Effect of Kamiinsamyangyoung-tang on Immune Response and Blood Formation

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Kamiinsamyangyoung-tang(KIT) has been widely used to treat amenorrhea and bradymenorrea caused from vital energy and blood deficit. KIT was composed of Insamyangyoung-tang, Cervi Cornu Pantotrichum and Hominis Placenta. The aim of this study is to investigate effect of KIT on Immune response and Blood formation. We investigated thymocytes, splenocytes population, γ-interferon, IL-2, IL-4, NO and phagocytic activity. We found that KIT had no effect on the Th and Tc cell population of thymocytes, Th cell population of splenocytes and γ-interferon quantity was decreased. KIT decreased the formation of Nitric Oxide from abdominal macrophage, on the other hand, it had no influence on the quantity of IL-2, IL-4.

Key words : Kamiinsamyangyoung-tang, Immune response, Blood formation

Introduction

Insamyangyoung-tang has an effect of supplementing vital energy and blood deficit. Kamiinsamyangyoung-tang was composed of Insamyangyoung-tang, Cervi Cornu Pantotrichum and Hominis Placenta. Its effect of replenishing Yang-qi and supplementing blood was more intensified, so it has been provided for the patient having menolipsis and palpitation, especially in the case of postpartum bleeding. Many experimental study about Insamyangyoungtang's immune response have been performed and proved to increase immune response.

But the effect of Insamyangyoung-tang supplemented with Cervi Cornu Pantotichum and Hominis Placenta never has been demonstrated.

In this study, to prove immune response and blood formation effects, we surveyed the rise and fall of thymocytes, splenocytes and macrophage, and the results are as follows.

Materials and Methods

1. Animals and treatments

Animals were 8 week-old mice(female) 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.

2. Prescription of KIT

Table 1. KIT was consisted of following 14 herbs

Materials	Volume(g)
Ginseng Radix	6
Atractylodis Rhizoma	12
Angelicae Gigantis Radix	9
Hoelen Alba	12
Astragali Radix	12
Polygalae Radix	6
Aurantii nobilis Pericarpium	6
Schizandrae Fructus	6
Paeoniae Radix	18
Rehmanniae Radix Vapratum	12
Cinnamomi Cortex Spissus	6
Glycyrrhizae Radix	6
Cervi Cornu Pantotrichum	6
Homonis Placenta	12
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KIT was placed in approximately 2,000ml of distilled water in round flask and boiled for 2 hrs using condense device. The KIT extract was filtered with Whatman filter and then concentrated with rotary vacuum evaporator. This flask was placed in deep freezer for 24 hr and then dried by freeze dryer for 12hrs. Yield was 15.1%. An appropriate amount of the powder obtained was dissolved with PBS.

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· Received : 2004/11/18 · Revised : 2004/12/20 · Accepted : 2005/01/20

3. 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, Kamiinsamyangyoung-tang(below this sentence using KIT) 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. KIT'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 3 7℃. 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.

4. 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)〕.

5. Cytokines measurement

Regarding 5 mice as a group, KIT 500㎎/㎏ was orally administered to an experimental group, PBS to a control 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.

6. Nitric oxide product measurement from the abdominal phagocyte

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 $NaNO_2$.

7. Lucigenin chemiluminescence 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.

8. Engulfment measurement by phagocytic activity of abdominal macrophage

FITC-conjugated E. coli particle was diluted by the density of 1㎎/㎖, trypan blue was melted with citrate

buffer(pH 4.4) by the density of 250㎍/㎖. Making divided macrophage at the density of 1×10^6cells/㎖, we used it in this experiment. To suppress the extracellular fluorescence, after adding trypan blue 100㎕, we observed with a inverted fluoromicroscope.

9. Anemia induced by phenylhydrazine

Regarding 5 mice as a group, phenylhydrazine · HCl 9㎎/㎏ was injected three times a day in vein of tail. KIT 500㎎/㎏ was orally administered to an experimental group, PBS to a control group once a day during experimental days. After injecting phenylhydrazine · HCl, on the day of 5th, 10th and 15th, we gather 0.5 ㎖ blood, manage with heparin, measured RBC, hemoglobin, hematocrit.

10. 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 was a rise to experimental group at the percent of 115.9±1.3% carried out with concanavalin A(Con A), in the condition that the survival rate of control group converted into 100 percent. In a case not carried with Con A, the survival rate of control group is 90.9±0.8% and the experimentals' 107.4±1.5%. There was a fall(Table 2).

Table 2. Effect of the administration of Kamiinsamyangyoung-tang water extract(KIT) on the cell viability of *Concanavalin A* treated-thymocytes in C57BL/6 mice

Samples	Cell Viability (%)	
	Non-treated of Concanavalin A	Treated of Concanavalin A
Control	100.0±1.1	115.9±1.3
KIT	90.9±0.8*	107.4±1.5*

KIT (500 mg/kg) was administered *p.o.* once a day for 7 days, and the separated thymocytes (1×10^7 cells/ml) were cultured for 48 h in RPMI1640 media mixed with an activating mitogen of concanavalin A. The data represents the mean±SE of 5 mice. *: Significantly different from control group (p<0.001).

2. Effect on the propagation of splenocytes

As a result of measuring splenocytes, the survival rate of control group converted into 100 percent, Experimental group was increased at the rate of 135.9±2.0% in a case carried with LPS. In a case not carried with LPS, the survival rate of control group was 101.8±0.8%, experimental group's 134.8±1.0%, there was no difference(Table 3).

Table 3. Effect of the administration of KIT on the cell viability of lipopolysaccharide treated-splenocytes in C57BL/6 mice

Samples	Cell Viability (%)	
	Non-treated of lipopolysaccharide	Treated of lipopolysaccharide
Control	100.0±0.8	135.9±2.0
KIT	101.8±0.8	134.8±1.0

KIT (500 mg/kg) was administered *p.o.* once a day for 7 days, and the separated splenocytes (1×10^7 cells/㎖) were cultured for 48 h in RPMI1640 media mixed with an activating mitogen of lipopolysaccharide. The data represents the mean±SE of 5 mice.

3. Effect on the propagation of thymocytes (*In vitro*)

Measuring thymocytes survival rate not carried with Con A, there was a decrease to experimental group at the percent of 98.1±1.3%, 97.0±1.0%, 93.2±1.1% carried out with KIT 1, 10 and 100㎍/㎖, in the condition that the survival rate of control group converted into 100 percent. In a case carried with Con A, the survival rate of control group is 113.9±1.3% and the experimentals' 114.0±0.9%. 111.4±1.0%. 106.1±0.8%. There's a decrease, too(Table 4).

Table 4. Effect of KIT on the cell viability of Concanavalin A reated-thymocytes *in vitro*

Samples	Dose (μg/ml)	Cell viability (%)	
		Non-treated of Concanavalin A	Treated of Concanavalin A
Control	-	100.0±0.6	113.3±1.3
KIT	1	98.1±1.3	114.0±0.9
KIT	10	97.0±1.0	111.4±1.0
KIT	100	93.2±1.1*	106.1±0.8*

The separated thymocytes (1×10^7 cells/ml) were cultured for 48 h in RPMI1640 media mixed with KIT (1, 10 and 100 μg/ml). The data represents the mean±SE of 3 experiments. *: Significantly different from control group (p<0.001).

4. Effect on the propagation of splenocytes (*In vitro*)

As a result of measuring splenocytes, in a case not carried with LPS, the survival rate of control group converted into 100 percent, experimental group was 100.6±1.4%, 99.6±0.7%, 100.1±0.4% carried out with KIT 1, 10 and 100㎍/㎖. In a case carried with LPS. the survival rate of control group was increased at the rate of 151.2±1.7%, experimental group's 149.8±1.7%, 150.4±1.9%, 148.9±1.5%. There was no difference(Table 5).

Table 5. Effect of KIT on the cell viability of lipopolysaccharide treated-splenocytes *in vitro*

Samples	Dose (μg/ml)	Cell viability (%)	
		Non-treated of lipopolysaccharide	Treated of lipopolysaccharide
Control	-	100.0±0.8	151.2±1.7
KIT	1	100.6±1.4	149.7±1.7
KIT	10	99.6±0.7	150.4±1.9
KIT	100	100.1±0.4	148.9±1.5

The separated splenocytes (1×10^7 cells/ml) were cultured for 48 h in RPMI1640 media mixed with KIT (1, 10 and 100 μg/ml). The data represents the mean±SE of 3 experiments.

5. Effect on the subpopulation of the thymocytes

CD4 single positive($CD4^+$) cell of thymocytes is 11.9±0.2% in a control group and 11.4±0.2 in an experimental group. CD8 single positive($CD8^+$) cell of the thymocytes is 3.0±0.3% in a control group and 2.9±0.2% in an experimental group. There was no change in both CD4 and CD8(Table 6, Fig. 1).

Table 6. Effect of KIT on the subpopulation of murine thymocytes

Samples	Cell Subpopulation (%)	
	$CD4^+$	$CD8^+$
Control	11.9±0.2	3.0±0.3
KIT	11.4±0.2	2.9±0.2

KIT (500 mg/kg) 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.

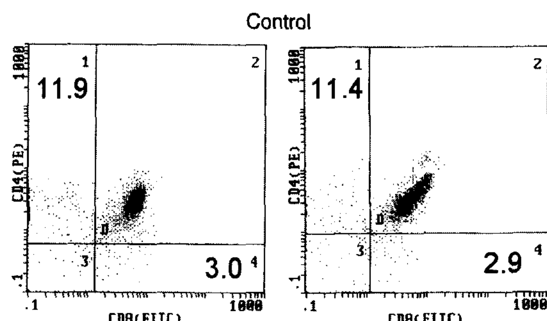

Fig. 1. Cytofluorometric pattern of thymocytes subpop ulation change

6. Effect on the subpopulation of the splenocytes

As a consequence of measuring splenocytes, Thyl positive cell($Thy1^+$) of control group was 22.7±0.9% and the experimental group's was 21.5±1.8%. There was no difference. In a case of B220 positive cell($B220^+$), control group was 31.8±1.1% and experimental group was 23.9±1.4%, which means a significant decrease.

Of the splenic T-lymphocytes, $CD4^+$ cell of control group was 15.1±1.2% and experimental group was significantly decreased by the rate of 12.5±0.5%. $CD8^+$ cell of control group was 8.7±0.7% and experimental group 8.2±0.5%(Table 7, Fig. 2).

Table 7. Effect of KIT on the subpopulation of murine splenocytes

Samples	Cell Subpopulation (%)			
	$B220^+$	$Thy1^+$	$CD4^+$	$CD8^+$
Control	31.8±1.1	22.7±0.9	15.1±1.2	8.7±0.7
KIT	23.9±1.4**	21.5±1.8	12.5±0.5*	8.2±0.5

KIT (500 mg/kg) was administered *p.o.* once a day for 7 days, and the separated splenocytes were stained with PE-conjugated anti-B220, 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 (*: $p<0.05$, **: $p<0.01$).

7. Effect on the secretion of γ-interferon

Measuring γ-interferon, control group was 350.8±12.5 pg/㎖ and experimental group was significantly increased at the numerical value of 497.4±11.3 pg/㎖(Table 8).

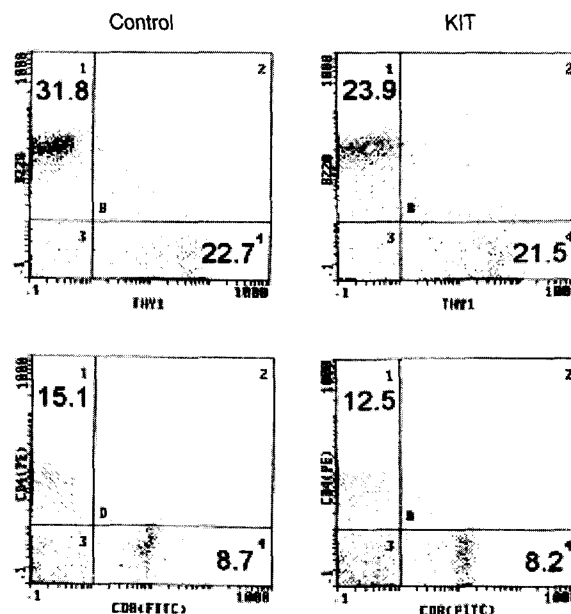

Fig. 2. Cytofluorometric pattern of splenocytes subpopulation change *in vivo*

Table 8. Effect of KIT on the production of γ-Interferon in murine splenocytes

Samples	γ-Interferon (pg/ml)
Control	350.8±12.5
KIT	497.4±11.3*

KIT (500 mg/kg) was administered *p.o.* once a day for 7 days, and the separated splenocytes (2×10^7 cells/ml) were cultured for 72 h in RPMI1640 media. The secretion of γ-Interferon was determined in supernatants of cultures with ELISA kit. The data represents the mean±SE of 5 mice. *: Significantly different from control group ($p<0.001$).

8. Effect on the secretion of Interleukin-2

As the result of measuring Interleukin-2, control group was 398.2±14.2 and experimental group was 379.8±12.8. There was no significance(Table 9).

Table 9. Effect of KIT on the production of interleukin-2 in murine splenocytes

Samples	Interleukin-2 (pg/ml)
Control	398.2±14.2
KIT	379.8±12.8

KIT (500 mg/kg) was administered *p.o.* once a day for 7 days, and the separated splenocytes (2×10^7 cells/ml) were cultured for 72 h in RPMI1640 media. The secretion of interleukin-2 was determined in supernatants of cultures with ELISA kit. The data represents the mean±SE of 5 mice.

9. Effect on the secretion of Interleukin-4

In a consequence of Interleukin-4, control group was 152.8±9.5 and experimental group 140.9±9.8. There was no difference, either(Table 10).

10. Effect on the nitric oxide production from the abdominal

macrophage

In the nitric oxide production quantity, control group not carried with LPS and ɣ-interferon was 1.6±0.1, 1.7±0.2㎛, on the condition that carried with LPS and ɣ-interferon was 17.3±0.9, 21.8±0.7㎛ each 24 hrs and 48 hrs later. Experimental group not carried with LPS and ɣ-interferon was 1.4±0.2, 1.5±0.1㎛, in a group carried with LPS and ɣ-interferon 11.7±0.2, 17.8±0.3㎛. Experimantal group's significantly decreased(Table 11).

Table 10. Effect of KIT on the production of interleukin-4 in murine splenocytes

Samples	Interleukin-4 (pg/ml)
Control	152.8±9.5
KIT	140.9±9.8

KIT (500 mg/kg) was administered *p.o.* once a day for 7 days, and the separated splenocytes (2 × 10^7 cells/ml) were cultured for 72 h in RPMI1640 media. The secretion of interleukin-4 was determined in supernatants of cultures with ELISA kit. The data represents the mean±SE of 5 mice.

Table 11. The production of nitric oxide from peritoneal macrophages in KIT-administered mice

Samples	Time (hr)	Nitric oxide(μM)	
		Non-treated of LPS and ɣ-IFN	Treated of LPS and ɣ-IFN
Control	24	1.6±0.1	17.3±0.9
	48	1.7±0.2	21.8±0.7
KIT	24	1.4±0.2	11.7±0.2*
	48	1.5±0.1	17.8±0.3*

KIT (500mg/kg) 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 hrs. adherence period were cultured in RPMI1640 media in the presence LPS and ɣ-interferon. *Significantly different from control group (p<0.05).

11. Effect on the nitric oxide production from the abdominal macrophage(*In vitro*)

In the nitric oxide production quantity, control group not carried with LPS and ɣ-interferon was 1.6±0.1, 1.7±0.3㎛, on the condition that carried with LPS and ɣ-interferon was 29.3±0.3, 42.4±0.4㎛ each 24 hrs and 48 hrs later. Experimental group carried with LPS and ɣ-interferon was 29.0±0.9, 27.2±1.3, 25.2±0.4㎛, 48 hts later 41.5±0.8, 40.7±1.2, 38.9±0.5㎛. Experimantal group's was significantly decreased(Table 12).

Table 12. Effect of KIT on the production of nitric oxide from peritoneal macrophages *in vitro*

Samples	Dose (μg/ml)	Nitric oxide (μM)	
		24 hr	48 hr
Control(-)	-	1.6±0.1	1.7±0.3
Control(+)	-	29.3±0.3	42.4±0.4
KIT	1	29.0±0.9	41.5±0.8
KIT	10	27.2±1.3	40.7±1.2
KIT	100	25.2±0.4**	38.9±0.5*

The separated splenocytes (1 × 10^7 cells/ml) were cultured for 48 h in RPMI1640 media mixed with KIT (1, 10 and 100 μg/ml). The data represents the mean±SE of 3 experiments. *: Significantly different from control group (*: p<0.05, **: p<0.01). Control(-): IFN-ɣ and LPS non-treated group, Control(+): IFN-ɣ and LPS treated group.

12. Effect on the phagocytic activity from the abdominal macrophage

CL quantity of experimental group produced from macrophages was 7.3×10^6 RLU, control group 6.5×10^6 RLU. CL quantity was increased in the experimental group(Table 13, Fig. 3, Fig. 4).

Table 13. The relative luminescence unit(RLU) of peritoneal macrophages separated from KIT-administered mice

Samples	RLU
Control	6.5 × 10^6
KIT	7.3 × 10^7

KIT (500mg/kg) 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 × 10^6 cells/ml) obtained after 2 hrs. adherence period were cultured in DME (without phenol red) mixed with opsonized zymosan. The chemiluminescence was measured for 30 min with luminometer.

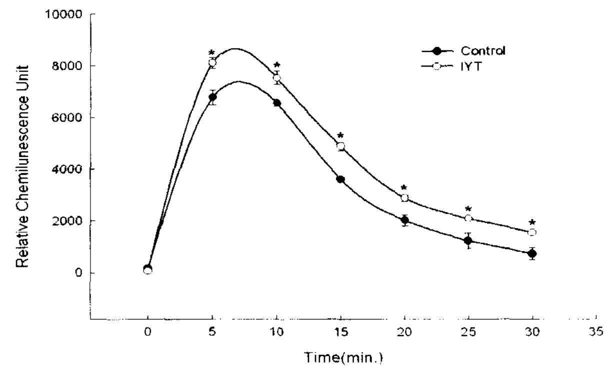

Fig. 3. Effect of the administration of KIT on lucigenin chemiluminescence in murine peritoneal macrophages. KIT (500 mg/kg) was administered *p.o.* once a day for 7 days and the separated peritoneal macrophages (2 × 10^6 cells/ml) were cultured in DME media (without phenol red) mixed with opsonized zymosan. The chemiluminescence was measured for 30 min with luminometer. Each bar represents the mean±SE of 5 mice. *: Significantly different from control group (p<0.001).

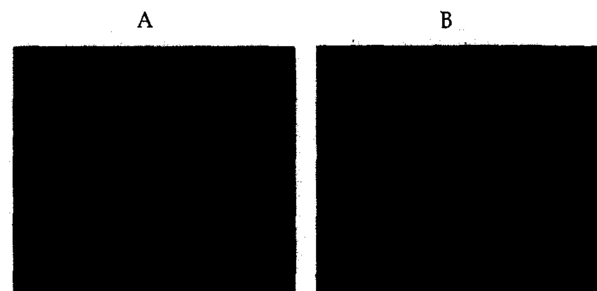

Fig. 4. Photomicrographs of engulfment of fluorescein conjugated *E.coli* particles in peritoneal macrophages obtained from KIT-administered mice. Inverted fluoromicroscope photomicrographs (100×) showing uptake of fluorescein conjugated *E. coli* particles in control (A), and peritoneal macrophages obtained from KIT-administered mice (B).

13. Effect on the phagocytic activity from the abdominal macrophage(*In vitro*)

In vitro, CL quantity of control group produced from macrophages was 1.3×10^7 RLU, experimental group 1.1×10^7 ,

1.0×10^7, 9.0×10^6 RLU by the KIT density of 1, 10 and 100㎍/㎖. CL quantity was decreased in the experimental group(Table 14, Fig. 5).

Table 14. The relative luminescence unit (RLU) of peritoneal macrophages treated with KIT *in vitro*

Samples	Dose (μg/ml)	RLU
Control	-	1.3×10^7
KIT	1	1.1×10^7
KIT	10	1.0×10^7
KIT	100	9.0×10^6

The separated peritoneal macrophages (2×10^6 cells/ml) obtained after 2 hrs. adherence period were cultured in DME (without phenol red) mixed with KIT. The chemiluminescence was measured for 30 min with luminometer.

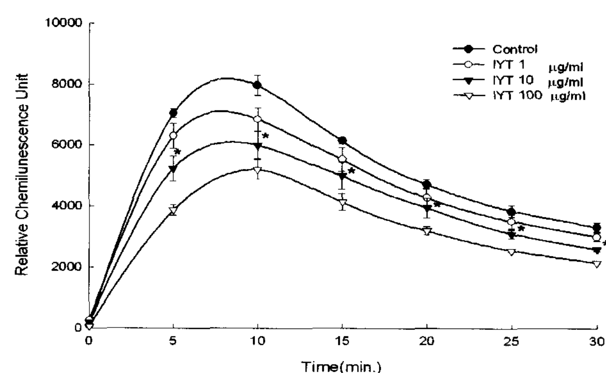

Fig. 5. Effect of KIT on lucigenin chemiluminescence in murine peritoneal macrophages *in vitro*. The cells (2×10^6 cells/ml) were cultured in DME media (without phenol red) mixed with opsonized zymosan 2h after was cultured with KIT (1, 10 and 100 μg/ml). The chemiluminescence was measured for 30 min with luminometer. Each bar represents the mean±SE of 3 experiments. *: Significantly different from control group ($p<0.001$).

14. Effect on the RBC numerical value

RBC number of control group, before administering phenylhydrazine, was 5.8±0.2, after 2 days of administering phenylhydrazine, 3.6±0.2, after 10days 4.0±0.2. Experimental group, 5days later 3.8±0.1, 10 days later 4.7±0.2, 15days later 5.4±0.2, in a comparison of control group, was rapidly recovered(Table 15).

Table 15. Effect of KIT on the change of RBC number in anemic rats induced by phenylhydrazine · HCl

Mice Condition	Samples	RBC Number (10^4/mm^3)			
		0 day	5 day	10 day	15 day
Normal	Control	5.8±0.2	3.6±0.2	4.0±0.2	4.6±0.2
	KIT	-	3.8±0.1	4.7±0.2*	5.4±0.2*

Phenylhydrazine · HCl (9 mg/kg) was injected *i.p.* for 3 days and KIT (500 mg/kg) was administered *p.o.* once a day for 15 days. The number of RBC was measured at 0, 5, 10 and 15 days. Each bar represents the mean±SE of 5 rats. *: Significantly different from control group ($p<0.05$).

15. Effect on the Hemoglobin quantity

Hemoglobin quantity of control group, before administering phenylhydrazine, was 13.5±0.4, after 5 days of administering phenylhydrazine, 7.6±0.3, after 10days 8.9±0.3, after 15days 11.0±0.2g/dℓ. Experimental group, 5 days later administering phenylhydrazine 7.8±0.2, 10 days after 10.4±0.3, 15days later 13.1±0.2g/dℓ, in a comparison of control group, was rapidly recovered(Table 16).

Table 16. Effect of KIT on the change of hemoglobin in anemic rats induced by phenylhydrazine · HCl

Mice Condition	Samples	Hemoglobin (g/dl)			
		0 day	5 day	10 day	15 day
Normal	Control	13.5±0.4	7.6±0.3	8.9±0.3	11.0±0.2
	KIT	-	7.8±0.2	10.4±0.3*	13.1±0.2*

Phenylhydrazine · HCl (9 mg/kg) was injected *i.p.* for 3 days and KIT (500 mg/kg) was administered *p.o.* once a day for 15 days. The volume of hemoglobin was measured at 0, 5, 10 and 15 days. Each bar represents the mean±SE of 5 rats. *: Significantly different from control group ($p<0.01$).

16. Effect on the Hematocrit value

Hematocrit quantity of control group, before administering phenylhydrazine, was 42.7±2.5%, after 5 days of administering phenylhydrazine 25.6±2.0%, after 10days 31.8±1.9%, after 15days 35.3±2.2%. Experimental group, 5 days later administering phenylhydrazine, 27.8±1.8%, 10 days after 37.2±2.1%, 15days later 41.5±2.3%, in a comparison of control group, was increased(Table 17).

Table 17. Effect of KIT on the change of hematocrit in anemic rats induced by phenylhydrazine · HCl

Mice Condition	Samples	Hemoglobin (g/dl)			
		0 day	5 day	10 day	15 day
Normal	Control	42.7±2.5	25.6±2.0	31.8±1.9	35.3±2.2
	KIT	-	27.8±1.8	37.2±2.1*	41.5±2.3*

Phenylhydrazine · HCl (9 mg/kg) was injected *i.p.* for 3 days and KIT (500 mg/kg) was administered *p.o.* once a day for 15 days. The value of hematocrit was measured at 0, 5, 10 and 15 days. Each bar represents the mean±SE of 5 rats. *: Significantly different from control group ($p<0.05$).

Discussion

Insamyangyoung-tang is initially originated from 《Taepyunghyeminhwajekukbang》 and is composed of Ginseng Radix, Astragali Radix, Atractylodis Rhizoma Alba, Hoelen Alba, Polygalae Radix, Aurantii nobilis Pericarpium, Schizandrae Fructus, Angelicae gigantis Radix, Paeoniae Radix, Rehmanniae Radix Vapratum, Cinnamomi Cortex Spissus, Glycyrrhizae Radix. The object of this prescription is to supplement vital energy and blood, to strengthen the general weakness. Kamiinsamyayngyoung-tang is made up added Cervi Cornu Pantotrichum and Hominis Placenta to Insamyangyoung-tang. So it can be used to amenorrhea caused to postpartum bleeding and palpitation.

Cervi Cornu Pantotrichum is a young horn of deer, has been known to have an effect of reinforcing kidney and liver, supplementing blood and vigor, strengthening bone and

muscle. A woman in chilbed's placenta is collected and cleaned several times, after that, it is called as Hominis Placenta. Placenta is a complex of vital energy, so it can raise a small fetus to a new born baby. It has a strong cell revival effect and maintains a life always in healthy condition.

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 KIT, there is an effect on the decrease of thymocytes but no effect of splenocytes' propagation. In a control group, the multiplication of thymocytes are decreased in a case of KIT-administration while LPS(mitogen) treated case was increased. This shows that KIT is more potential in decreasing thymocytes 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 γ-interferon(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 control group Th cell(CD4 single positive cell) is 11.9±0.2%, Tc cell(CD8 single positive cell) 3.0±0.37% while in thymus of KIT-adaministerd group, $CD4^{+}$ cell was 11.4±0.2%, $CD8^{+}$ cell 2.9±0.2%.

Of splenocytes of control group, T-lymphocytes(Thy1 positive cell) is 22.7±0.9% and B-lymphocytes(B220 positive cell) 31.8±1.1% but in a case KIT was administered T-lymphocytes were significantly decreased by 23.9±1.4%. Th cell was 12.5±0.5% and Tc cell 8.2±0.5% of splenic T-lymphocytes, in the KIT medication case, Th cell was significantly decreased. That the population of T-lymphocytes and Th cell's population of thymocitic T-lymphocytes was decreased suggests that KIT can control the thymocytic immune reaction. That Th cell was decreased only in thymocytes but That it didn't show any change in splencytes is the hint KIT promotes the Th cell's specialization during T-lymphocytes differentiation.

In a KIT-medication case, the quantity of cytokines was no difference compared with the control group. A result of measuring γ-IFN only increased. In the front result that there is no change with Th cell of splenocytes and Th cell of thymocytic T-lymphocytes is decreased, 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 KIT medication was decreased comparison with the control group.

These results suggest that NO was associated with the increase of Th cell population in a case administerd with KIT. 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 expriment 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 KIT medication. While primarily NO suppresses pseudopodia formation of activated macrophages[10], in this experiment KIT 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 KIT-medication into a living body, it is thought that KIT has the immunoregulation effect which promotes the cytokines' secretion from Th cell of T-lymphocytes, macrophages' phagocytosis and the propagation of thymocyte.

Making blood means a production of blood cell which consists of blood. In the blood there are RBC, WBC and platelets. RBC transports O_2 and CO, WBC obstructs the outside trespassers to protect our body, platelets do a important role in the blood coagulation.

Anemia is caused by the decrease of RBC number and hemotocrit, is represented as pale skin, dizziness and palpitation. Phenylhydrazine directly destroys RBC memebrane and causes melting- blood anemia. After induced by Phenylhydrazine, 5 days later RBC and hemoglobin was decreased in comparison with control group, 15 days later it tends to be recovered. But KIT administered group's RBC, hemoglobin and hematocrit was increased 10 days later. It means that KIT have an effect of making blood.

Synthesizing all these results, in an example of KIT-medication into a living body, it is thought that KIT has the immunoregulation effect which promotes the cytokines'

secretion, macrophages' phagocytosis, the propagation of thymocytes, and making blood effect of augmenting RBC, hemoglobin and hematocrit.

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