Effects of cosmetics containing purified honeybee (Apis mellifera L.) venom on acne vulgaris

Sang Mi Han¹, Kwang Gill Lee¹, Sok Cheon Pak²
1. Department of Agricultural Biology, National Academy of Agricultural Science, Suwon 441-100, Korea
2. School of Biomedical Sciences, Charles Sturt University, Bathurst, New South Wales 2795, Australia

OBJECTIVE: Acne vulgaris is a chronic dermatologic problem with multiple factors involved in its pathogenesis. Alternative solutions to acne treatment were instigated by antibiotic resistance despite of its extensive use. Purified bee venom (PBV) has been proposed as a promising candidate for that purpose. The present study was designed to confirm the antibacterial effect of PBV and access the efficacy of cosmetics containing PBV in subjects with acne vulgaris.

METHODS: The skin bacterium Propionibacterium acnes was incubated with PBV at various concentrations and bacterial growth was evaluated using the colony forming unit (CFU) assay. The mechanism of PBV employed in killing P. acnes was examined by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). In addition, a total of 12 subjects were randomized in a double-blind, controlled trial to receive either cosmetics containing PBV or cosmetics without PBV for two weeks. Evaluations included lesion counts and skin microorganism.

RESULTS: PBV exhibited antimicrobial activity in a concentration-dependent manner, reducing the number of P. acnes CFU by approximately 6 logs at a concentration of 0.5 mg. When PBV concentration was higher than 1.0 mg, no P. acnes colonies were spotted on an agar. TEM and SEM of untreated P. acnes illustrated the normal pleomorphic structure, whereas the PBV-treated bacterium lost the integrity of surface architecture. Significant difference (P=0.027) in the grading levels based on numbers of lesion counts for inflammatory and noninflammatory was observed in favour of the PBV group compared with the control group. In terms of average decrement of skin microorganism, subjects receiving cosmetics containing PBV experienced a significant 57.5% decrease of adenosine triphosphate levels, whereas participants receiving cosmetics without PBV experienced a nonsignificant decrease of 4.7%.

CONCLUSION: These results show that the in vitro actions of antimicrobial activity of PBV were translated in vivo. Cosmetics containing PBV provided a certain degree of efficacy in terms of lesion counts and skin microorganism concentration compared with cosmetics without PBV in subjects with acne vulgaris. PBV may be a good candidate compound for developing therapeutic drug for the treatment of acne vulgaris.

KEYWORDS: bee venoms; acne vulgaris; Propionibacterium acnes; skin; cosmetics

1 Introduction

Bee venom from the honeybee (Apis mellifera L.) contains a variety of different peptides including melittin, apamin, adolapin, and mast cell-degranulating peptide[1,2]. Bee venom has been used as a complementary medicine to treat conditions such as rheumatoid arthritis and cancerous tumors[1,3,4]. Recently, bee venom also has been used as a cosmetic ingredient in antiaging, anti-inflammatory,
and antibacterial products. Purified bee venom (PBV) is generally obtained by collecting a large amount of bee venom from honeybees by electric stunning using a bee venom collector (a process that does not harm the honeybees), removing impurities from the collected bee venom, and lyophilizing purified venom. We previously reported the skin-photoprotective effect of PBV that resulted from the reduction in the protein levels of matrix metalloproteinases, which are the major contributors to photoaging processes[5].

Acne vulgaris is one of the most common dermatologic problems, affecting mainly adolescents and often continuing into adulthood with women being affected at higher rates than men[6]. Although multiple factors are involved in acne pathogenesis, the underlying mechanism for its onset and subsequent development are not fully elucidated yet. However increasing amounts of data seem to confirm the involvement of abnormalities in sebum production, follicular keratinization, bacterial proliferation and inflammation[7]. According to the current concept of acne pathogenesis, sebaceous hyperplasia and follicular hyperkeratinization cause a change of follicular milieu with consecutive proliferation of bacteria, mainly Propionibacterium acnes[8]. This leads to increased production of pro-inflammatory cytokines which stimulate comedogenesis at the level of follicular keratinocytes. Further inflammatory responses via the activation of cell-mediated immune processes lead to the increasing severity of acne.

There are currently a number of proven effective treatments for acne vulgaris with various degrees of success. Early and effective therapy based on the treatment of pathogenic causes described above should be implemented because scarring caused by severe inflammatory acne is an extreme concern to the patients. As long as clinical symptoms can be alleviated and scarring can be prevented by intervention, it needs to be promoted. This could be apparent from the use of bee venom since it has recently been reported to possess antibacterial effect against acne-inducing bacteria[9]. The aim of the present study was to confirm the antibacterial effect of bee venom, and evaluate the efficacy of cosmetics containing PBV in the treatment of acne vulgaris.

2 Materials and methods

2.1 Purified honeybee venom collection

All experimental procedures used in the present study were approved by the Animal Care and Use Committee at the National Academy of Agricultural Science (NAAS) and conform to the US National Institutes of Health Guidelines for the care and use of laboratory animals. Experimental colonies of natural honeybees (Apis mellifera L.) used in this study were maintained at NAAS, Suwon, Korea. Bee venom was collected with a bee venom collector (Chungjin Biotech, Korea) in a sterile manner under strict laboratory conditions. In brief, the bee venom collector was placed on the hive, and the bees were given enough electric shock to cause them to sting a glass plate from which dried bee venom was later scraped off. The collected venom was diluted in cold sterile water and then centrifuged at 10 000×g for 5 min at 4 ℃ to discard residues from the supernatant. PBV was lyophilized by freeze dryer and refrigerated at 4 ℃ for later use.

2.2 Colony forming unit assay

The colony forming unit (CFU) assay was performed as described by Ernst et al[10]. P. acnes (ATCC 6919) was obtained from the Korean Culture Center of Microorganisms, Seoul, Korea. P. acnes was cultured at 37 ℃ on reinforced clostridial medium (BD, MD, USA) under anaerobic conditions for 2 d and collected in the mid-log phase. The bacterium was washed three times with the assay buffer, which consisted of 10 mmol/L sodium phosphate, supplemented with 0.03% trypticase soy broth (TSB, BD, MD, USA) and counted by applying a conversion factor of 7.5×107 bacteria per milliliter = 1 optical density unit at 600 nm. Various concentrations of PBV were incubated with 3.75×107 bacteria in a final volume of 30 μL at 37 ℃ for 4 h. After incubation, 10-fold dilutions were prepared and plated on solid media composed of Brucella broth (BD, MD, USA) with 5% sheep red blood cells. The plates were incubated for 2 d at 37 ℃ under anaerobic conditions, then individual colonies were counted, and the number of CFU per tube was calculated.

2.3 Electron microscopy

P. acnes at a concentration of 2.08×107/mL was suspended in 10 mmol/L phosphate buffer sodium (PBS, pH 7.2) supplemented with 0.03% TSB and incubated with 100 ng PBV. Samples were fixed for 30 min with 2% glutaraldehyde in PBS at room temperature and then washed and suspended in PBS. Samples for scanning electron microscopy (SEM) were filtered through a micropore filter. The filters coated with P. acnes were dehydrated in a graded ethanol series (50%, 75%, 95% and 100% with 15 min each), followed by similar exposure to a hexamethyldisilazane reagent (50%, 75%, and 95% with 30 min each). After this process, the samples were incubated in 100% hexamethyldisilazane reagent overnight. SEM samples were coated with gold, viewed and imaged with a Hitachi scanning electron microscope (S-2460N, Hitachi, Japan). Transmission electron microscopy (TEM) samples were dehydrated in graded ethanol as above, embedded in Epon and sectioned. TEM samples were stained with uranyl acetate, viewed and imaged using a Carl Zeiss transmission electron microscope (LEO912AB, CarlZeiss, Germany).

2.4 Clinical study design and treatment

Korean female and male subjects aged from 12 to 35 years
participated in a double-blind, placebo-controlled, split face study with left-right randomization that was carried out June 2012. Twelve subjects would complete the study for the home-use test procedure to ensure. To be enrolled, all subjects must have had mild to moderate acne, as graded by the Korean Acne Grading System (KAGS), grade of 1 to 3. The severity of acne was assessed using KAGS, proposed by the consensus conference in 2004[11]. The severity score (grades 1 to 6) was as follows: grade 1, papules ≤ 10; grade 2, papules 11-30; grade 3, papules ≥ 31, nodules ≤ 10; grade 4, nodules 11-20, ± mild ongoing scars; grade 5, nodules 21-30, ± moderate ongoing scars; grade 6, nodules ≥ 30, ± severe ongoing scars and ± sinus tract. To be enrolled, all subjects had to have signed a certification that they had no allergy or sensitivity to bees or bee sting. All subjects were required to read, understand and sign a written informed consent and complete a brief demographic medical history form. Subjects under the age of 18 had to have a parent or a legal guardian who signed the informed consent form. This randomized, double-blind controlled study was performed to evaluate the efficacy and safety of cosmetics containing PBV in the treatment of acne vulgaris as defined by the KAGS.

Participants with dermatological conditions which could interfere with treatment or evaluation were excluded. The study protocol was approved by institutional review boards. All volunteers provided written informed consent prior to entering the study. This study was conducted in accordance with Good Clinical Practice. Subjects were randomized in a 1:1 ratio to receive either cosmetics containing PBV or cosmetics without PBV twice daily in the morning and evening for two weeks. Cosmetics were applied topically on the whole face with an amount of 4 mL per day. The concentration of PBV is 0.06 mg/mL in PBV-containing cosmetics (Table 1).

<table>
<thead>
<tr>
<th>Table 1 Formulation of cosmetics</th>
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<tbody>
<tr>
<td>Ingredient</td>
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<tr>
<td>Control</td>
</tr>
<tr>
<td>Water</td>
</tr>
<tr>
<td>Butylene glycol</td>
</tr>
<tr>
<td>Alcohol</td>
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<tr>
<td>Polyethylene glycol-60 hydrogenated castor oil</td>
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<tr>
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</tr>
<tr>
<td>Allantoin</td>
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<tr>
<td>Citric acid</td>
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<tr>
<td>Bee venom</td>
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</table>

The data are expressed as the mean ± standard error of mean. Statistical differences among groups were calculated by analysis of variance by SPSS (SPSS 18.0 version, Chicago, IL, USA). The significant differences with a value less than 0.05 were determined by independent t-test.

3 Results

3.1 Antimicrobial effects of PBV

3.1.1 Bacterial growth

To confirm whether the antimicrobial PBV kills *P. acnes*, the activity of PBV at various concentrations against bacterial growth was tested. Bacterial growth was evaluated using the CFU assay. PBV exhibited antimicrobial activity in a concentration-dependent manner, reducing the number of *P. acnes* CFU by approximately 6 logs at a concentration of 0.5 μg (Figure 1A). When PBV concentration was higher than 1.0 μg, no *P. acnes* colonies were spotted on an agar during the incubation for 4 h at 37 °C (Figure 1B).

3.1.2 Observation by electron microscopy

PBV exerts its antimicrobial activity against Gram-negative and Gram-positive organisms[5]. To determine what mechanism is employed in the killing of *P. acnes* by PBV, we examined bacteria that had been treated with PBV using both SEM and TEM. Both SEM and TEM of untreated *P. acnes* illustrate the bacterium’s normal pleomorphic structure. By SEM, the surface of the untreated bacteria appeared smooth and rounded, whereas the PBV-treated bacteria demonstrated a recessed and withered surface (Figure 2A). Also, differences between untreated and PBV-treated bacteria were appreciated by TEM, which demonstrated many “ghost” cells after 12 h of PBV treatment and few surviving bacteria with...
darker and more condensed cytoplasms compared to untreated bacteria (Figure 2B). As can be seen in Figure 2B, the untreated bacteria had fimbriae while PBV-treated bacteria had surface with an absence of fimbriae. TEM revealed the untreated \( P.\ acnes \) had a cell wall with well demarcated outer and inner dark, lipophilic layers and a lighter, hydrophilic peptidoglycan layer. In contrast, after incubation with PBV, \( P.\ acnes \) lost the integrity of this surface architecture as the lighter, more hydrophilic, peptidoglycan layer as well as the darker more lipophilic layers of the cell appear disturbed, losing their crisp, well-defined structures (Figure 2B). In addition, we observed a wider, likely edematous, space inside of the cell wall, further suggesting its disruption as well as peripheral clumping of nuclear material within the cell. In whole, these images reveal that PBV perturbs the surface integrity of \( P.\ acnes \) in a manner that may make it porous, suggesting that this is the likely mechanism by which PBV kill this organism.

### 3.2 Observed efficacy

A total of 12 subjects, between 12 and 35 years of age and in general good health were to be empanelled (Figure 3). The grading levels based on numbers by lesion counts for inflammatory and noninflammatory at baseline and after two weeks of treatment are shown in Table 2. Statistically significant difference from the treatment was not shown in subjects receiving cosmetics without PBV \( (P = 0.363) \). Treatment with cosmetics containing PBV caused a significant success rate \( (P = 0.001) \) during the two-week period.
In terms of average decrement of skin microorganism from baseline (Table 3), subjects receiving cosmetics containing PBV experienced a significant 57.5% decrease of ATP level during two-week study period ($P=0.007$ from $17.825 \pm 3.911$ to $7.566 \pm 6.082$), whereas participants receiving cosmetics without PBV experienced nonsignificant decrease of 4.7% from $17.539 \pm 3.202$ to $16.708 \pm 2.268$ ($P=0.448$).

### Table 2 The change of KAGS score based on numbers of inflammatory and noninflammatory lesions

<table>
<thead>
<tr>
<th>KAGS score</th>
<th>Control group (n=6)</th>
<th>PBV group (n=6)</th>
<th>Comparison of group (P value)$^*$</th>
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<tbody>
<tr>
<td></td>
<td>Week 0 Week 2</td>
<td>Week 0 Week 2</td>
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<td>0 0</td>
<td>0 3</td>
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<tr>
<td>6</td>
<td>0 0</td>
<td>0 0</td>
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</table>

$^*$Analyzed by independent $t$-test; $^\text{**}$analyzed by paired $t$-test. KAGS: Korean Acne Grading System; PBV: purified bee venom.

### Table 3 The average decrement of skin microorganism level

<table>
<thead>
<tr>
<th>Class</th>
<th>Control group (n=6)</th>
<th>PBV group (n=6)</th>
<th>Comparison of group (P value)$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 0</td>
<td>17.539 ± 3.202</td>
<td>17.825 ± 3.911</td>
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<tr>
<td>Week 2</td>
<td>16.708 ± 2.268</td>
<td>7.566 ± 6.082</td>
<td>0.012</td>
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</table>

$^*$Analyzed by independent $t$-test; $^\text{**}$analyzed by paired $t$-test. PBV: purified bee venom.

Comparison between two groups by independent $t$-test confirmed the effect of PBV on *P. acnes* counts ($P=0.012$). Figure 4 illustrates the effect of cosmetics containing PBV on facial lesions after 2 weeks of treatment.

**Figure 4** Photographs of subjects at baseline and week 2 of treatment for (A) control group and (B) purified bee venom group

### 4 Discussion

As therapeutic agents for acne vulgaris, antibiotics are normally administered to inhibit inflammation or kill bacteria. According to the systemic review, topical antibiotics such as clindamycin and erythromycin are an effective treatment for predominance of inflammatory lesions in mild to severe acne$^{[11]}$. Oral antibiotics are recommended for moderate acne patients whose acne severity has not been adequately reduced by topical antibiotics. Oral erythromycin demonstrated a similar efficacy to oral tetracycline in total lesion count reduction. However, the development of antibiotic resistance is of concern and limiting use of antibiotics seems to be a better eco-responsible acne management$^{[12]}$. To overcome the problem of antibiotic resistance, natural products with antimicrobial properties have been extensively studied for a possible application in acne treatment. PBV has been proposed as a promising candidate for an alternative treatment for antibiotic therapy of acne vulgaris$^{[9]}$.

PBV possesses a variety of different peptides with melittin as the main component$^{[13]}$. Repetitive chemical therapy with PBV was able to produce a robust analgesic effect on chronic neuropathic pain$^{[14]}$. PBV has been reported to have anticancer activities. Cancerous tumor growth was hampered by PBV through induction of apoptosis in lung cancer cells$^{[15]}$, breast cancer cells$^{[16]}$, hepatocellular carcinoma cells$^{[17]}$ and prostate cancer cells$^{[18]}$. We previously found that PBV possessed antifibrogenic properties that were mediated by suppression of pro-inflammatory cytokines and fibrogenic gene expression$^{[19]}$. 
We also demonstrated that PBV\(^{20}\) and apamin\(^{21}\) prevent the combined lipopolysaccharide and atherogenic diet-induced atherosclerosis via down-regulation of endothelial adhesion molecules. Recently PBV has also been experimented for antiaging and antibacterial functions making it ideal for use in skin care and cosmetic preparation. We reported skin-photoprotective action of PBV through reduction of protein levels of matrix metalloproteinases which are main contributors to photoaging processes\(^{22}\). Antimicrobial activity of PBV against skin bacteria in human monocytic cells was also found in our another study\(^{23}\). Furthermore, PBV augmented wound healing with concomitant inhibition of cytokines associated with fibrosis, which resulted in decreased wound size and increasing of epithelial proliferation in a mouse full-thickness excision wound model\(^{24}\).

In the assessment and evaluation of the safety of substance, determination of irritant effects on skin and eyes is an important initial step\(^{24}\). PBV was well tolerated and exhibited no dermal and ocular irritation potential in rabbits\(^{25}\), rats and guinea pigs\(^{26}\). This randomized, double-blind controlled study was designed to determine the clinical benefit of a cosmetics containing PBV twice daily in the treatment of subjects with acne vulgaris. Results of this study showed that the addition of PBV to cosmetics improved the outcome of acne vulgaris in terms of reduction of both inflammatory and noninflammatory lesions and decrement of skin microorganism. Seborrhea in patients with acne leads to a proliferation of bacteria particularly \(P.\) \textit{acnes} by providing an ideal anaerobic environment for their growth\(^{30}\). Subsequently lymphocytes are attached and inflammatory cytokines are expressed to cause comedogenesis. We reported that PBV reduced the \(P.\) \textit{acnes}-induced secretion of interleukin-8 and tumor necrosis factor-\(\alpha\)\(^{30}\). It implies that this anti-inflammatory activity of PBV is the key mechanism for its antibacterial property. Our current study confirms the antimicrobial activity of PBV against \(P.\) \textit{acnes} with cell lysis, indicating the cell membrane appears to be the primary site of PBV action.

Our findings suggest that cosmetics containing PBV provided a certain degree of efficacy in terms of lesion counts and skin microorganism concentration compared with cosmetics without PBV in subjects with acne vulgaris. Long-term treatment with cosmetics containing PBV could be safe because the irritation potential of PBV is negligible. In this study, the \textit{in vitro} actions of antimicrobial activity of PBV were translated \textit{in vivo}. Cosmetics containing PBV provided a certain degree of efficacy in terms of lesion counts and skin microorganism concentration compared with cosmetics without PBV in subjects with acne vulgaris. PBV may be a good candidate compound for developing therapeutic drug for the treatment of acne vulgaris.

5 Acknowledgements

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6 Competing interests

The authors declare that they have no competing interests.

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