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원 저

Combined Treatment of Colchicine and Herbal Medicines (*Gamichunghyulbohyul-tang* or *Gamiyongdamsagan-tang*) Attenuate the Behcet's Disease Symptoms in Mice

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베체트병 마우스 모델에서 증상의 호전에 대한 콜키친과 한약 (가미청혈보혈탕 또는 가미용담사간탕)의 복합 투여 효과

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목적 : 단순포진바이러스로 유발한 베체트병 마우스 모델에서 가미청혈보혈탕과 가미용담사간탕을 colchicine과 함께 투여할 경우, 한방 혹은 양방 단독 투여의 경우와 비교하여 베체트병 증상의 호전에 어떠한 영향을 주는지 알아보고자 하였다.

방법 : colchicines, 가미청혈보혈탕, 가미용담사간탕을 각각 또는 colchicines+가미청혈보혈탕, colchicines+가미용담사간탕을 20일간 복합 투여하여 베체트 증상이 호전 되는 정도를 살펴보고, 호전 되는 증상의 종류를 파악하고, 이때 동반되는 싸이토카인을 RT-PCR, FACS 등으로 확인하고자 하였다.

결과 : 단독투여군보다 복합투여군에서 증상이 호전되는 율이 높았으며, 호전 시기를 앞당겼고, 이때 싸이토카인 interleukin-4의 발현이 증가하였다.

결론 : 베체트병 마우스 모델에서, 양방과 한방의 복합투여가 단독투여보다 증상의 호전에 보다 더 효과적이었다. *(J Korean Oriental Med 2001;22(2):102-108)*

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Introduction

Behcet's disease (BD) is a recurrent, chronic, and multisystemic disorder with mucocutaneous, ocular, arthritic, vascular, gastrointestinal and central nervous system involvement. The etiology of BD has been

linked to viral infection, autoimmune disease, streptococcal-related antigens, specific alleles of the human major histocompatibility complex, and hazardous chemicals[1-4]. Since Hul si Beh et first propounded viral etiology in 1937[5], many studies have suggested a viral involvement in the disease[6,7]. By DNA-DNA dot blotting and polymerase chain reaction, HSV DNA was also detected in patients' leukocytes and saliva[8,9]. However, treatment with acyclovir, which is of proven efficacy in the treatment of HSV infection, did not much alleviate the frequency or severity of orogenital ulceration or other clinical features of BD[10]. So, immune cell dysfunction triggered by HSV might be related to the pathogenesis of this disease, and has been reported by several research groups[11-14]. Until now, the drugs used for the treatment of BD have not been effective enough for the complete management of the symptoms. To find other possibilities for alleviation of the symptoms of BD, we applied *Gamichunghyul-bohyul-tang* and *Gamiyongdamsagan-tang* combined with colchicine, a drug that is most widely used as a medication for BD, to the HSV-induced BD mouse model.

Materials & Methods

A. Animals and induction of BD symptoms

Four to 5-week-old male ICR mice were used for this study. Using the method of Hirata *et al.*[15], the earlobes of the mice were scratched with a needle, then inoculated with 1.0 x 10^6 plaque forming units/ml of HSV type 1 (F strain). Virus inoculation was performed twice with 10 day interval, followed by 16 weeks of observation[16]. Mice were bred in temperature- and light-controlled conventional rooms (20-22 ℃, 12 hr light cycle starting at 8:00 a.m.). The mice had free access to food and water. During the experimental period, the animals were closely observed and photographed. The animals were handled in accordance with a protocol approved by our institutional animal care committee.

B. Gross observation of BD symptoms

In order to classify the symptomatic mice as having BD, we followed the revised Japanese classification with minor modifications. Oral, genital and other skin ulcers (including bulla & crust) and eye symptoms were classified as major symptoms. Arthritis, gastrointestinal ulcers and neurologic involvement were identified as minor symptoms. Mice with at least one major and one minor symptom were classified as having BD. Symptomatic mice were photographed with a Nikon FM2 camera equipped with a 105 mm microlens.

C. Major ingredients and dosages of colchicines and herbal medicines

D. Water extraction of herbal medicine

Mixed herbs were boiled in 3 times weight of water for 3 hours, and filtered with gauze, then lyophilised. Water solved lyophilised powder was oral administered to BD mice or control mice.

E. RNA isolation & RT-PCR

Total RNA was isolated by acid guanidium thiocyanate-phenol-chloroform extraction[17]. Spleen tissues were homogenized in 1 mL of extraction buffer, composed of 4 M guanidine solution (Aldrich, Milwaukee, WI, USA), 25 mM sodium citrate pH 7.0, 0.5 % sodium N-lauroyl sarcosinate (Fisher, Pittsburgh, PA), and 0.1 M 2-mercaptoethanol (Sigma, St. Louis, MO). A 1/10 volume of chloroform : isoamyl alcohol (49 : 1) was added to the samples, which were incubated on ice for 5 minutes, then centrifuged at 10,000 xg for 15 minutes at 4 ℃. RNA contained in the upper aqueous phase was collected, precipitated with

Major Ingredients		Dosage
Colchicine		2 μg/day/mouse 20 days
Gamichunghyulbohyul-tang	*paeoniae radix alba* *angelicae gigantis radix* *atractylodis macrocephalae* *astragali radix* *artemisiae capillaries herba* *lonicerae flos* *poligoni cuspidate rhizoma*	8 mg/day/mouse 20 days
Gamiyongdamsagan-tang	*gentianae radix* *bupleuri radix* *forsythiae fructus* *scrophulariae radix* *gardeniae fructus*	6 mg/day/mouse 20 days
Colchicines+ *Gamichunghyulbohyul-tang*		2 μg/day/mouse+ 8 mg/day/mouse 20 days
Colchicines+ *Gamiyongdamsagan-tang*		2 μg/day/mouse+ 6 mg/day/mouse 20 days

the equal volume of isopropanol, and washed twice in 70 % ETOH. RNA pellets were dissolved in distilled water, quantified by OD 260/280 determination, and visualized in an ethidium bromide stained agarose gel. Two micrograms of total RNA were reverse transcribed using a cDNA kit (Gibco BRL, Grand Island, NY, USA), using oligo dT primers and AMV reverse transcriptase to generate cDNA for use as a template in PCR amplifications. Two microliters from the reverse transcriptase reaction were added to 50L reaction mixtures containing 50 mM KCl pH 8.4, 20 mM Tris-HCl, 2.5 mM $MgCl_2$, 200μM dNTPs, 2.5 U of Taq polymerase (Gibco BRL), and 1.2 μM primers. Specific primers for cytokines and beta-actin were as follows :

β-actin[18]
sense: 5' -TGGAATCCTGTGGCATCCATGAAAC-3'
antisense : 5' -TAAAACGCAGCTCAGTAACAGTCCG-3'
IL-2[19]
sense : 5' -TGATGGACCTACAGGAGCTCCTGAG-3'
antisense : 5' -GAGTCAAATCCAGAACATGCCGCAG-3'
IL-4[20]
sense : 5' -ACGCCATGCACGGAGATGGAT-3'
antisense : 5' -CAAGCATGGAGTTTTCC-3'
IL-10[21]
sens : 5' -AGACTTTCTTTCAAACAAAGGACCAGCTGGA-3'
antisense : 5' -CCTGGAGTCCAGCAGACTCAATACACACTGC-3'
IFN-γ[18]
sense : 5' -AGCGGCTGACTGAACTCAGATTGTAG-3'
antisense : 5' -GTCACAGTTTTCAGCTGTATAGGG-3'

The amplification was processed in a Perkin Elmer Thermo Cycler 900 with an initial 5 minutes denaturation at 94 ℃, followed by 35 cycles of the profile : 94 ℃ for 30 seconds ; 56 ℃ for 30 seconds ; and 72 ℃ for 1 minute. The products were subjected to electrophoresis on a 1.8 % agarose gel and visualized under UV light.

F. Flow cytometric analysis of intracellular cytokines

Before intracellular cytokine staining, splenocytes were freshly isolated. Brefeldin A (5 g/ml) (Sigma, St.

Louis, MO) was added for the last 4 hours of incubation to accumulate cytokines in the Golgi complex. Cells were harvested, washed in culture medium containing brefeldin A and fixed with 4 % formaldehyde in 1 % fetal bovine serum containing PBS for 20 min at room temperature. Then, cells were permeabilized with 0.1 % saponin in PBS containing 1 % fetal bovine serum and 0.1 % sodium azide (saponin buffer) for 10 min at room temperature. Cell suspensions were then treated with FITC- or PE-conjugated anti-IL-4, IFN-γ antibody (CalTag, Burlingame, CA) suspended in permeabilized buffer. Samples were analysed on a flow cytometer FACS Vantage (Becton Dickinson) collecting at least 20,000 gated lymphocytes.

Results

Multisystemic disorder with mucocutaneous, ocular, arthritic, vascular, gastrointestinal and central nervous system involvement were appeared in HSV-inoculated mice. A mouse with at least one major and one minor symptom were classified as having BD. In order to classify the symptomatic mice as having BD, we followed a revised Japanese classification with minor modifications. Oral, genital and other skin ulcers (including bulla & crust) and eye symptoms were classified as major symptoms, and arthritic, vascular, gastrointestinal and central nervous system involvement were classified to minor symptoms[16]. HSV-induced BD mice treated with colchicine combined with *Gamichunghyulbohyul-tang* and *Gamiyongdamsagan-tang* showed improvement in symptoms(Fig. 1). Colchicine combined with *Gamichunghyulbohyul-tang* and *Gamiyongdamsagan-tang* treatment were more effective to BD-like symptoms compare to colchicine treated alone. The treatment of herbal medicines without colchicine also improved the BD symptoms, and improved ratio was similar to with colchicine (Table 1). The beginning of improvement was noted with combined treatment of colchicine with herbal medicines at 3-25 days, compare to 6-29 days with single treatment of colchicine or herbal medicine. Most improved symptoms by treatment of colchicine and/or herb medicines were muco-cutaneous manifestations (Table 2).

Improved mice by treatment of herb medicines expressed Interleukin-4 (IL-4) mRNA in spleen tissues. Improved mice by treatment of colchicine alone or colchicine combined with herb medicines expressed raised intracellular IL-4 in splenocytes by FACS, though IL-4 mRNA were not expressed by RT-PCR, compare to no-treated BD mice (Table 3,4).

As a whole, improvements administered by colchicine combined with herbal medicines accompanied the alterations of cytokine expression, IL-4. Improved lesions mainly limited in mucocutaneous manifestation.

Fig. 1. Improved skin lesions of mouse with combined treatment of colchicine and *Gamiyongdamsagan-tang* (a. before treatment, b. after treatment).

Discussion

Colchicine is effective and frequently used drug for BD patients, but only shows limited successes. Azathioprine, indomethacin, cyclophosphamide, chlorambucil, levamisole, transfer factor, fibrinolytic therapy, dapsone, and systemic corticosteroids also show limited successes. Recently, cyclosporine, thalidomide, interferon-, interferon-, acyclovir, and FK-506 have been tried but the effectiveness of those drugs did not show any significant differences from the previous ones[22]. Acyclovir, antiviral agent, was tried as a therapeutic drug for BD. But, it showed only negative results as it failed to alleviate the frequency and severity of orogenital ulceration and ocular manifestation[23,24]. Famciclovir treatment decreased mortality and prolonged the survival time in HSV-1 infected mice[25,26]. But administration of Famciclovir was effective only in 40 % of BD mice[27]. In oriental medicine, the cause of BD is considered to downward flow of damp-heat(濕熱下注) and extream heat due to defiency of *yin*(陰虛熱毒). Therefore eliminating dampness and heat(淸熱利濕) and replenishing the vital essence and removing heat(養陰淸熱) are applied to oriental medical treatment for BD[28]. Clinically *Chunghyulbohyul-tang* and *Yongdamsagan-tang* are generally used[28]. In this study, *Gamichunghyulbohyul-tang* and *Gamiyongdamsagan-tang* combined with colchicine are used. Colchicine combined with herb medicines were effective in 50-67 % of BD mice compare to showing 40 % effectiveness of Famciclovir or 33 % effectiveness of colchicine treatment alone. Herb medicines induced IL-4 mRNA,

Table 1. Improvement of HSV-Induced BD Mice by Combined Treatment of Colchicine and Herbal Medicines

Treatment	Improved mice/ Total mice (%)
Colchicine	2/6 (33.3)
Gamichunghyulbohyul-tang	5/8 (62.5)
Gamiyongdamsagan-tang	5/9 (55.6)
Colchicines+ *Gamichunghyulbohyul-tang*	4/6 (66.7)
Colchicines+ *Gamiyongdamsagan-tang*	4/8 (50.0)

Table 2. Improved Symptoms of BD Mice Treated with Colchicine and/or Herbal Medicines

Treatment	Improved Symptoms
Colchicine	Skin crust, skin ulcer
Gamichunghyulbohyul-tang	Gastrointestinal involvement, erythema, skin crust, oral ulcer
Gamiyongdamsagan-tang	Skin crust, skin ulcer
Colchicine + *Gamichunghyulbohyul-tang*	Peri-ocular crust, erythema, skin ulcer
Colchicine + *Gamiyongdamsagan-tang*	Skin crust, skin ulcer

Table 3. Cytokine Expressions of BD Mice Analyzed by RT-PCR & FACS (n=5)

RT-PCR			FACS	
IFN-γ	IL-4	β-actin	IFN-γ	IL-4
+	-	+	23	8

RT-PCR data represent one of three replicate samples from two representative experiments and FACS demonstrate mean of three replicate samples from two representative experiments.

Table 4. Analysis of Improved BD Mice Treated with Colchicine and/or Herbal Medicines (n=3)

Treatment	RT-PCR		FACS
	IFN-γ	IL-4	IL-4
Colchicine	+	-	26
Gamichunghyulbohyul-tang	+	+	
Gamiyongdamsagan-tang	+	+	
Colchicine + *Gamichunghyulbohyul-tang*	+	-	11
Colchicine + *Gamiyongdamsagan-tang*	+	-	45

one of Th2 cytokines that has been known for improving inflammatory diseases. Colchicine increased protein level of IL-4. Combined treatment of colchicine and herb medicines expressed an increased protein level of IL-4. An increased protein level of IL-4 could be helpful for improvement of BD symptoms. Induced IL-4 mRNA by herb medicine alone is also helpful to improve the BD symptoms, but might be needed more time to act. The effect of colchicine combined with herb medicines was advanced the beginning of improvement. These combined treatment also accompany with the recurrence of BD symptoms 2-3 weeks later showed improvement about 30 %. And effectiveness of colchicine combined with herb medicines limited in mucocutaneous manifestations in BD mice. The side effect of herb medicines was weight loss in 10-18 % of body weight treated by 3 times concentrated herb medicines for 10 days administer to normal healthy mice.

In conclusion, combined treatment of colchicine and herbal medicine was more effective in improving BD symptoms than a single treatment of colchicines in HSV-induced BD mouse model. The improvement accompanied the cytokine, IL-4 expression.

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