Abstract

Beneficial effects of bee pollen on oral/intestinal environment and skin aging

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Introduction

Bee pollen (BP) is a pollen ball or pellet that is carried by the honey bee while collecting honey. The chemical components of BP depend on the type of flowers. BP is rich in proteins, amino acids, nucleic acids, vitamins, lipids, fiber, minerals, and various types of phenolic compounds (Campos et al., 2008). As it was called to be a "life-giving dust" in ancient Greece and Egypt, bee pollen has many beneficial effects including antioxidant, antifungal, antimicrobial, antiviral, anti-inflammatory, immuno-stimulating, and antitumor activities on human health (Khalifa et al., 2021). Based on these reports, several BP products are commercially available in the form of supplements, ingredients, candies, and cosmetics. Furthermore, many of these products can be orally administered, often repeatedly, and the cosmetics are often applied to the skin. Nonetheless, the effects of BP on oral or skin health have not been demonstrated so far. The purpose of the study is to demonstrate the effect of BP on the oral environment and the protective effect of BP on skin damage. Two different experiments were carried out to demonstrate them. One experiment was to examine the effect of BP on the oral environment, including the bacterial flora, and on the expression of antimicrobial peptides (AMPs) in the oral mucosa of mice. Another experiment was to examine whether BP alleviates the harmful effects of ultraviolet B rays (UVB) on the skin keratinocytes.

Materials & Methods

1. Determination of the antimicrobial activity of BP

To demonstrate the antimicrobial activity of BP, the minimum inhibitory concentration (MIC) of BP on two common oral pathogens, *Streptococcus mutans* (ingbritt strain) and *Porphyromonas gingivalis* (ATCC–33277 strain) were determined. *S. mutans* and *P. gingivalis* were pre-cultured in BHI liquid medium with supplements; cells were collected, washed, and the optimal density (OD) of the bacterial suspension was measured with a spectrophotometer wavelength. The BP extract was diluted with the BHI medium. The dilution series of BP was prepared in a 96-well plate, and the bacterial suspension was inoculated at a dilution of 1:100 in a BHI liquid medium. The MIC was determined after 72 h of anaerobic culture.

2. Effect of BP on the oral environment of mice

Six-week-old C57BL/6J male mice were divided into the BP group and the control group. The BP diet was prepared by mixing 5% BP obtained from API Co. Ltd. (Gifu, Japan) with standard laboratory chow (Oriental Yeast, Tokyo, Japan). Experiment BP group and control group mice were fed with BP diet and standard laboratory chow, respectively. After 1 month, mice were sacrificed to collect the oral swab and buccal mucosa.

2.1. Effect of BP on oral microbe (16S rRNA sequencing)

The bacterial DNA was extracted from the oral swab using DNeasy Blood & Tissue Kit (Qiagen, Germany) following the manufacturer's instructions. The extracted DNA was used for the 16S rRNA sequencing in a next-generation sequencer (Illumina, USA). A two-step PCR, Amplicon PCR targeting V3-V4 regions of bacterial 16S rRNA gene and Index PCR was performed. The PCR products were cleaned after each PCR and then the library was quantified, normalized, and then pooled. The pooled library was subjected to 16S rRNA sequencing. The sequencing data was analyzed using the Quantitative Insights into Microbial Ecology2 (QIIME2, v2020.2). Greengenes (v13.8) database was used to assign the sequenced data to bacterial name. The data was analyzed for the differences in relative taxonomic abundance, alpha diversity, and beta diversity. Analysis of comparison of the microbiome (ANCOM) was used to test the differences in relative taxonomic abundance. Kruskal-Wallis test and Permutational multivariate analysis of variance (PERMANOVA) were used to test the significant differences in alpha diversity and beta diversity, respectively.

2.3. Effect of BP on antimicrobial peptides (AMPs) [Quantitative reverse transcription PCR (qRT-PCR)]

Total RNA was extracted from the oral tissue using a TRIzol reagent. The RNA was reverse transcribed to cDNA. qRT-PCR was performed to evaluate the mRNA expression level of common AMPs; *beta-defensin-1, -2, -3*, *Cathelicidin, S100A8,* and *S100A9* using LightCycler® 96 (Roche Diagnostics, Switzerland). Data were expressed as the ratio of the target mRNA to the GAPDH mRNA. $p < 0.05$ was considered statistically significant.

3. Effect of BP on skin aging

The human keratinocyte cell, PHK16-0b was cultured in a Prime-CnT epithelial medium. Cells were divided into 4 groups; a) untreated cells (control), b) cells exposed to UVB radiation (UVB) at a dose of 20 mJ/cm^2), c) cells treated with bee pollen extract (BP) at a concentration of 4 mg/ml, and d) cells exposed to 20 mJ/cm^2 UVB and treated with 4 mg/ml of bee pollen extract (UVB+BP). The optimum doses of UVB radiation and bee pollen treatment were assessed from cell viability results performed using the CyQuant cell viability assay kit. qRT-PCR was performed to examine the alteration in the mRNA expression of age-related genes (*p16, IL-6*). The level of intracellular ROS was measured by dihydroethidium (DHE) staining and assessed by confocal microscopy. ATP level was measured by using an ATP detection kit (Dojindo, Japan). Oxidative DNA damage was assessed by the determination of the 8-hydroxydeoxyguanosine (8-OHdG) level with an ELISA kit (Cell BIOLABS, San Diego, CA, USA).

Results

1. Determination of the antimicrobial activity of BP

The growth of *P. gingivalis* was inhibited at concentrations ranging from 2.5 to 10.0%; therefore, 2.5% was considered the MIC of BP for *P. gingivalis*. While *S. mutans* showed no growth inhibition when the BP concentrations ranged from 0 to 10.0%.

2. Effect of BP on the oral/intestinal environment

2.1. Effect of BP on oral microbe

The alpha diversity of the oral bacterial flora was evaluated by Observed OTUs and Shannon index. The Shannon index was significantly higher in the control group than in the BP group ($p = 0.045$) while no significant differences were observed in Observed OTUs between the two groups. The beta diversity was evaluated using weighted and unweighted UniFrac distance on the principal coordinate axis (PCoA) plot. The weighted UniFrac distances were significantly different between the two groups ($p<0.05$). In contrast, no significant differences were observed in the unweighted UniFrac distances between the two groups. The taxonomic abundance analysis

showed a total of 221 different bacterial genera between the two groups. The most abundant genus among all the samples was *Lactobacillus*, followed by *Lactococcus* and *Staphylococcus*. The ANCOM test revealed one differentiating genus, *Lactococcus* to be altered between the two groups (W=82). This genus had a higher proportion in the BP group than in the control group.

2.2 Effect of BP on antimicrobial peptides (AMPs)

The mRNA expression levels of *beta-defensin-2* and *beta-defensin-3* were significantly upregulated, whereas *CRAMP* was significantly downregulated in the buccal mucosa of mice of the BP group as compared to the control group ($p < 0.05$). The mRNA expression levels of other antimicrobial peptides, such as *beta-defensin-1, S100 A8*, and *S100 A9*, showed no significant differences between the two groups.

3. Effect of BP on skin aging

Based on the cell viability assay, a UVB dose of 20 mJ/cm^2 and a BP concentration of 4 mg/ml were determined to be the optimal doses for further experiments. The mRNA level of age-related genes *(p16, IL-6)* expression was significantly upregulated in the UVB group as compared to the control ($p<0.05$). However, after bee pollen treatment in the UVB+BP group, the mRNA expression of those genes was significantly downregulated as compared to the UVB group ($p<0.05$). On immunocytochemistry, DHE staining in the nucleus was significantly higher in the UVB group as compared to other groups. However, DHE staining after bee pollen treatment was significantly decreased in the UVB+BP group as compared to the UVB group ($p<0.05$). DNA damage as assessed by the 8-OHdG level was significantly higher in the UVB group compared with other groups. However, the 8-OHdG level significantly decreased in the UVB+BP group when compared with the UVB group. Intracellular ATP level was significantly higher in the UVB group as compared with other groups ($p < 0.05$).

Discussion

The present study showed a beneficial effect of bee pollen on the oral environment including oral microbes and antimicrobial peptides of the oral mucosa of mice. Also, the beneficial effect of BP on alleviating the harmful effects of UVB on the skin keratinocytes was shown.

The results showed that BP inhibited an oral pathogen, *S. mutans* at MIC of 2.5%. The results of 16S rRNA sequencing showed an increased abundance of beneficial bacteria such as *Lactococcus* in the oral cavity of BP diet-fed mice. Various species of *Lactococcus* have been used as a probiotic because of their therapeutic properties such as the balance of normal microbiota, maintenance of gut barrier function, and modulation of an immune response. *Lactococcus lactis* has an inhibitory effect on *S. mutans*; in addition, it prevents the formation of biofilms that contain *P. gingivalis*. As such, bee pollen might have a beneficial effect on oral health by increasing the proportion of *Lactococcus* genus.

BP upregulated the expression levels of *defensin-2* and *defensin-3* and downregulated the expression of *CRAMP* in the oral mucosal tissues of mice. *Beta-defensin-2* and *-3* are inducible by stimulation with inflammatory cytokines and certain types of bacteria. Two mechanisms are thought to be involved in the upregulated expression levels of *beta-defensin-2* and *-3* in the present study: the effectiveness of BP on the oral mucosa, and the alterations in the proportions of the genera due to the bee pollen. Although *CRAMP* can be found in the gingival epithelium, its expression appears to be a product of neutrophil migration in epithelial cells, rather than production. The anti-inflammatory effect of BP on neutrophils might result in a decrease in the number of neutrophils and lead to a downregulation in the expression level of *CRAMP*.

BP showed a protective effect against aging and oxidative stress-induced DNA damage in human keratinocytes. UVB radiation is known to produce reactive oxygen species (ROS) and generate oxidative stress. The oxidative stress may cause early aging. The upregulated mRNA expression of age-related genes such as *p16* and *IL-6* in the UVB-exposed keratinocytes was downregulated after treatment with BP. The ROS level as shown by DHE staining was significantly higher in UVB-exposed keratinocytes. However, after BP treatment the level of ROS decreased. The oxidative stress-induced DNA damage was significantly higher in UVB-treated keratinocytes

which were reduced after BP treatment. These findings suggest the ability of BP to protect the human keratinocyte against UVB-induced photoaging.

Conclusion

In conclusion, BP has a beneficial effect on oral health by modulating oral microbes and anti-microbial peptides. Also, BP showed anti-aging effect from UVB damage by reducing ROS and oxidative stress.