ABSTRACT

Objectives: A traditional herbal prescription, Kyung–Ok–Ko (KOK), has long been used in oriental medicine as an invigorant for age–related diseases, such as amnesia and stroke. However, the beneficial value of KOK for immune responses is largely unknown. Based on the above mentioned effects of KOK, other previous reports, and its use in traditional medicine, we hypothesized that KOK displays beneficial effects against methotrexate (MTX)–induced immune suppression.

Methods: We investigated the effects of KOK (0.6 g/kg/day, oral (p.o.)) on deteriorated immunity caused by MTX (2 mg/kg/day, p.o.) in an immune suppression mouse model. MTX was fed to mice once a day for 7 days. After the immune responses of the mice deteriorated by MTX treatment, KOK in water was fed to the mice once a day for 14 days. We then measured the expression levels of various cytokines, such as T helper cell (Th1, Th2) cytokines, and the number of immune cells, such as spleen T cells, B cells, and macrophages.

Results: The data showed that MTX decreased Th1 profiles (interferon (IFN)–γ, interleukin (IL)–2, IL–12) and the number of immune cells, and increased Th2 profiles (IL–4, IL–5, IL–13), which were normalized significantly by post–administration of KOK. However, there was no significant difference in body–weight gain between MTX– and KOK–treated mice.

Conclusion: These results indicate that KOK has immune–enhancing functions and reduces immunotoxicity of MTX, suggesting that supplementation with KOK will improve immune responses clinically and be useful for the prevention of immune–related diseases.

Key words: Kyung–Ok–Ko, methotrexate, Th1, Th2, immune suppression

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I. Introduction

Two subsets of T helper cells, types 1 (Th1) and 2 (Th2), have distinct roles in the immune system. Th1 cells modulate cellular immunity by producing interleukin (IL)-2, IL-12, and interferon-γ (IFN-γ), whereas Th2 cells are implicated in humoral responses by secreting IL-4, IL-5, IL-10, and IL-13. In addition, IFN-γ suppresses Th2 immune responses, while IL-4 and IL-10 downregulate Th1 responses. Methotrexate (MTX) is a folate antagonist first developed for the treatment of malignancies and, subsequently, used in nonneoplastic diseases as an anti-inflammatory and immune-suppressive drug. MTX was the most commonly used treatment of rheumatoid arthritis and other chronic inflammatory disorders. MTX has also been used as an adjunct therapy for persistent mild cardiac allograft rejection.

Kyung-Ok-Ko (KOK), also known as Qiong-yu-gao in Chinese and Kei-gyoku-kou in Japanese, is a traditional herbal prescription consisting of a decoction of the following six ingredients: Wolfiporia extensa, Rehmannia glutinosa, Panax ginseng C.A. Meyer, and Mel. In eastern Asian countries, such as Korea, China, and Japan, KOK is prescribed as a tonic medicine to maintain health and increase longevity. According to previous studies, KOK has antityrosinase, antiplatelet, antithrombotic, and antioxidant activities. In addition, some studies have examined the effects of KOK on age-related disorders, as well as the biological properties of KOK, including its antioxidant, anti-inflammatory, anti-fatigue, and immunological activities. Therefore, the aim of the present study was to investigate whether KOK affected the pattern of Th1 and Th2 cytokine secretion and regulated the Th1/Th2 balance.

II. Materials and Methods

1. Reagents

MTX was purchased from Sigma (St. Louis, MO). MTX (Sigma, p.o.) at 2 mg/kg/day was used to suppress immune responses according to a previous report. KOK and RG extract were provided by Kwang Dong Pharmaceutical Company (Seoul, Republic of Korea); chemical profiling and standardization of KOK and red ginseng (RG) extract were performed by Kwang Dong Pharmaceutical Company. Its preparation was described previously.

2. Animals and husbandry

Male C57BL/6 mice (6–7 weeks old, approximately 27 g) were purchased from Orient Bio Co., (Sungnam, Republic of Korea) and used after 12 days of acclimatization. The mice were housed (5 per polycarbonate cage) under controlled temperature (20–25°C) and humidity (40–45%), and a 12:12 h light: dark cycle was maintained. All mice were treated in accordance with the "Guidelines for the Care and Use of Laboratory Animals," which were issued by Kyungpook National University (IRB No, KNU 2017– 0093-1).

3. MTX administration-mediated immune suppression mouse model

For the MTX–induced immune suppression test, 4 groups of mice (n = 10 per group) were used: (i) distilled water (vehicle control, VC) for 3 weeks, (ii) MTX–treated (2 mg/kg/day, p.o.) for 1 week, (iii) MTX was administered for 1 week and then KOK (0.6 g/kg/day, p.o.) was administered for 2 weeks, (iv) MTX was administered for 1 week and then RG extract (45 mg/kg/day, p.o.) was administered for 2 weeks. Body weight and food intake were determined twice a week. Mean total food intake during the total experimental period of 3 weeks was calculated as well as daily food consumption.

4. Measurement of serum levels of Th1 and Th2 cytokines

Mouse blood samples were collected by cardiac puncture and placed in tubes containing ethylenediaminetetraacetic acid (EDTA, 10 mg/mL). Mouse cytokines (IFN-γ, IL-2, IL-4, IL-5, and IL-12) were measured using a mouse cytokine 10-Plex Panel kit (Thermo Fisher Scientific, Waltham, MA), and the mouse IL-13 ELISA kit was purchased from R&D Systems (Minneapolis, MN), ELISA assays were performed according to the manufacturer’s instructions.

5. Determination of the number of immune cells

Spleen tissues were isolated, minced and then digested with collagenase–D (1 mg/mL) in the presence of 5 mM EDTA for 30 min at 37°C. The undigested fibrous material was removed by filtration through a 70-μm cell strainer (Falcon; BD Biosciences, San Jose, CA). Cells were subjected to flow cytometric analysis with the following monoclonal antibodies: peridinin–chlorophyll–protein (PerCP)– conjugated anti–mouse
CD3e, fluorescein isothiocyanate (FITC)–conjugated anti-mouse CD4, R–phycoerythrin (R–PE)–conjugated anti-mouse CD8a, R–PE–conjugated anti-mouse CD45R/B220, or FITC–conjugated anti-mouse CD11b (BD Biosciences). Stained cells were resuspended in 1 mL of PBS, and the fluorescence was quantified using a FACScan™ flow cytometer.

6. Statistical analyses
All experiments were independently performed at least three times and the values are expressed as means ± SD. The Student’s t-test was used to calculate significant differences and statistical significance was accepted at p-values < 0.05. SPSS for Windows, version 16.0 (SPSS, Chicago, IL) was used to perform all statistical analyses.

III. Results
In the current study, RG extract was used as a positive control because of its immune-stimulating effects. Based on previous reports, KOK at 0.6 g/kg/day and RG extract at 45 mg/kg/day were used in the current study. Administration of MTX or KOK did not change body weight and food intake (Table 1).

Mice were administered vehicle control (VC, p.o.) for 3 weeks, MTX (2 mg/kg/day, p.o.) for 1 week, or KOK (0.6 g/kg, p.o.) and RG extract (45 mg/kg/day, p.o.) for 2 weeks. Results are shown as means ± SD (n = 10).

1. Effects of MTX on the immune responses
First, we confirmed the immune suppression responses by MTX (Table 2). Serum levels of Th1 cytokines, such as IFN-γ, IL-2, and IL-12, in the vehicle control (VC) group were 405.6 ± 35.98, 26.2 ± 2.41, and 307.2 ± 27.54 pg/mL, respectively. Seven days after MTX administration, serum IFN-γ, IL-2, and IL-12 were 71.5 ± 6.35, 6.7 ± 0.62, and 74.9 ± 6.73 pg/mL, respectively. Serum levels of Th2 cytokines, such as IL-4, IL-5, and IL-13, in the VC group were 51.1 ± 4.87, 43.3 ± 4.05, and 76.9 ± 6.98 pg/mL, respectively. Seven days after MTX administration, serum IL-4, IL-5, and IL-13 were 95.5 ± 9.12, 79.3 ± 7.49, and 143.9 ± 12.94 pg/mL, respectively. These results were consistent with those of previous reports, which demonstrated that healthy immunity is dependent on immune balance and coordination between Th1 and Th2 systems, and the greater the imbalance between

| Table 1. Effects of MTX or KOK on mouse body weight and food intake. |
|-----------------|-----------------|-----------------|
| VC              | MTX             | KOK             | RG extract      |
| Body weight (g) | 26.8 ± 1.7      | 27.1 ± 1.4      | 26.8 ± 1.3      |
| Food intake (g/mouse/day) | 5.2 ± 0.3 | 5.8 ± 0.6 | 5.3 ± 0.3 |

Fig. 1. Effects of KOK on Th1 cytokine levels. Mice were administered MTX (2 mg/kg/day, p.o.) for 1 week, followed by VC (p.o.), KOK (0.6 g/kg, p.o.), or RG extract (45 mg/kg/day, p.o.) for two weeks. Concentrations of interferon (IFN)–γ(A), interleukin (IL)–2(B), and IL–12(C) (pg/mL) in serum were measured by mouse cytokine 10–Plex Panel kit. Results are presented as means ± SD (n = 10). *p < 0.01 versus week 0 (Control) and †p<0.01 versus week 1 (MTX treatment).
the more inflammation our body produces and the less effective and efficient our immune responses become.\textsuperscript{1, 3}

2. Effects of KOK on Th1 cytokines secretion

To investigate the role of KOK in the secretion into the blood of Th1 cytokines, KOK in water was fed to the mice once a day for 14 days after immune responses of the mice deteriorated by MTX treatment. We then measured the expression levels of Th1 cytokines in serum, such as IFN-$\gamma$, IL-2, and IL-12. Data showed that when KOK (0.6 g/kg, p.o.) was added after MTX treatment, decreased IFN-$\gamma$, IL-2, and IL-12 by MTX was recovered significantly by KOK treatment (Fig. 1). KOK showed the similar activity as RG extract.
3. Effects of KOK on the expressions of Th2 cytokines

The secretion into the blood of the Th2 cytokines, such as IL-4, IL-5, and IL-13 in response to KOK after MTX treatment in mice was examined. Increased levels of IL-4, IL-5, and IL-13 in the blood of mice by MTX were decreased after KOK treatment time-dependent manner (Fig. 2). KOK showed the similar activity as RG extract.

4. Effects of KOK on splenic cellularity

Further, we determined the effects of KOK on splenic cellularity. As shown in Table 4, VC did not affect the splenic cellularity. However, the number of total spleen cells, T cells, B cells, and macrophages were significantly decreased by MTX, which was ameliorated by KOK (Fig. 3).

IV. Discussion

Recently, the population of the elderly has increased sharply, and there is a high interest in increasing immune system by taking healthy functional foods. Declines in the immune function of humans can be reduced by aging and increase the risk of infection. Anticancer agents and immunosuppressants for transplantation are well known as side effects causing immune system failure and immune cell proliferation and function reduction. Diverse immunosuppressant drugs are often used to treat autoimmune disorders such as lupus, psoriasis, and rheumatoid arthritis.

Many research teams have researched the immune system for the past few decades, and their understanding of the function of the immune system has increased. Focus on accurately and deeply studying the mechanism of action of drugs in developing their immune modulators. Specifically, investigate the specific components of the cytokine network and the signal transduction pathway and the role of immune response regulation particularly intensively.

Th1/Th2 balance is the major framework used to address adaptive immunity, and Th1 and Th2 cells are characterized by specific cytokine signatures. IL-2, IL-12, and IFN-γ are considered to be hallmark Th1 cytokines, while IL-4, IL-5, and IL-13 are the hallmark Th2 cytokines. Some studies have demonstrated that KOK had immunological activities.

In the present study, after the immune responses of the mice deteriorated by MTX treatment, KOK was fed to the mice in order to investigate the role of KOK in Th1/Th2 balance. According to our results, decreased expression of Th1 cytokines, such as IFN-γ, IL-2, and IL-12, by MTX was recovered by KOK, while increased expression of Th2 cytokines, such as IL-4, IL-5, and IL-13, by MTX was decreased by KOK. Moreover, the number of total spleen cells, T cells, B cells, and macrophages were significantly decreased by MTX, which was ameliorated by KOK. KOK showed the same activity as RG extract when RG extract, whose efficacy is known for regulating immune and inflammatory responses, was compared using it as a positive control. These results suggest that KOK has immune-enhancing functions and reduces immunotoxicity of MTX without affecting body-weight gain.

V. Conclusions

In conclusion, our results suggest that KOK regulates Th1 and Th2 immune parameters of vascular function. Noting that the imbalance between Th1 and Th2 systems and the disordered splenic cellularity by MTX could be coordinated by KOK, our findings also present novel evidence about the potential clinical implications of KOK treatment not only in normal vascular immune function but also in preventing immune-related diseases.

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