Immunoregulation Effect of KamiBohuh-tang

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<Abstract>

The purpose of this study is to prove the efficacy of KamiBohuh-tang(KBT) on immunoregulation and the possibility of KBT as an immunoadjuvant. KBT with solid feed was administered orally once a day for 7 days to an experimental group, a solution of salt and solid feed without KBT to a control group. After a week T cell, B cell, cytokines, nitric oxide and phagocytic activity are measured. KBT enhanced the proliferation of splenocytes and the subpopulation of Th cells in splenic T-lymphocytes, but did not affect the proliferation of thymocytes. KBT decreased the subpopulation of T-lymphocytes in splenocytes. KBT enhanced the production of interferon-γ, interleukin-2, interleukin-4 in mice serum and the phagocytic activity in peritoneal macrophages but it suppressed the production of nitric oxide. These results suggest that KBT is a potent prescription on immune response via the increase of the proliferation of splenocytes, the production of cytokines from splenic Th cells and the phagocytic activity in vivo.

Key words : KamiBohuh-tang, splenocytes, thymocytes, nitric oxide, phagocytic activity, cytokines.

Introduction

The disease of women are classified into 4 groups, i.e. menstruation, hysterorrhea, fetus and delivery[21]. Menstruation includes all kinds of disease associated with catamenia, hysterorrhea is related with female genital organ. Fetus comprises pregnancy disease and delivery covers birth and postpartum disease. In spite of above all the criteria, women experiencing the delivery are exhausted so much that they are inclined to have a lot of postpartum disease. As the vital energy and blood are almost consumed during pregnancy and delivery, their tolerance to the disease drops so rapidly[11]. Bohuhtang is originated from 《EuiHakIpMun》[14] initially and made for the object of filling up the vital energy and blood after delivery. About the treatment of postpartum disease, Ju[8], in his book 《DankyeSimBup》, gave prominence on the supplement of the vital energy & blood and Jin told "After delivery, the primary vital energy nearly doesn't exit. So precede supplementing the energy. In case of dyslochia, add the circulation-promoting medicines to the restorative." In 《SoMun》[7] 〈TongPyungHer -SilLon〉, there is a phrase "If the noxious vapor flourishes, it is a disease condition. If vital energy is derived, it is a weak condition.", in 〈PyungYeol ByungLon〉 "If the noxious vapor flourishes, the vital energy of the body is certainly weak." Ancient time there is a phrasing that the happening of disease is intimately associated with the relation between noxious vapor and vital energy. Especially they thought the vital energy is the most important of all the other factors and the vital energy corresponds with the immunity in a general idea[24]. KamiBohuh-tang is composed of blood and vital energy supplying medicines, adding several ones which are promoting the blood circulation and removing the blood clot to Buhuhtang.. It has been often used in treating the postpartum abdominal pain caused by the weakness and lumbago. It relaxes the tensioned muscle and motivates the small channel of the skin and muscle to move dynamically. This study will report the remarkable results acquired from observing improving effects on the immunity cell and reduction of nitric oxide. Here is the report based on the results of this experiment.

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Received: 2001/11/24 · Revised: 2002/01/20 · Accepted: 2002/02/04

Materials and Methods

1. Material and Animal

The materials used in this test are carefully selected from Oriental Medicine Hospital of Daejeon University and prescription[18] is as follows(Table 1). Animals were 8 week-old mice(male) in BALB/C and were purchased at the Korean Experimental Animal. They were fed with enough solid feed, water and used after fully adapted to temperature 20±2℃, for a week, humidity 50±5% and dark/light 12 hours.

Table 1. Prescription of KamiBohuh-tang

Materials	Volume(g)
Ginseng Radix	6
Atractylodis Rhizoma	6
Angelicae Gigantis Radix	6
Cnidii Rhizoma	6
Astragali Radix	6
Cibotir Rhizoma	6
Typhae Pollen	6
Pteropi Faeces	6
Lycopi Herba	6
Fraxini Cortex	4
Glycyrrhizae Radix	4
Schizonepetae Herba	4
Hoelen Alba	4
Eucommiae Cortex	4
Phlomidis Radix	4
Achyranthis Radix	4
Chaenomeils Fructus	4
Persicae Semen	4
Carthami Flos	4
Corydalis Tuber	4
Zingiberis Rhizoma	2
	100

2. Preparation of Sample

Three packs of prescription were boiled with 2,000㎖ distilled water and extracted. After filtration, concentrating with a rotary evaporator, KamiBohuh-tang 80g was obtained through the final process of freeze drying. It was fed, dissolved in a solution of salt.

3. Method

1) Thymocytes, splenocytes and macrophages separation

Separation of the mouse's thymocytes and splenocytes was done with Wyscocki[25] and Mizel[15] way. Regarding 5 mice as a group, KamiBohuh-tang(below this sentence using KBT) was administered by a dose of 500㎎/㎏, once in a day for 7 days. At the 8th day, those mice were killed shattering thymus and spleen in a petri dish containing DPBS-A and filtrating with a stainless mesh, cell-floating solution was obtained. Washing two times with DPBS-A, solution having thymocytes and splenocytes were used. For macrophage separation, on the 4th day of drug-administration, 3% thioglycollate 2㎖ was injected in the mouses's abdomen. On the 8th day, after killing those mice, cold PBS 10㎖ was injected in the abdomen, later abdominal cells were collected. Centrifuging with a velocity of 1,300 rpm, 4℃ for 10 minutes, it was cultivated in a CO_2 incubator and separated into each petri dish of 120㎜ diameter. Removing unsticked cells after 2 hours, sticked macrophages gathered with a cell scraper were used. RPMI 1640 culture medium was used for thymocytes, splenocytes and macrophages and it was made adding 10% FBS, penicillin-streptomycin(100units/㎖, 100㎍/㎖).

2) Multiplication measurement of thymocytes and splenocytes

KBT's effect on the propagation of the separated thymocytes and splenocytes was measured by the MTT method[16]. Diluting thymocytes and splenocytes separated into each 96-well plate, adding concanavalin A(Con A) 5㎍/㎖ to thymocytes and lipopolysaccharide(LPS) 10㎍/㎖ to splenocytes. We cultivated each well in a CO_2 incubator of 37℃. When the culturing was ended, 10% SDS 100㎕ dissolved in 0.1N HCl was added to each well. After culturing in a shade for another 18 hours, extinction degree of color- generated cells was measured in 570㎚ by a microplatreader. Extiction degree of experimental group was converted into percentage, compared with a contrasting group.

3) Subpopulation measurement of thymocytes and splenocytes

Separated thymocytes and splenocytes were washed three times with RPMI 1640 culture medium. T cell population was dyed with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 monoclonal antibody. T and B cell's subpopulation was two times dyed with PE-conjugated anti-B220, FITC-conjugated anti-Thy1 monoclonal antibody at the temperature of 4℃ for 30 minutes and was measured with a flow cytometer[23] [excitation; 488㎚, emission; 525 ㎚(FITC), 575㎚(PE)] .

4) Cytokines measurement of the blood serum

Regarding 5 mice as a group, KBT 500㎎/㎏ was orally administered to an experimental group to a contrasting group once a day for 7 days. On the 8th day the blood serum was divided from centrifuged blood which was obtained from the mice' heart. Taking 50㎕ blood serum, cytokines were measured using each mouse immunoassay kit. Blending blood serum with assay diluent 50㎕, it was washed four times after 2 hour's incubation at the normal temperature. Finishing a wash, anti-mouse cytokines conjugated concentrate 100㎕ was added. After 2 hours' incubation at the normal temperature, it was washed 5 times, mingled with substrate solution 100㎕ and cultured for 30 minutes. After appending Stop solution 100㎕, extinction extent was measured at the 450㎚ with a microplate reader and cytokines' measure was converted by the pre-formed galvanometer.

5) Nitric oxide product measurement from the abdomen

After planting separately divided macrophages on the 24 well plate(2×10^6 cells per well), nitric oxide(NO) product was measured by the Griess method[20]. Appending LPS 1㎍/㎖, γ-IFN 25 units/㎖ to each well and culturing for 24 hours, culturing solution 100㎕ was mixed with Griess's reagent 100㎕ (1%sulfanilamide+0.1%N-naphthylenediamine 2HCl+2.5%

H_3PO_4). Leaving alone at the temperature of 37℃ for 10 minutes, extinction extent of NO_2^- density was measured at 570 ㎚ with a microplate-reader converted by the preformed galvanometer of $NaNO_2$.

6) Phagocytic activity measurement of abdominal macrophage

Making divided macrophage at the density of 2×10^6cells/㎖, we used it in this experiment. Manufacture of Lucigenin solution was done afetr dissolving in 10㎖ DPBS-A and reserved at the temperature of -20℃(Stock solution). Lucigenin stock solution was used after DME cultural medium's dilution by the 10% density. Chemiluminescence was measured at the 37℃ with a luminometer[2,3]. Uniting macrophage floating solution 50㎕, lucigenin solution 50㎕ and zymosan solution 30㎕, we made the final volume of this serum 200㎕. At the 5 minutes' interval, lucigenin chemiluminescence was measured for 30 minutes.

7) Statistical analysis

All the experimental data was expressed as Mean±SE and statistical analysis was performed using student's t-test. A probability level of 0.05 was used to establish significance.

Results

1. Effect on the propagation of thymocytes

As a result of measuring thymocytes, there isn't any change to experimental group at the percent of 102.2±2.7% carried out with concanavalin A(Con A), in the condition that the survival rate of contrasting group converted into 100 percent. In a case not carried with Con A, the survival rate of contrasting group is 50.2±1.5% and the experimentals' 52.8±1.1%. There was no change, either(Table 2).

Table 2. Effect of KBT Water Extract on the Proliferation of Murine Thymocytes

Samples	Cell Proliferation (%)	
	Treated of Concanavalin A	Non-treated of Concanavalin A
Control	100.0±1.2	50.2±1.5
KBT	102.2±2.7	52.8±1.1

KBT (500㎎/㎏) was administered p.o. once a day for 7 days, and the separated thymocytes (1.2×10^6cells/㎖) were cultured for 48 hour in RPMI1640 media mixed with an activating mitogen of concanavalin A. The data represents the mean±SE of 5 mice.

2. Effect on the propagation of splenocytes

As a result of measuring splenocytes, the survival rate of contrasting group converted into 100 percent, Experimental group was increased at the rate of 168.0±2.7% in a case carried with LPS. In a case not carried with LPS, the survival rate of contrasting group was decreased at the rate of 77.3±1.0%, experimental group's was significantly increased at the rate of 144.3±2.3%, comparing with contrasting group(Table 3).

Table 3. Effect of KBT on the Proliferation of Murine Splenocytes

Samples	Cell Proliferation (%)	
	Treated of Lipopolysaccharide	Non-treated of Lipopolysaccharide
Control	100.0±1.8	77.3±1.0
KBT	168.0±2.7*	144.3±2.3*

KBT (500㎎/㎏) was administered p.o. once a day for 7 days, and the separated splenocytes (1.2×10^6cells/㎖) were cultured for 48 hour in RPMI 1640 media mixed with an activating mitogen of lipopolysaccharide. The data represents the mean±SE of 5 mice. *: Significantly different from control group ($p<0.001$).

3. Effect on the subpopulation of the thymocytes

CD4 single positive($CD4^+$) cell of thymocytes is 11.5±0.3% in a contrasting group and 11.7±0.6 in an experimental group. CD8 single positive($CD8^+$) cell of the thymocytes is 2.7±0.3% in a contrasting group and 2.2±0.3% in an experimental group. There was no change both CD4 and CD8(Table 4).

Table 4. Effect of KBT on the Subpopulation of Murine Thymocytes

Samples	Cell Subpopulation (%)	
	$CD4^+$	$CD8^+$
Control	11.5±0.3	2.7±0.3
KBT	11.7±0.6	2.2±0.3

KBT (500㎎/㎏) was administered p.o. once a day for 7 days, and the separated thymocytes were stained with PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 monoclonal antibody for 30 minutes at 4℃. The subpopulation was determined with a flow cytometer. The data represents the mean±SE of 5 mice.

4. Effect on the subpopulation of the splenocytes

As a consequence of measuring splenocytes, Thy1 positive cell($Thy1^+$) of contrasting group was 39.7±1.6% and the experimental group's was 35.3±1.2%. In both cases Thy1 cells were significantly decreased. In a case of B220 positive cell($B220^+$), contrasting group was 23.2±1.4% and experimental group was 22.9±1.5%, which was decreased in the aspect of T cell compared with contrasting group. Of the splenic T-lymphocytes, $CD4^+$ cell of contrasting group was 13.8±1.3% and experimental group was significantly increased by the rate of 19.1±1.4%. $CD8^+$ cell of contrasting group was 3.7±0.2% and experimental group 3.4±0.1%(Table 5).

Table 5. Effect of KBT on the Subpopulation of Murine Splenocytes

Samples	Cell Subpopulation (%)			
	$Thy1^+$	$B220^+$	$CD4^+$	$CD8^+$
Control	39.7±1.6	23.2±1.4	13.8±1.3	3.7±0.2
KBT	35.3±1.2*	22.9±1.5	19.1±1.4*	3.4±0.1

KBT (500 mg/kg) was administered p.o. once a day for 7 days, and the separated splenocytes were stained with PE-conjugated anti-B220 및 FITC-conjugated anti-Thy1 monoclonal antibody or PE-conjugated anti-CD4 and FITC-conjugated anti-CD8 monoclonal antibody for 30 minutes at 4℃. The subpopulation was determined with a flow cytometer. The data represents the mean±SE of 5 mice. *: Significantly different from control group ($p<0.05$).

5. Effect on the secretion of cytokines in blood serum

For the outcome of measuring interferon-γ, contrasting

group was 32.7±3.9 and experimental group was significantly increased at the numerical value of 120.7±10.6. As a result of measuring Interleukin-2, contrasting group was 45.6±3.5 and experimental group was 57.9±1.8 with the significance. Contrasting group was 45.2±1.0 and experimental group was significantly increased at the numerical value of 94.6±7.1 in a consequence of measurement of Interleukin-4(Table 6).

Table 6. Effect of KBT on the Production of Cytokines in Mice Serum

Samples	Production of Cytokine (pg/ml)		
	interferon-γ	Interleukin-2	Interleukin-4
Control	32.7±3.9	45.6±3.5	45.2±1.0
KBT	120.7±10.6**	57.9±1.8*	94.6±7.1*

KBT (500 mg/kg) was administered p.o. once a day for 7 days, and the production of cytokines was determined in separated serum with ELISA kit. The data represents the mean±SE of 5 mice. *Significantly different from control group (*; p<0.05, **; p<0.01).

6. Effect on the nitric oxide production from the abdominal macrophage.

In the Nitric Oxide production quantity, contrasting group was 12.1±0.2㎛ and experimental group was significantly decreased, comparing with the former's value of 9.9±0.2㎛(Table 7).

Table 7. The Production of Nitric Oxide from Peritoneal Macrophages in KBT-administered Mice.

Samples	Nitric oxide(㎛)
Control	12.1±0.2
KBT	9.9±0.2*

KBT (500㎎/㎏) was administered p.o. once a day for 7 days, and then 3% thioglycollate was injected i.p. at the 4th day. Peritoneal macrophages obtained after 2 hours. adherence period were cultured in RPMI1640 media in the presence LPS and interferon-γ. *; Significantly different from control group(p<0.01).

7. Effect on the phagocytic activity from the abdominal macrophage

CL quantity of experimental group produced from macrophages was increased much than the one of contrasting group(Fig. 1).

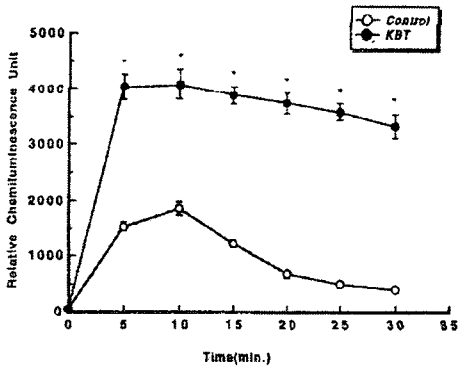

Fig. 1. Effect of KBT on lucigenin chemiluminescence in murine peritoneal macrophages. KBT(500㎎/㎏) was administered p.o. once a day for 7 days, and then 3% thioglycollate was injected i.p. at the 4th day. Peritoneal macrophages(2×10^6cells/㎖) obtained after 2 hours' adherence period were cultured in DME media(without phenol red) containing opsonized zymosan. The chemiluminescence was measured at 5 min. intervals for 30 min. Other procedures were described as detailed in the materials and method section. Each point represents the mean±SE of 5 mice. *; Significantly different from control group (p <0.001).

Discussion

Women's diseases are intimately related with all the type of phenomenon and disease from 14 years old to 50. So women's diseases are largely classified into 4 groups, i.e. menstruation, hysterorrhea, fetus and delivery[21]. Of all the postpartum disease, though it doesn't have anything to do with the delivery, delivery itself might worsen the passage of disease and condition of post-delivery, are distinguished with other 3 type's disease. At the postpartum periods, the change of genital organ and the whole body condition were returned at the time of nonpregnancy, so this period is called as "a puerperium". There is a difference between each individual but it comes within the purview of 6~8 weeks after delivery[11]. Bohuhtang is initially originated from 〈EuiHakIpMun〉 [14] and is composed of Ginseng Radix, Atractylodis Macrocephalae Rhizoma, Hoelen, Angelicae Sinensis Radix, Cnidii Rhizoma, Fraxini Cortex, Glycyrrhizae Radix and Zingiberis Rhizoma. The object of this prescription is to supplement vital energy and blood, to strengthen the general weakness. About the remedy for postpartum disease, Ju[8] told in 〈DanKyeSimBup〉 "At the postpartum periods there is no profit in a weak condition. Doctor must supplement vital energy and blood before anything else. Though there is other type of disease, don't treat that disease. Every disease is caused by the bloody exhaution, so it is impossible to vapor the noxious one away." Jin[22] told like this "After delivery the primary vital energy is almost exhausted but new blood doesn't produce. Supplementing vital energy and blood is advised first of all." and Her "After delivery, you must provide a supplement for vital energy and blood." As an object described above, Bohuhtang has been used for a long time and is used a lot until now. The KBT is organized removing Rehmanniae Radix Vapratum, Hoelen and Paeoniae Radix from Paljintang and adding Astragali Radix, Fraxini Cortex. The prescription's efficacy is that Ginseng Radix is supplementing the vital energy and producing resin that Atractylodis Macrocephalae Rhizoma is helping the difestive system's function and harmonizing the blood and strengthening the heart's function. Besides Cnidii Rhizoma is to produce and to breed new blood, Astragali Radix to strengthen the vital energy and the skin, Fraxini Cortex to revolve the vital energy and Glycyrrhizae Radix to harmonize all the medicines. The final object of this prescription is to fill up the storage of vital energy and blood, so this prescription can be used for intensifying and replenishing the blood & vital energy. In 〈SoMun〉 〈SangKoChunJInLon〉 [7]there is a phrase "The primary energy is sincere and the spirit is sound, how can the disease

happen?" also in 〈PyungYeolByungLon〉 "If the noxious vapor flourishes, the vital energy of the body is certainly weak." Ancient people thought that if the vital energy flourishes, the noxious vapor coudn't make a disease and that they can protect the disease generation using that way. They put the importance on the resistance of the healthy body and called the related factor as a primary vital energy. We can find out the correlation between the immunity and the noxious vapor-vital energy theory. Vital energy seems to contain the immunologic function. The shortage of vital energy is a stipulation of noxious vapor's invasion, so the disease was thought to be a fight between the noxious vapor's invasion and vital energy's protection[9,12]. The immunity was taken to be the protective reaction of an infectious disease at the beginning[6]. But these days the concept of the immunity has been enlarged to have a resistance of a congenital disease[24]. Consequently to judge whether KBT effectively controls the immunity system or not, the function of thymocytes, splenocytes and macrophages which are very important factors in a living body was observed. An immune reaction is classified as two groups. One is a specific immunity related with T and B-lymphocyte and the other is non-specific immunity related with macrophages[6].

In this experiment using KBT, there is no effect on the propagation of thymocytes but a promoting effect of splenocytes' propagation. In a contrasting group, the multiplication of splenocytes are increased in a case of KBT-administration by 67% degree while LPS(mitogen) treated case was only increased by 27%. This shows that KBT is more potential in promoting the splenocytes' propagation than mitogen. Thymocytes are differentiated through the specialization process in the medulla and cortex of thymus into helper T lymphocyte(Th) and cytotoxic T lymphicyte(Tc). Differentiated Th1 cell secretes interferon-γ (γ-IFN), Interleukin(IL-2) and Th2 cell such cytokines as IL-4, IL-5, IL-6 and IL-10[17]. This kind of cytokines accelerates the specialization and multiplication of T cell, B cell and macrophages. Cytotoxic T cell disintegrates tumor cell and activates macrophages. In contrasting group Th cell(CD4 single positive cell) is 11.5%, Tc cell(CD8 single positive cell) 2.7% while in thymus of normal mice group, $CD4^+$ cell was 12%, $CD8^+$ cell 3%. This result are similar with other cases already reported[1]. Of splenocytes of contrasting group, T-lymphocytes (Thy1 positive cell) is 39.7% and B-lymphocytes(B220 positive cell) 23.2% but in a case KBT was administered T-lymphocytes were significantly decreased by 35.3%. Th cell was 13.8% and Tc cell 3.7% of splenic T-lymphocytes, in the KBT medication case, Th cell was significantly increased by 19.1%. That the population of T-lymphocytes was decreased but Th cell's population of splenic T-lymphocytes was increased suggests that KBT can control the splenocytic immune reaction. That Th cell was decreased only in splenocytes but didn't show any change in thymocytes is the hint of this idea KBT promotes the Th cell's specialization during T-lymphocytes differentiation. In a KBT-medication case, the quantity of cytokines was altogether increased compared with the contrasting group. As a result of measuring IFN-γ, IL-2 and IL-4 secreted from the Th1 cell. In the front of result that there is no change with Th cell of thymocytes and Th cell of splenic T-lymphocytes is increased, cytokines' augmentation from the secretion of Th cell is originated from the Th cell increase of T-lymphocytes. Nitric oxide(NO) is known to be a control factor of T-lymphocyte's life span and to regulate cytokines produced by T-lymphocytes[13]. Besides NO suppressed the multiplication of helper T cell and an autoimmunity system[19]. In this experiment NO production of KBT medication was decreased comparison with the contrasting group. These results suggest that NO was associated with the increase of Th cell population in a case administerd with KBT. If a different thing invades from the outside, a living body promotes the phagocytosis to protect a living body itself. Phagocytosis like these is happening in polymorphonuclear leukocytes[4]. Phagocytes are important in the immunologic side but more important in the process of healing wounds. So phagocytic activity of macrophages was measured in this experiment using the chemiluminescence measurement. The principle of this method is that during the macrophages' phagocytosis of particle it produces oxygen radical and this produced oxygen radical activates with lucigenin and produces lucigenin chemiluminescence, which is measured. As a result of measuring chemiluminescence(CL), CL quantity was increased in a case of KBT medication. While primarily NO suppresses pseudopodia formation of activated macrophages[10], in this experiment KBT suppresses NO production and increases phagocytic activity. It gives suggestions that NO is partially related with the phagocytic activity increase of macrophages. Synthesizing all these results, in an example of KBT-medication into a living body, it is thought that KBT has the immunoregulation effect which promotes the cytokines' secretion from Th cell of splenic T-lymphocytes, macrophages' phagocytosis and the propagation of splenocytes.

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