20 mg/kg for 7 days). After co-cultured with Con A (5  $\mu$ g/ml) for 24 h or 48 h, the spleen cells were tested by MTT using an automated plate reader at 570 nm. The values of data were shown as mean  $\pm$  SD (n = 4).

## Discussion

The T-cell cytokines play important roles in immune homeostasis (18, 19). The patterns of T-cell cytokines maintain the stability of immune microenvironment that is essential for the normal body condition. As an end effector of HPA axis, GC can induce extensive effects on immune system. The lymphocyte differentiation, proliferation and polarization can be affected by the changes of internal GC level (20-22). T lymphocytes are more sensitive to immunosuppressive GC than other lymphocytes. As a result, the secretion of cytokines is accordingly disturbed. Disturbance of T-cell cytokine paradigm induced by GC will induce disorder of life with various symptoms (23, 24). It is poorly understood how the production of T-cell cytokines is connected with exterior phenomenon. A large number of studies on IFN- $\gamma$ , IL-2, IL-4, and IL-10 pay more attention to mechanisms of diseases than to symptoms or behavioral signs. Since symptoms can provide earliest information for patients, the TCM emphasizes the detection and treatments of disease symptoms to decrease the chance of developing the complication of diseases and improve the quality of life. Symptoms are defined as 'Zheng' and served as the clinical evidence for evaluating the diagnosis and prescription in TCM. Yin-yang theory is the essential part of TCM, which deals with many symptoms as witness for clinical practices. Yang-deficiency is one side of *vin-yang* disturbance; a series of symptoms are ascribed to yang-deficient such as anorexia, cool-blooded, fatigue and dysfunction. In the present study, we established the *yang*-deficient model with HC injection to explore T-cell cytokine expression under vang-deficient condition. In order to detect the role of HC-induced yangdeficient status, we injected BALB/c mice with different doses of HC, and only the high-level-treated mice developed overt standard yang-deficient phenotypes in short-term duration (data not shown). The profile of T-cell cytokines under this condition might provide incipient information about T-cell activity responding to the acute alteration of HC at early stage of some diseases including acute malnutrition. The results showed that the mice treated with high-dose of HC suffered from typical yang-deficient symptoms with reduced activity and decreased appetite, body weight and temperature. The high-level HC administrations at circadian nadir in mice must have disturbed the rhythm of murine HPA axis action and consequently might result in disorders of endocrine system, which initiated the impaired appetite and weight-loss in mice. Along with the evident yang-deficient symptoms, the mice showed obvious inhibition on IFN- $\gamma$ , IL-2, IL-4 and IL-10 production in transcription level. The decreased proliferation of lymphocytes responding to Con A stimulation demonstrate that T lymphocytes are more sensitive to HC inhibition both in silent and activated states, at least partly, by inhibiting the transcription of cytokine genes of IFN- $\gamma$  and IL-2. IL-4 and IL-10. The results suggested that the *yang*-deficient symptoms were related to HC-induced serious immunosuppressive condition, and the impaired T-cell cytokine production is involved in the yang-deficient process. Therefore, novel strategy through

regulating cytokine production might be considered as potent approach to patients with *yang*-deficiency.

In summary, this study showed the first look in describing the immune mechanisms under *vang*-deficient status. However, although this yang-deficient murine model can not mimic the general mechanisms for clinical induction of yang-deficient patients, these results potentially provided one mechanism by which weakly Th1/Th2-related cytokine expression is involved in processes of yang-deficient developments, and it makes possible to analyze the clinical symptoms of *yang*-deficiency. Therefore, the strategy to boost Th1/Th2-related cytokine expression will benefit the recovery of *yang*-deficient patients. However, although we have detected the suppression of high-level HC in Th1/Th2related cytokine production under yang-deficient conditions, the modifications observed in steroid hormone-treated mice were not only the results of alterations of cytokine expression profile but also a consequence of metabolic disorder on central system. For all that, the elaborate mechanisms related to human diseases with debilitating syndromes in vangdeficient status remain to be further studied.

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