

The effects of Saiko-keishi-to and Juzen-taiho-to on Th1-Th2 balance in different age mice

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Abstract

It has been reported that traditional Japanese herbal medicines (Kampo medicines) are effective to treat patients with atopic constitution. These allergic responses are characterized by the Th2-mediated helper T lymphocytes. However, it seems that effects of Kampo medicines are different according to the patient's age. In this study, we investigated the effects of Kampo medicines, Saiko-keishi-to (SKT; 柴胡桂枝湯) and Juzen-taiho-to (JTT; 十全大補湯), on Th1-Th2 balance in splenic T cells of BALB/c neonate mice, and at 8 and 25 weeks old.

The alterations of splenic T lymphocyte subpopulations were observed both in neonate and 8 week old mice, where the ratios of CD4/CD8 decreased significantly both in SKT treated neonate and SKT treated 8 week old mice compared to corresponding control groups. The ratio of CD3/CD19 decreased significantly only in SKT treated neonate mice. However, in JTT treated groups, no significant difference was observed at any age.

Splenocytes were cultured with anti-mouse CD3 mAb, and the resulting supernatant was subjected to the determination of cytokine production using ELISA. Neither SKT nor the JTT treated group showed significant change of IFN- γ production. However, IL-4 productions in SKT and JTT treated groups significantly reduced both in neonate and 8 week old mice compared to corresponding control mice, and IL-12 production in SKT treated group decreased in neonate mice. In contrast, no change of IL-4 production was observed in splenic lymphocyte subpopulations and cytokine productions in 25 week old mice. These results indicated that oral administration of Kampo medicines (SKT and JTT) on Th1-Th2 balance were different according to aging.

Key words cytokines, Kampo medicines, T lymphocytes, Th1-Th2 balance, traditional herbal medicines.

Abbreviations SKT, Saiko-keishi-to, 柴胡桂枝湯; JTT, Juzen-taiho-to, 十全大補湯; IL-4, interleukin-4; IL-12, interleukin-12; IFN- γ , interferon- γ .

Introduction

Kampo medicines (Japanese traditional herbal medicines) are widely used for the treatment of the many kinds of acute and chronic diseases in eastern Asia. Recently, various biological activities of Kampo medicines have been reported, namely cytokine induction,¹⁾ augmentation of the host resistance to infection,^{2,3)} anti-

inflammatory effect⁴⁾ and therapeutic effect on autoimmune animal models.⁵⁻⁷⁾

In Japan, the types of diseases have largely changed from infectious diseases to constitutional diseases in this decade. Twenty percent of patients who visited our hospital have constitutional diseases, such as atopic dermatitis, bronchial asthma, and allergic rhinitis. Many reports have shown that Kampo medicines are effective clinically for the treatment of patients with atopic derma-

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titis.^{8,9)} These studies, taken together, suggested that one of the pharmacological effects of Kampo medicines might be applied by immunomodulation and by affecting the Th1-Th2 balance.^{10,11)} In mice, CD4⁺ helper T cell (Th) populations have been divided into two subpopulations according to their different cytokine production patterns.^{12,13)} CD4⁺ T cells producing interferon- γ (IFN- γ) and IL-2 but no IL-4 are defined as Th1 cells that are believed to be responsible for cellular immunity.^{14,15)} In contrast, T cells producing IL-4, IL-5, and IL-13, which mediate humoral immunity, are defined as Th2 cells.^{16,17)} It has been suggested that Th1-Th2 balance is important for keeping homeostasis, though failure of Th1-Th2 balance underlines various immune diseases.^{18,19)} Examples of Th1-dominant reactions include delayed type hypersensitivity (DTH) responses,^{20,21)} contact hypersensitivity, experimental autoimmune encephalomyelitis and rheumatoid arthritis.^{22,23)} In contrast, Th2 cells are responsible for atopic diseases, bronchial asthma, allergic rhinitis^{24,25)} and other immunoglobulin-mediated autoimmunity.^{12,13)} It seemed that these Th2-dominant reactions were caused by decreased cellular immunity and high concentrations of serum IgE Abs.

It is of interest to investigate whether it is possible to manipulate Th1-Th2 balance by orally administering Kampo medicines, and the effects of Kampo medicines were different according to aging. In this study, we indicated the murine model that administration of Kampo medicines Saiko-keishi-to (SKT; 柴胡桂枝湯) and Juzen-taiho-to (JTT; 十全大補湯) to BALB/c neonate mice, and at 8 and 25 weeks old applied with drinking water for 4 weeks. Before and after administration, we compared immunological features of treated mice to those of non-treated mice. We analyzed cell surface phenotype by a flow cytometer, and cytokine productions that associated with Th1-Th2 balance.

Materials and Methods

Crude drugs: The Kampo medicines used in this study, Saiko-keishi-to (SKT; Chai-Hu-Gui-Zhi-Tang in Chinese) and Juzen-taiho-to (JTT; Shi-Quan-Da-Bu-Tang in Chinese) are basically plant source medicines, and each prescription was a combination of several different medicinal plants (Crude drugs). The recipes of formulations are as follow, with the dosage (g) given in

parentheses: Saiko-keishi-to (SKT); Bupleuri Radix (5.0), Pinelliae Tuber (4.0), Cinnamomi Cortex (2.0), Scutellariae Radix (2.0), Ginseng Radix (2.0), Paeoniae Radix (2.0), Zizyphi Fructus (2.0), Glycyrrhizae Radix (1.5), Zingiberis Rhizoma (0.5); Juzen-taiho-to (JTT); Ginseng Radix (3.0), Angelicae Radix (4.0), Atractylodis Rhizoma (4.0), Astragali Radix (3.0), Glycyrrhizae Radix (2.0), Hoelen (4.0), Cinnamomi Cortex (3.0), Paeoniae Radix (3.0), Rehmanniae Radix (4.0), Cnidii Rhizoma (3.0). These crude drugs were purchased from Uchida Wakan-Yaku Co Ltd. (Tokyo, Japan), Tochimoto Tenkaido Co Ltd. (Osaka, Japan) and Tsumura & Co. (Tokyo, Japan). Astragali Radix used is classified into extra high-grade in Japanese market, and quality of other crude drugs is controlled by the Japanese Pharmacopoeia (JP XIII).

Preparation of Kampo prescriptions: The Kampo prescriptions were administered to mice in a form of decoction. The procedure used for the preparation was as follows: Combined ingredients were mixed with 600 ml of distilled water, and the whole was boiled for 40 min until the volume was reduced to 300 ml. The extracted solution was centrifuged at 6,000 rpm for 20 min, and then the supernatant was filtered and frozen until use.

Animals and treatments: Specific pathogen-free BALB/c female mice were purchased from Japan SLC Co Ltd. (Shizuoka, Japan). The animals were maintained in SPF condition and were housed with a lighting schedule (12 hr of light and 12 hr of darkness) at a controlled temperature ($22 \pm 1^\circ\text{C}$). Neonates, 8 and 25 week old BALB/c mice were used in this experiment. The experiments were conducted in accordance with the Guideline for Animal Use and Experimentation of the Kitasato Institute.

Eight week and 25 week old mice were administered SKT or JTT orally with drinking water for 4 weeks. The control mice were provided tap water alone. The daily doses of Kampo medicines were controlled corresponding to 20-fold dose per kg body weight in human adults by regulating its concentration in relation to water consumption.

For administering Kampo medicines to neonate mice, the mothers started receiving SKT or JTT with drinking water at embryonic day 18-20. After birth, neonate mice fed on mother's milk. During the lactation period, Kampo medicines were administered to mother mice. Neonate mice were received Kampo medicines

through milk for 3 weeks, and then received Kampo medicines through drinking water for 1 week after weaning.

Reagent and chemicals: RPMI-1640 medium, purified hamster anti-mouse CD3 monoclonal antibody (clone 145-2C11), fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD8 (53-6.7), R-phycoerythrin (PE)-conjugated rat anti-mouse CD4 (RM4-5), FITC-conjugated hamster anti-mouse CD3 (145-2C11), and PE-conjugated rat anti-mouse CD19 (1D3) were obtained from Pharmingen (CA, USA). Fetal bovine serum (FBS), penicillin, streptomycin and lipopolysaccharide (LPS, *Escherichia coli* serotype 0127, B8) were from Sigma (MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits for IL-4 and IFN- γ were from Amersham International PLC (Buckinghamshire, UK), and kit for IL-12 was from Biosource International (CA, USA).

Preparation of splenic lymphocytes: At autopsy, the spleens were immediately removed and pressed with a slide glass in phosphate buffered saline (PBS). The cell suspension was passed through a #200 stainless steel sieve. Red blood cells were removed by Tris-NH₄Cl hemolysis buffer, and splenic lymphocytes were washed 3 times with PBS, and then re-suspended in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin at a density of 5×10^6 cells/ml.

Cell culture and cytokine production measurements: Splenic lymphocytes (5×10^6 cells/well) were stimulated by purified hamster anti-mouse CD3 Ab (1.0 μ g/ml) for IL-4 and IFN- γ measurements, and stimulated by LPS (10 μ g/ml) to measure IL-12, in 24-well culture plate (FALCON 3097, Becton-Dickinson & Co., NJ, USA). After incubation for 42 hrs at 37°C, the supernatant was collected and assessed cytokine production by ELISA kit.

Flow cytometric analysis: Cells were double stained with combination of FITC- and PE-conjugated antibodies, and analyzed by the two-color immunofluorescence test. Briefly, splenic lymphocytes were incubated with 1 μ g/million cells of fluorescein-conjugated Abs for 1 hr at 4°C in the dark place. After incubation, fluorescein-stained cells were washed 3 times with cold PBS and analyzed by flow cytometry on EPICS ELITE with logarithmic amplifier (Coulter, Hialeah, FL, USA).

Lymphoid cells were gated by the forward- and side-scatter gating method for the analysis of the lymphocytes population. A fluorescein histogram of 10,000 counts was collected in each sample.

Statistics and data analysis: The data were analyzed using Fishers' PLSD test. A value of $p < 0.05$ was accepted as statistically significant.

Results and Discussion

Japanese traditional herbal medicines (Kampo medicines) are widely used for the treatment of the many kinds of acute and chronic diseases in eastern Asia. Many reports have also shown that Kampo medicines are effective for treating patients with atopic dermatitis.^{8,9)} Increment of Th2 type cytokine production is observed in patients with allergic disease such as atopic dermatitis, allergic rhinitis, systemic lupus erythematosus (SLE), and bronchial asthma.^{28,29)} It was reported that the effect of Kampo medicines toward to these diseases was seen in a decrease of subjective symptom.^{30,31)} It is considered that decreased IgE levels and immunoregulation mediated these healing mechanisms.^{32,33)} Clinically, we often experience cases in which atopic dermatitis of infants improves in a rather short time compared to that of adults. Therefore, it is considered that Kampo medicines might act *via* affecting the Th1-Th2 balance,^{10,11)} and their effects might differ depending on the age of the patients.³⁴⁾ In this study, our endeavor to determine the influence of age, male BALB/c mice of different ages were treated with Kampo medicines (SKT and JTT) for 4 weeks. Then before and after administration, we compared immunological features of treated mice to those of non-treated mice by flow cytometric analysis of cell surface phenotype, and cytokine productions.

At first, we examined whether oral administration of Kampo medicine modulates lymphocytes population, the phenotype of cells were analyzed with a flow cytometer. Splenic lymphocyte subpopulations were determined in neonates, and in 8 and 25 week old BALB/c mice after administration of SKT or JTT for 4 weeks. The obtained results were shown in Table I. No significant differences of splenic lymphocyte subpopulation were observed in the JTT treated group compared to the control group. In contrast to JTT, several changes of lymphocytes population were observed in SKT treated

mice. By treatment of SKT in neonates, the population of CD3⁺CD19⁻ cells decreased, and this was due to decrement of both CD4⁺CD8⁻ and CD4⁺CD8⁺ cells. The CD4/CD8 ratio was decreased by SKT. Whereas, the population of CD3⁺CD19⁺ cells increased significantly, so the ratio of CD3/CD19 cell decreased significantly in neonates. Thus, in neonates SKT increased B lymphocyte population, and reduced helper T population. In 8 week old mice, the CD3/CD19 ratio was not different compared with control. However, the population of CD4⁺CD8⁻ cells and the ratio of CD4/CD8 significantly decreased in SKT treated group, compared to the control group. In 25 week old mice, no significant changes were observed by administration of SKT. These results suggest that SKT had a potential alteration of T lymphocyte populations in neonates and at younger ages.

SKT contains Bupleuri radix as a component crude drug. Previously, we reported that a part of orally administered polysaccharide from Bupleuri radix was absorbed from the intestine,^{10,35)} and the oral administration of polysaccharide fraction from Bupleuri radix activated murine B cells *in vivo*.³⁶⁾ Therefore, it is presumed that the increment of B cells in neonates might be partially due to polysaccharide of Bupleuri radix. However, details of the mechanisms on the modulation of lymphocytes population by SKT is not known at present, and must await further investigation.

The realization that murine and human CD4⁺ helper T cells can be subdivided into Th1 and Th2 subsets is based on their profile of cytokine production.³⁷⁾ Th1 cells

produce interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), interleukin-2 (IL-2), and stimulate the cellular immune response. In contrast, Th2 cells produce IL-4, IL-5, IL-6 and IL-13, and stimulate the humoral immunity. Furthermore, much evidence suggested that the Th1 and Th2 types of immune response are reciprocally regulated *in vivo*.^{14,38)} It has also been suggested that many diseases were partially caused by a skewed Th1 and Th2 cytokine balance.^{18,19)} As mentioned above, the increment of Th2 type cytokine production is observed in patients with allergic disease.^{28,29)} Conversely, Th1 cells mediate inflammatory diseases such as graft versus host disease (GVHD).³⁹⁾ These data indicated that Th1-Th2 balance plays a pivotal role in keeping the homeostasis in the immune system.

Next, therefore, we examined on the effect of SKT and JTT from a viewpoint of modulation of Th1-Th2 balance. It is known that IL-4 and IFN- γ are a major determinant of the differentiation of naive T cells into Th1 and Th2 cells, respectively.⁴⁰⁾ To investigate the influence of Kampo medicines on Th1-Th2 balance, we examined the productions of IL-4 and IFN- γ as well as IL-12 in the supernatants of the spleen cell. The results are shown in Figures 1 and 2, no significant difference in IFN- γ , Th1 type cytokine, production in mice of any age was observed in SKT or JTT treated groups (Fig.1). However, IL-4, which is Th2 type cytokine, productions in SKT treated groups and JTT treated groups were significantly reduced in neonates and in 8 week old mice, but not in 25 week old mice (Fig.2).

Table 1 Effects of SKT and JTT on splenic lymphocyte subpopulations in different age mice. The splenic lymphocytes were stained with fluorescein-conjugated antibodies, measured percentages of lymphocyte subpopulations by flow cytometer as described in Materials and Methods. Each value represented as mean \pm S.E. from neonate mice (n= 8-11), 8 week old mice (n= 7), 25 week old mice (n=7). Significantly different from control group, *: $p<0.05$, **: $p<0.01$.

		CD4+ (%)	CD8+ (%)	CD4/CD8	CD3+ (%)	CD19+ (%)	CD3/CD19
neonate	Cont.	23.8 \pm 1.0	14.3 \pm 0.6	1.67 \pm 0.06	37.0 \pm 1.7	54.0 \pm 1.6	0.70 \pm 0.06
	SKT	17.0 \pm 1.1**	12.3 \pm 0.5*	1.39 \pm 0.09**	30.2 \pm 1.3**	61.0 \pm 1.2**	0.50 \pm 0.03**
	JTT	24.0 \pm 1.0	14.7 \pm 0.4	1.63 \pm 0.06	39.4 \pm 1.2	52.3 \pm 1.2	0.76 \pm 0.04
8 week old	Cont.	43.8 \pm 2.2	22.8 \pm 0.7	1.91 \pm 0.05	62.0 \pm 2.4	24.4 \pm 2.2	2.72 \pm 0.42
	SKT	39.3 \pm 1.5*	23.5 \pm 1.1	1.68 \pm 0.06*	59.9 \pm 1.4	25.8 \pm 1.1	2.36 \pm 0.14
	JTT	42.9 \pm 1.2	23.9 \pm 0.7	1.82 \pm 0.09	65.0 \pm 1.6	21.4 \pm 1.9	3.23 \pm 0.37
25 week old	Cont.	47.7 \pm 2.0	20.4 \pm 1.5	2.38 \pm 0.14	58.7 \pm 2.4	29.9 \pm 2.6	2.08 \pm 0.27
	SKT	44.4 \pm 2.0	20.1 \pm 1.9	2.29 \pm 0.16	61.1 \pm 3.5	27.2 \pm 3.7	2.60 \pm 0.48
	JTT	42.3 \pm 2.6	20.4 \pm 1.5	2.10 \pm 0.10	58.6 \pm 4.7	28.7 \pm 5.2	2.55 \pm 0.61

mean \pm S.E. * $p<0.05$ vs.cont.
** $p<0.01$ vs.cont.

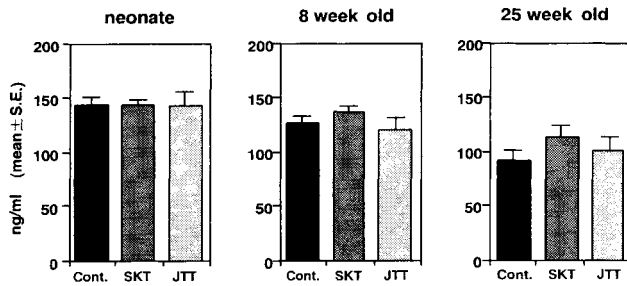


Fig. 1 The effects of SKT and JTT on the IFN- γ production from splenic lymphocytes in different age mice. Splenic lymphocytes (5×10^6 /ml) were cultured with anti-CD3 mAb (1μ g/ml) in 24-well plate for 42hr and culture supernatants were harvested and IFN- γ productions in the supernatants were detected as described in Materials and Methods. Each value represented as mean \pm S.E. from neonate mice (n=8~11), 8 week old mice (n=7), 25 week old mice (n=7).

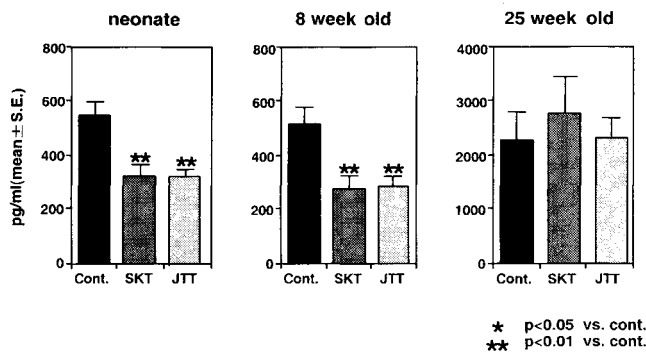


Fig. 2 The effects of SKT and JTT on IL-4 production from splenic lymphocytes in different age mice. Splenic lymphocytes (5×10^6 /ml) were cultured with anti-CD3 mAb (1μ g/ml) in 24-well plate for 42hr and culture supernatants were harvested and IL-4 productions in the supernatants were detected as described in Materials and Methods. Each value represented as mean \pm S.E. from neonate mice (n=8~11), 8 week old mice (n=7), 25 week old mice (n=7). Significantly different from control group, **: p<0.01.

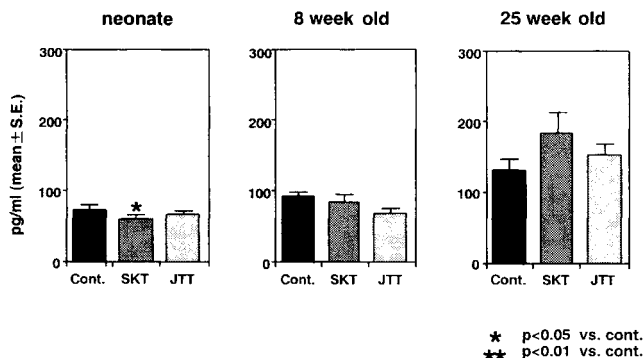


Fig. 3 The effects of SKT and JTT on the IL-12 production from splenic lymphocytes in different age mice. Splenic lymphocytes (5×10^6 /ml) were cultured with LPS (10μ g/ml) in 24-well plate for 42 hr and culture supernatants were harvested and IL-12 productions in the supernatants were detected as described in Materials and Methods. Each value represented as mean \pm S.E. from neonate mice (n=8~11), 8 week old mice (n=7), 25 week old mice (n=7). Significantly different from control group, *: p<0.05

It has been reported that IL-12, produced by antigen-presenting cell (APC), such as dendritic cell or macrophage, stimulate the differentiation of naive T cells into Th1 cells.⁴¹⁾ Figure 3 indicates that SKT and JTT did not affect the IL-12 production from the splenocytes stimulated with LPS at any age, except SKT treated neonates. As described above, no significant differences in IFN- γ productions was observed in mice of any age, therefore it is speculated that the modulation ability of SKT and JTT on Th1-Th2 balance might not be through modulation of the IL-12 production.

It has been known that BALB/c mice showed Th2 dominant immune response, and the differentiation of helper T cells was modified by a lot of factors, *i.e.* expression of co-stimulatory molecules on APC and humoral factors such as IL-13 or IL-18.^{42,43)} On the other hand, modulation of Th1-Th2 balance is one of possible strategies for treatment of allergic diseases. Recently, a new Th2 cytokine production inhibitor, suplatast tosilate, has been reported, and showed a wide variety of anti-allergic actions in various experimental animal models.⁴⁴⁻⁴⁷⁾ Several clinical trials of suplatast tosilate have come to the conclusion that these Th2 cytokine production inhibitors have overall beneficial effects in allergic diseases.⁴⁸⁾

The results obtained here indicated that oral administration of SKT and JTT reduced IL-4 production in neonates and 8 week old mice, and resulted in a shift into Th1 domination. Therefore, it is presumed that clinical effectiveness of SKT and JTT on atopic dermatitis may partly be explained by this modulation of Th1-Th2 balance. But, it is not known which factor(s) contribute to the effects of Kampo medicines, and further investigations are required.

According to the dose of medicines, it is widely known that humans have a low metabolic ability on drugs.⁴⁹⁾ Inversely, drugs are metabolized in mice more quickly than in humans. Indeed, it was reported that the hepatic clearance of drugs in humans was approximately one-seventh that of other mammals.⁵⁰⁾ Furthermore, it was reported that when comparing renal clearance rate between human and mice, clearance of mice is about 10-fold greater than that of humans.^{51,52)} Therefore, we used a relatively high dosage in this study; the dosage of SKT or JTT was equal to 20-fold dose per kg body weight corresponding to human adults.

Kampo medicine is used according to the patient's condition and age. In this study, the changes of lymphocytes population was observed in SKT treated mice and the reduction of IL-4 production were observed in both SKT treated mice and JTT treated neonate and 8 week old mice. However, such influences were not observed at the age of 25 weeks old. From these results, it presumed that younger age mice were more sensitive on immunomodulation by Kampo medicines than aged mice. Our results may provide a plausible explanation for the clinical experience that atopic dermatitis of infants improves in a rather short time compared to that of adults. So far as we know, this is the first description that the modulation of lymphocytes population and Th1-Th2 balance by oral administration of Kampo medicines was different according to aging. To elucidate the action mechanisms of SKT and JTT on Th1-Th2 balance and active components of these Kampo medicines, further investigations are necessary.

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和文抄録

アトピー性皮膚炎、気管支喘息などのアトピー体質を有する患者の治療に漢方方剤が有効であるが、こうした漢方方剤の効果は患者の年齢により影響を受けることを臨床経験する。今回Th2優位のBALB/cマウスに対する漢方方剤（柴胡桂枝湯および十全大補湯）の作用がマウスの週齢（仔マウス、8週齢、25週齢）によってどのように異なるかについて比較検討した。脾臓リンパ球サブポピュレーションにおいてはCD4/CD8比は仔マウスおよび8週齢マウスの柴胡桂枝湯投与群でコントロール群と比較し低下していた。CD3/CD19比は仔マウスの柴胡桂枝湯投与群で低下していた。脾臓リンパ球IL-4産生能は仔マウスおよび8週齢マウスにて、柴胡桂枝湯投与群、十全大補湯投与群のいずれでも有意に低下していた。IFN- γ 産生能は変化を認めなかった。IL-12産生能は仔マウスでは柴胡桂枝湯投与群で有意に低下していた。25週齢マウスでは柴胡桂枝湯投与群と十全大補湯投与群の

いずれにおいても有意な変化は観察されなかった。以上の結果から柴胡桂枝湯や十全大補湯などの漢方方剤の作用は週齢によって異なり臨床応用上も考慮に入れるべきと考えられた。

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