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The effect of bee pollen and its flavonoids on immune-modulating in mice

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Abstract : Bee pollen is a valuable apitherapeutic product and has been known to have diverse biological activities, including antimicrobial, anti-inflammatory, and even anticancer activity. However, its effect on the immune system is not well studied and is rather controversial. This study intended to elucidate the biological activity of bee pollen on immunity. For this purpose, we used lyophilized bee pollen after wet grinding, which shows increased extraction of bioactive components and enhanced biological activity. First, lyophilized bee pollen after wet grinding significantly increased the proliferation of splenocytes isolated from normal mice. On the other hand, lyophilized bee pollen after wet grinding dose-dependently reversed splenocyte proliferation by concanavalin A or lipopolysaccharide. To clarify the activity of bee pollen on immunity lyophilized bee pollen after wet grinding was administered daily to mice for five weeks and isolated splenocytes. In this study, there was no significant difference in the population of immune cells and the size of spleen between bee pollen- and sterile water-treated groups. However, proliferation of splenocyte isolated from bee pollen-administered animals was boosted by both concanavalin A and lipopolysaccharide. Finally, kaempferol, a well-known flavonoid from bee pollen, dose-dependently increased splenocyte proliferation by both Con A and LPS. On the other hand, naringenin, another flavonoid in the bee pollen, dose-dependently inhibited the proliferation of splenocytes by Con A and LPS. Together, these data indicate that bee pollen may be able to prime the immunity to boost immune reaction after inflammation.

Keywords : Bee pollen, kaempferol, naringenin, concanavalin A, lipopolysaccharide, splenocyte

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1. Introduction

Bee pollen is a bee product and is accepted as a valuable apitherapeutic product due to its potential therapeutic value[1]. For example, ethanol or hydromethanol extracts from bee pollen inhibit NO production and COX-2 activity, and carrageenan-induced rat hind paw edema[2,3]. In addition, bee pollen showed antimicrobial activity; thus, bacteria such as Staphylococcus aureus, Agrobacterium tumefaciens, and A. tumefaciens are very susceptible to the extract of bee pollen[4,5]. Further, pollen extract showed a cytotoxic effect on Lewis lung carcinoma cells[6]. However, the effect of pollen on the immune system is rather controversial. First, a diet supplemented with bee pollen boosted the early development of thymus and cloacal bursa, and promoted the immune response of spleen[7]. In addition, bee products that include bee pollen were found to increase the levels of antibody titer in Japan quails, thereby indicating that bee products have immunostimulant activity[8]. On the other hand, ethanol extract of pollen Typhae suppressed the mitogen-stimulated proliferation of splenocyte[9]. Further, bee pollen inhibited mast cell degranulation by inhibiting IgE-mediated mast cell activation[10,11]. Considering the wide user population of bee pollen, including patients with benign prostatic hyperplasia and atopic dermatitis, there is an urgent need to elucidate the precise role of bee pollen in the immune system.

Bee pollen is surrounded by the outer wall, exine which is resistant to digestive enzymes, mechanical pressure, and even most acidic and basic solutions[12]. Therefore, after oral intake, bee pollen begins to swell in the digestive tract and, thus, only a small proportion of its active components permeates through osmosis[13,14]. To overcome this demerit, we recently introduced the wet-grinding process, due to which the extraction of phenolic contents from bee pollen increased by 11.9 times compared to those from homogenized bee pollen. In addition, the antioxidant activity increased by 6.78 or 3.24 times, as examined by ABTS assay or DPPH radical scavenging assay, respectively[12]. Therefore, these data indicate that wet grinding may enhance the usefulness of bee pollen for nutritional as well as therapeutic purposes.

As mentioned above, bee pollen clearly demonstrated beneficial effects in diverse conditions such as infection and cancer, but its biological effect on the immune system remains rather controversial. Of note is its low bioavailability, which is such that when bee pollen is accurately chewed before swallowing, its bioavailability is only approximately 10-15%. Therefore, this poor absorption may disturb the in vivo evaluation of its biological role in the immune system. In this study, we test the immune modulatory effect of bee pollen degraded by wet grinding.

2. Research methods

2.1. Animals

Male C57BL/6 mice (Koatech, Kyungki-do, South Korea) were housed at a standard temperature (22 ±1℃), humidity (50 ±5%) conditions with light controlled from 8:00 a.m. to 8:00 p.m. (12hr interval), and access to food and water *ad libitum*. All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978) and approved by the Institutional Animal Care and Use Committee of the Kyunsung University (Study-20-016A).

2.2. Splenocyte isolation and proliferation assay

Splenocyte isolation and proliferation assay were performed as introduced in Cho et al.,[15] with minor modification. In detail, spleens were isolated from the mice sacrificed

under aseptic condition and washed with RPMI 1640 medium (Gibco, Grand Island, NY, USA). After crushing the spleen cell mass was passed through a cell strainer (BD Bioscience, Bedford, MA, USA) to obtain a homogeneous cell suspension. Cells were washed twice with medium containing 10% fetal bovine serum (FBS, GenDEPOT, Barker, TX, USA) and centrifugation at 1000 rpm for 5 min. The recovered spleen cells were resuspended in tris-buffered ammonium chloride solution (NH_4Cl, pH 7.2) for 5 min to remove erythrocytes. After centrifugation, harvested cells were resuspended in RPMI 1640-FBS medium. After cell counting with C-Chip (Incyto, Chungcheongnam-do, South Korea) under microscope, cell was seeded in 96-well plates at a density of 1×10^5 cells/well. For the assay of splenocyte proliferation, thiazolyl blue tetrazolium bromide (MTT, 1 mg/mL, Sigma, St. Louis, MO, USA) was added to each well and incubated for 3 hours, and then dimethyl sulfoxide (DMSO, Sigma) was added to resolve formazan. The absorbance was measured in a microplate reader (INNO, South Korea).

2.3. Splenocyte proliferation by bee pollen

Male C57BL/6 (8-weeks old, Koatech) were sacrificed after asphyxia with carbon dioxide. Isolated splenocytes were incubated with 0.019, 0.037, or 0.074% bee pollen (wet-grinded or lyophilized after wet grinding, Korea Beekeeping Association in Sokcho, South Korea) for 48 hours. To induce splenocyte proliferation, concanavalin (Con A, 5 μg/ml, Sigma) or lipopolysaccharide (LPS, 15 μg/ml, Sigma) was added during the splenocyte culture for 48 hours. The extent of splenocyte proliferation was determined by MTT assay. The absorbance was measured at 540 nm. The method of wet grinding was introduced in our previous report[12] and the lyophilized bee pollen was obtained by lyophilization of wet-grinded bee pollen.

2.4. Splenocyte proliferation by chronic administration of bee pollen in mice

Lyophilized bee pollen after wet grinding (1 g/kg, daily) or sterile water was orally administered to C57BL/6 mice (male, 8-weeks old, Koatech) for five weeks. Forty-eight hours after the last oral administration, mice were sacrificed after asphyxia with carbon dioxide and splenocytes were isolated as described above. Isolated splenocytes were then cultured with proliferation Con A (5 μg/ml, Sigma) or LPS (15 μg/ml, Sigma) for 48 hours, and splenocyte proliferation was determined by MTT assay.

2.5. Splenocyte proliferation by flavonoids

Normal C57BL/6 mice (male, 8-weeks old, Koatech) were sacrificed after asphyxia with carbon dioxide. Splenocytes were isolated as described above, and the isolated splenocytes were incubated with kaempferol or naringenin for 48 hours. Splenocyte proliferation was induced by the addition of Con A (5 μg/ml, Sigma) or LPS (15 μg/ml, Sigma). The extent of splenocyte proliferation was determined by MTT assay. The absorbance was measured at 540 nm.

2.6. Statistical Analysis

Data were analyzed using GraphPad Prism 3.0 (GraphPad Software, Inc., San Diego, CA, USA) and presented as mean ± SEM, significance was assessed by a One-Way Analysis of Variance (ANOVA), and post hoc tests were performed using Bonferroni correction.

3. Results and Discussion

3.1. Proliferation of splenocytes by bee pollen

First, we tested whether nano-sized bee pollen induces proliferation of splenocytes in vitro. For this study, we prepared two types of

nano-sized bee pollen—wet-grinded bee pollen and lyophilized bee pollen after wet grinding. Splenocytes were isolated and cultured for 48 hours. As shown in Figure 1A, we found that lyophilized bee pollen after wet grinding (0.07%) significantly increased the proliferation of splenocytes. On the other hand, this low concentration of wet-grinded bee pollen did not increase splenocyte proliferation. Next, we examined the dose-dependency of lyophilized bee pollen. As shown in Figure 1B, splenocyte proliferation dose-dependently increased up to 0.037%, and there was no significantly different between 0.037% and 0.074%. To clarify the potential involvement of bee pollen on the absorbance of wavelength used in the experiment, we analyzed the absorbance up to 1% of bee pollen. However, we did not find any difference in the light absorbance (data not shown).

3.2. Effect of bee pollen on proliferation of splenocytes induced by mitogens

We tested the effect of bee pollen on splenocyte proliferation by mitogens. Interestingly, as shown in Figure 2A and 2B, both wet-grinded bee pollen (w. grinded) and lyophilized bee pollen after wet grinding (lyophilized) significantly reduced splenocyte proliferation induced by Con A or LPS. Considered the data presented in Figure 1 indicate that bee pollen has antithetical effects on splenocyte proliferation, depending on the conditions—we found increased proliferation under normal conditions and reduced proliferation when splenocyte proliferation is induced by mitogen. Next, we tested the dose-dependency of bee pollen in modulating splenocyte proliferation by mitogens. As shown in Figure 2C, 0.074% lyophilized bee pollen after wet grinding significantly reduced the increased splenocyte proliferation by Con A, and there was clear dose-dependency from 0.019% to 0.074% of bee pollen. In addition, there was a more dramatic dose-dependent decrease of splenocyte proliferation by LPS (Fig. 2D).

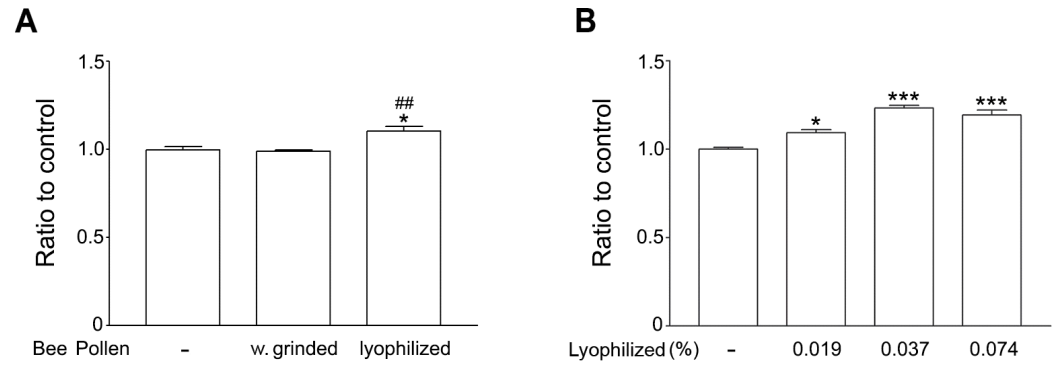

Fig. 1. The effect of bee pollen on splenocyte proliferation. (A) Splenocytes from normal mice were incubated with 0.07% wet-grinded bee pollen (w. grinded) or lyophilized bee pollen for 48 hours (lyophilized). The proliferation of splenocytes was analyzed using MTT assay. (B) Dose-dependent effects of lyophilized bee pollen after wet grinding on the proliferation of splenocytes was tested. Splenocytes were isolated from normal mice. Data were represented as relative ratio to control (without bee pollen) and as mean ± SEM (n=4). $*p<0.05$, $***p< 0.001$ compared with control, $##p<0.01$ compared with wet-grinded bee pollen.

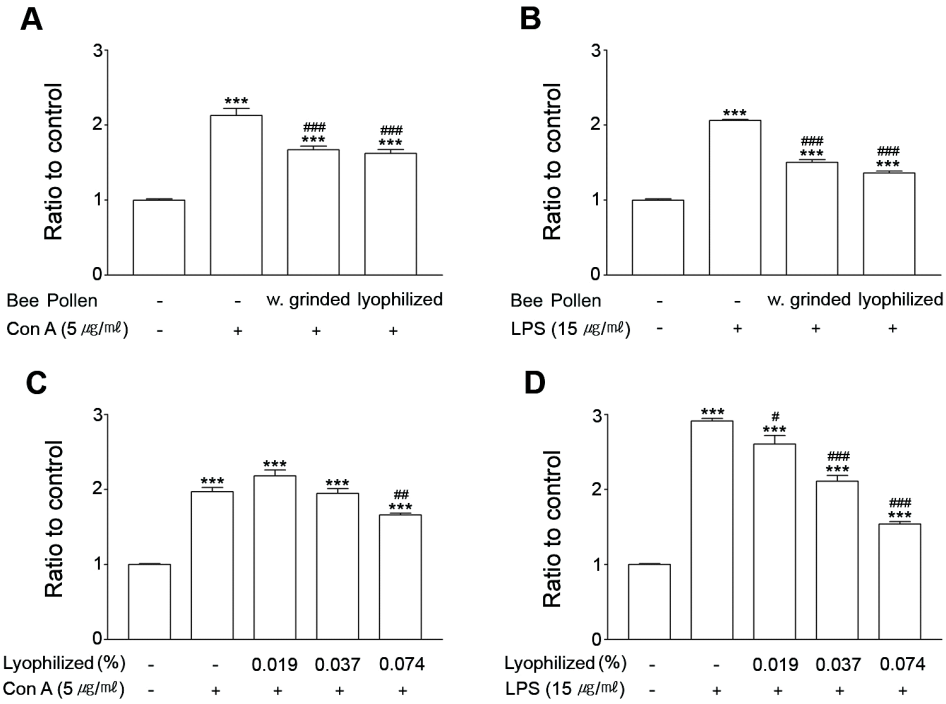

Fig. 2. The effect of bee pollen on splenocyte proliferation induced by mitogen. (A-B) Splenocytes from normal mice were incubated with 0.07% wet-grinded bee pollen (w. grinded) or lyophilized bee pollen after wet grinding (lyophilized) for 48 hours. The proliferation of splenocytes was induced by Con A (A) or LPS (B) treatment and analyzed using MTT assay. Data were represented as relative ratio to control and as mean ± SEM (n=4). ***p<0.001 compared with control, ###p< 0.001 compared with Con A- or LPS-treated control. (C-D) Dose-dependent effects of lyophilized bee pollen after wet grinding on the proliferation of splenocytes by Con A (C) or LPS (D) was tested. Data were represented as relative ratio to control and as mean ± SEM (n=4). ***p<0.001 compared with control, #p<0.05, ##p<0.01, ###p <0.001 compared with Con A- or LPS-treated control.

3.3. Effect of bee pollen when chronically administered to mice

As discussed above, the effect of bee pollen on the immune system is rather controversial. To elucidate the effect of bee pollen in greater detail, we administered lyophilized bee pollen after wet grinding by oral gavage for five weeks. We found that there was no difference in body weight gain between bee pollen-administered and water-administered animals (Fig. 3A). At 48 hours after the last administration, spleens were isolated and cultured to identify the splenocyte proliferation. First, we studied the basal potential of splenocyte proliferation without any mitogen. For this experiment isolated splenocytes were cultured for 48 hours without any mitogen and then MTT analysis was performed. In this experiment, there was no significant difference in the splenocyte proliferation between two groups (Fig. 3B). Next, we tested the potential of splenocyte proliferation by mitogens. To our surprise, splenocyte proliferation by both Con A and LPS was significantly potentiated by the

prior chronic administration of lyophilized bee pollen after wet grinding (Fig. 3C, 3D). These data clearly indicate that chronic administration of bee pollen is able to boost the immune response to mitogen.

3.4. Effect of flavonoids on splenocytes proliferation

Lastly, we tested the effects of kaempferol and naringenin, the representative flavonoids in bee pollen, on the proliferation of splenocytes. For this study, we isolated splenocytes from normal mice and cultured for 48 hours with kaempferol or naringenin. As shown in Figure 4A, 4B and 4C, kaempferol reinforced the proliferation of splenocytes dose-dependently in the condition without mitogen. In addition, when kaempferol was incubated with Con A or LPS, the proliferation of splenocytes increased dose-dependently. However, it is important to note that at lower concentration, kaempferol diminished the enhanced

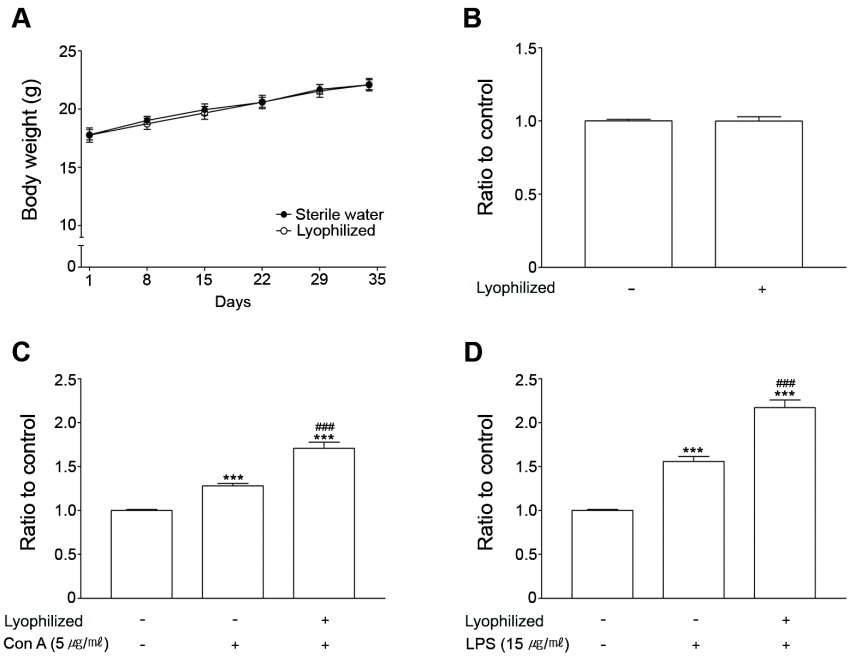

Fig. 3. Chronic administration of bee pollen boosts lymphocyte proliferation by mitogen. (A) The mice were administered lyophilized bee pollen after wet grinding or sterile water daily for five weeks. There was no significant difference in body weight between the two groups. Data were represented as mean ± SEM (n=9). (B) Splenocytes were isolated after daily administration of lyophilized bee pollen after wet grinding or sterile water for five weeks and were cultured without mitogen for 48 hours. There was no difference in splenocyte proliferation between two groups. Data were represented as mean ± SEM (n=4). (C) Isolated splenocytes were incubated with Con A for 48 hours. The proliferation of splenocytes was analyzed by MTT assay. Data were represented as mean ± SEM (n=4). (D) Isolated splenocytes were incubated with LPS for 48 hours. The proliferation of splenocytes was analyzed using MTT assay. Data were represented as mean ± SEM (n=4). ***$p<0.001$ compared with control, ###$p<0.001$ compared with Con A- or LPS-treated group.

proliferation by both Con A and LPS. In case of naringenin, it reduced splenocyte proliferation at condition without mitogen. In addition, naringenin dose-dependently reversed the enhanced proliferation of splenocytes by both Con A and LPS (Fig. 4D-F).

3.5. Discussion

Con A and LPS are known as activating factors for T-lymphocytes and B-lymphocytes, respectively[16]. Therefore, Con A- and LPS-mediated proliferation of splenocytes are regarded as parameters for cellular and humoral immunity, respectively[17]. Our in vitro data from normal mice are consistent with reports presented by Qin and Sun[9], in which they presented that ethanol extract of pollen Typhae significantly suppressed both Con A- and LPS-simulated proliferation of splenocytes in vitro. However, we provided new evidence showing that contrary to the condition with mitogens, bee pollen induces splenocyte proliferation independently. This data indicate that bee pollen may be able to play a role as a mitogen to splenocytes. Supporting this idea, polysaccharide fraction of bee pollen increased lymphocyte proliferation [18].

Contrary to the *in vitro* data with mitogens, chronic administration of bee pollen for five

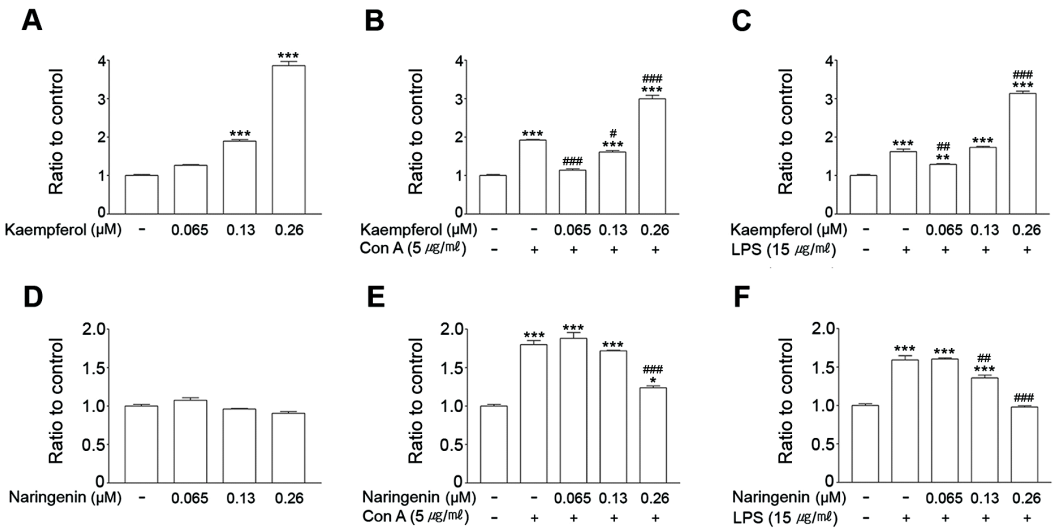

Fig. 4. The effect of flavonoids on splenocyte proliferation. (A) Splenocytes from normal mice were incubated with kaempferol (0.065, 0.13 or 0.26 μM) for 48 hours and the proliferation of splenocytes was analyzed using MTT assay. (B-C) Dose-dependent effects of kaempferol on splenocyte proliferation induced by Con A (B) or LPS (C) was examined using MTT assay. (D) Splenocytes from normal mice were incubated with naringenin (0.065, 0.13 or 0.26 μM) for 48 hours and the proliferation of splenocytes was analyzed using MTT assay. (E-F) Dose-dependent effects of naringenin on splenocyte proliferation induced by Con A (E) or LPS (F) was examined using MTT assay. Data were represented as relative ratio to control and as mean ± SEM (n=3). *p<0.05, **p<0.01, ***p<0.001 compared with control (flavonoid negative and/or mitogen negative), #p<0.05, ##p<0.01, ###p<0.001 compared with Con A- or LPS-treated control (flavonoid negative).

weeks boosted the proliferation of splenocytes by Con A or LPS. However, there was no difference in basal potential of splenocyte proliferation between lyophilized bee pollen- and sterile water-administered groups. Since splenocytes were not exposed to the bee pollen after splenocyte isolation, it can be assumed that splenocytes were primed to boost immune reaction during chronic in vivo exposure to bee pollen and thus, chronic administration of bee pollen may facilitate both cellular and humoral immune reaction. When this hypothesis is valid, reinforcement of immunity is expected when bee pollen is chronically administered. Supporting this concept, chronic administration of ethanolic extract of bee products, including bee pollen, increased antibodies titer against avian influenza in Japanese quails[8]. Similarly, it was reported that a high pollen diet enhanced the survival of honey bees against infection[19]. Taken together, it is believed that although the molecular mechanism is unveiled, chronic administration of bee pollen is able to prime the immune reaction to boost cellular and humoral immunities against infection in the physiological condition. However, it also should be remembered that bee pollen suppressed the immune reaction by the same mitogens when splenocytes were isolated from normal mice. This discrepancy and its molecular mechanism remains to be studied in the future.

In isolated splenocytes, naringenin dose-dependently reduced splenocyte proliferation by both Con A and LPS. On the other hand, kaempferol dose-dependently increased splenocyte proliferation. However, it is important to note that the lower concentration of kaempferol significantly reduced splenocyte proliferation by both mitogens. Since kaempferol exists not that rich in bee pollen, it is reasonable to infer that the composition of kaempferol in bee pollen at a concentration of under 0.074% may be very low. However, it is also noteworthy that without mitogen, kaempferol boosted splenocyte proliferation even at low concentration, and this result may support the enhanced proliferation of splenocytes by bee pollen without mitogen. In addition, it may be possible that, like bee pollen, naringenin boosts the proliferation of splenocytes by Con A and LPS when it is administered chronically and then splenocytes are isolated. Thus, it is evident that further study is needed to clarify the role of flavonoids from bee pollen in immunity.

Lyophilized bee pollen after wet grinding showed increased proliferation of splenocytes at concentration of 0.07%, but wet-grinded bee pollen without lyophilization did not. The lysophilization of bioactive compounds is a strategy frequently used since the stability of different substances in a solid state has some advantages over storage in liquid media[20]. In this study, the reason for the difference in activity between the two materials can be inferred that in case of wet grinding, the stability of active ingredients in aqueous phase gradually decreased immediately after manufacturing. However, further research is needed to prove this, which will be identified in the next study.

4. Conclusion

In the current study we identified that the bee pollen induces the proliferation of splenocytes isolated from normal rodents. On the other hand, bee pollen reverses the enhanced proliferation of splenocytes by Con A as well as LPS. However, and most importantly, we demonstrated that when bee pollen is chronically administered, it is able to boost both humoral and cellular immunity in rodents. In addition, there was no significant difference in the population of immune cells between chronically bee pollen-administered and control animals. Combined, these data may suggest that bee pollen is able to prime both cellular and humoral immunity. Further

studies are needed to elucidate the molecular mechanism of the bee pollen in immune modulation.

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