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ORIGINAL ARTICLE

Antiviral Assessments of Honeybee (*Apis mellifera* L.) Venom

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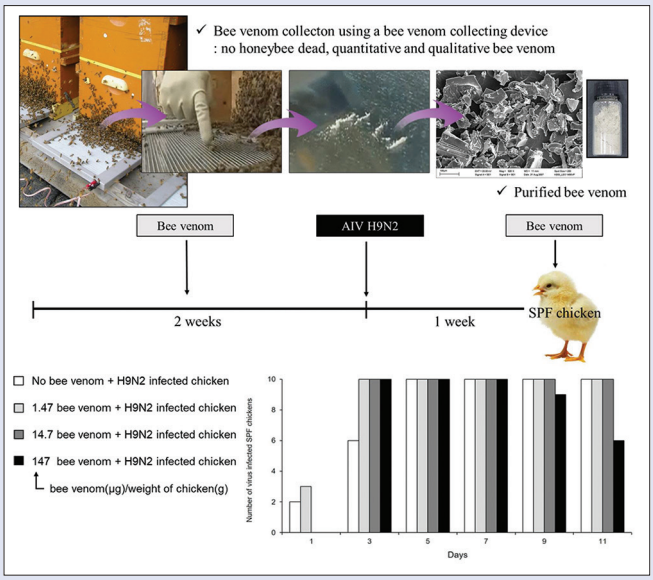
ABSTRACT

Background: The poultry industry has long been challenged by avian influenza which causes significant economic loss due to decreased egg production and quality. In addition, the notable ability of influenza viruses to develop resistance to conventional antibiotics is one of the biggest tasks that the industry currently faces. Attempts have been made to treat this bird flu with multiple approaches, but effective natural solutions remain elusive. Bee venom (BV) is used for the treatment of various human diseases due to its known anti-inflammatory and antibacterial properties. Recent studies suggest that antimicrobial peptides discovered in BV may be utilized as tools for the design of structurally novel antiviral agents effective against influenza viruses. **Materials and Methods:** In the present study, we purified BV containing 63.9% ± 5.4% melittin, 10.9% ± 1.6% phospholipase A_2, and 2.3% ± 0.3% apamin. BV was evaluated *in vitro* for its ability to inhibit the binding of H9N2 to the chicken red blood cells. **Results:** We found that anti-influenza activity of BV is equivalent to that of positive control. However, we observed the neutralization of H9N2 by BV as compared to the virus only group without BV. The hypothesized anti-influenza property of BV was further examined in chicken influenza infection model. The administration of BV through intranasal route resulted in no significant antiviral effect in chickens. **Conclusion:** This study does not support our hypothesis that BV can reduce the viral activity in chickens.

Key words: Anti-viral, *Apis mellifera*, avian influenza, bee venom, H9N2

SUMMARY

- We have purified bee venom (BV) from the honeybee and confirmed melittin as the main bioactive component by the ultra-performance liquid chromatography. BV was evaluated for its ability to inhibit the binding of H9N2 to chicken red blood cells (cRBCs). We have observed the neutralization of H9N2 by BV, implying that BV might possess a molecule to modulate the specific binding affinity of hemagglutination of H9N2 on the receptor of cRBCs.



Abbreviations used: AIV: Avian influenza virus; SPF: Specific pathogen-free; EID_{50}: 50% egg infective dose; BV: Bee venom.

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INTRODUCTION

Bird flu caused by avian influenza virus (AIV) keeps to appear mainly due to antigenic drift and antigenic shift characterized by multiple mutations, proving it notoriously variable.[1,2] Inevitably, large-scale occurrences of influenza infections by the new virus are not uncommon. The various types of avian species, as well as several kinds of mammalian hosts, are involved in the ecology and epidemiology of influenza viruses.[3] Due to the increased viral mutation rate, some existing vaccines are less likely to reduce the spread of influenza.[4] Furthermore, it takes years for the existing technologies to create a new vaccine for a newly mutated virus. To make things more difficult, newly mutated viruses are decreasing the ability of the vaccine-induced antibody. Realistically, there is no immediate vaccine to deal with newly emerging virus outbreaks.[4,5] To address these issues, there is a pressing need for the discovery of an alternative antiviral agent to control the spread of virus.

The therapeutic value of bee venom (BV) from honeybee (*Apis mellifera* L.) to improve the quality of life of patients is acknowledged for more than 100 years.[6] Modern approaches of venomics have allowed the discovery of venom constituents which were proven to be of pharmacological significance and have opened the way to optimization of therapeutic strategies through the use of active components such as melittin, apamin, and phospholipases.[7] In particular, melittin, as a major peptide component of BV, is known to possess the multiple biological functions against cancer,[8-10] liver fibrosis,[11,12] and atherosclerosis.[11,13,14] For example, melittin prevented the development of the late stage and early stage prostate for the inhibition of cancer cells through apoptosis.[10] This anticancer effect of BV was exerted the multiple effects on cellular

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functions of cancerous cells such as proliferation, apoptosis, metastasis, angiogenesis as well as cell cycle, and the anticancer processes involve diverse signal molecules and regulatory pathways. In addition, acute liver injury induced by lipopolysaccharide was attenudated by melittin treatment through the prevention of nuclear factor kappa Bactivation.[12] Furthermore, melittin inhibited tumor necrosis factor-α-induced matrix metalloproteinase-9 protein expression which is involved in the pathogenesis of atherosclerosis in human aortic smooth muscle cells.[11] However, the antiviral effect of BV has not been studied in AIV. Therefore, it was hypothesized that BV can reduce viral activity in chickens. To examine this hypothesis, the current study performed the anti-influenza activity of BV against chickens infected with AIV.

MATERIALS AND METHODS

Preparation of purified bee venom

The colonies of natural honey bees used in this study were maintained at the National Academy of Agricultural Science, Wanju, Korea. BV was collected using a BV collecting device (Chungjin, Korea) in a sterile manner under strict laboratory conditions. In brief, the BV collector was placed in the hive, and the bees were given enough electric shock to cause them to sting a glass plate from which dried BV was later scraped off. The collected BV was diluted in cold sterile water and then centrifuged at 10,000 g for 5 min at 4°C to discard the residues from the supernatant. BV was lyophilized by a freeze dryer and refrigerated at 4°C for later use. The amount of 3.2 mg BV was suspended in sterile distilled water and centrifuged at 3000 rpm for 10 min at 4°C. The supernatant was filtered through a 0.2 μm syringe filter (Corning, Germany) to remove any remaining particular material before use. The ultra-performance liquid chromatography analysis of the purified BV revealed three peaks matching those of the commercial standards, apamin, phospholipase A_2, and melittin, with retention times of approximately 1.8 min, 4.3 min, and 8.8 min, respectively. The purified BV contained 63.9% ± 5.4% melittin, 10.9% ± 1.6% phospholipase A_2, and 2.3% ± 0.3% apamin.

Virus preparation

AIV A/chicken/Korea/MS96/96 (H9N2) was propagated in 9-day-old specific pathogen-free (SPF) embryonated chicken eggs for 72 h in an incubator at 38°C. The eggs were then chilled at 4°C to kill the embryo and to reduce the contamination of the allantoic fluid with blood during harvesting. Allantoic fluids of the inoculated eggs were collected and centrifuged at 3000 rpm for 5 min and supernatants were diluted for use.

In vitro experiment

Prepared diluted working seed of AIV (10^{-1} ~ 10^{-8}) and BV (32, 16, 8, 4, 2, and 1 ppm) solution mixture was inoculated into 270 SPF embryonated chicken eggs at the rate of 0.1 ml per egg through intraallantoic way. All eggs were sealed with wax. Inoculated eggs were incubated for 72 h in an incubator at 38°C. Eggs were candled daily, and early death embryos in the first 24 h were removed. The allantoic fluid sample from each egg was withdrawn into the small measuring cylinder until the total volume of 1 ml was obtained in the harvesting stage. Allantoic fluids were then tested for 50% egg infective dose (EID_{50}). EID_{50} of the samples, determined by the dose of AIV that caused a cytopathic effect in 50% of the inoculated eggs, was calculated by the method of Reed and Muench mathematical technique.[15] EID_{50} was then used to calculate the log-scale reduction in infective dose. Assay for EID_{50} was performed in three replicates. The results were also included to analyze both positive (virus with sodium dichloroisocyanurate) and negative (virus without BV) controls.

Chickens

Three-week-old SPF chickens were purchased from Namduck Sanitek (Korea). Chickens were maintained the SPF laboratory conditions in a chicken isolator for 3 days before *in vivo* experiment.

In vivo experiment

Forty chickens were divided into four groups [Table 1]. Chickens were challenged with 10^6 EID_{50}/100 μl H9N2 viruses by the intranasal route. Before virus challenge, chickens were orally administered with three doses (10, 100, and 1000 ng/g) of BV daily for 1 week. For the following 2 weeks since the virus challenge, higher doses (20, 200, and 2000 ng/g) of BV were fed every other day. After the virus challenge, oral and cloacal swabs were collected every other day for the evaluation of viral shedding using the real-time polymerase chain reaction (RT-PCR). Swabs were allowed to stand in tubes containing 1.0 ml sterile phosphate-buffered saline, pH 7.2 frozen at −70°C for analysis. Following experimental infection, chickens were also checked daily for any clinical signs and mortality.

Real-time polymerase chain reaction assay

Culture RNA was extracted with the viral gene-spin RNA/DNA extraction kit (Intron, Korea) with a modified protocol for sample recommended by the manufacturer. The forward primer sequence was 5'-AGATGAGTCTTCTAACCGAGGTCG-3'; the reverse primer was 5'-TGCAAAAACATCTTCAAGTCTCTG-3'; and the probe sequence was 5'-TCAGGCCCCCTCAAAGCCGA-3'. Probe was labeled at the 5' end with the 6-carboxyfluorescein (FAM) reporter dye and at the 3' end with the 6-carboxytetramethylrhodamine (TAMRA) quencher dye. PCR mixtures were prepared in a total volume of 50 μl with the QuantiTect Probe RT-PCR kit (Qiagen, Valencia, CA, USA) under the following conditions: 25.5 μl of kit-supplied enzyme mixture, 0.4 μM each primer, 0.1 μM probe, 5 μl of RNA. The RT step conditions for all primers were 30 min at 50°C and 15 min at 95°C. A three-step PCR cycling protocol was used for the matrix gene primer as follows: 40 cycles of 94°C for 10 s, 55°C for 30 s, and 72°C for 10 s. Fluorescence data were acquired at the end of each annealing step. Real-time RT-PCR was performed with Applied Biosystems 7500 Fast real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis

Data are presented as mean ± standard deviation of at least three independent experiments. All analyses were conducted using the R is a free software environment for statistical computing. For evaluating statistical significance, one-way analysis of variance test along with Fisher's least significant difference was used. $P \leq 0.01$ is considered statistically significant.

Table 1: *In vivo* experimental design of H9N2 with specific pathogen-free chickens

Group	Number of SPF chickens	BV[a] (ng/g) dose
G1	10	10 and 20
G2	10	100 and 200
G3	10	1000 and 2000
G4	10	No BV feeding

[a]Doses (10, 100, and 1000) of BV were fed daily for 1-week before virus challenge which was continued with doses (20, 200, and 2000) following virus challenge for 2 weeks every other day. SPF: Specific pathogen-free; BV: Bee venom

Table 2: The anti-influenza virus activity of bee venom against A/chicken/Korea/MS96/96 (H9N2)

BV dose (μg/ml)	1	2	4	8	16	32	PC	NC
EID_{50}	3.14±0.99^{bc}	2.87±0.90^{bc}	2.78±0.9^{bc}	3.33±0.57^{b}	2.83±0.39^{bc}	2.89±0.72^{bc}	2.07±0.21^{c}	6.39±0.09^{a}
EID_{50} reduction	3.25±0.90^{a}	3.52±0.91^{a}	3.61±0.96^{a}	3.05±0.63^{a}	3.55±0.43^{a}	3.49±0.77^{a}	4.32±0.26^{a}	

Different letters indicate statistically significant differences between the groups (mean±SD). PC: Positive control (virus with sodium dichloroisocyanurate); NC: Negative control (virus without BV); EID_{50}: 50% egg infective dose; BV: Bee venom; SD: Standard deviation

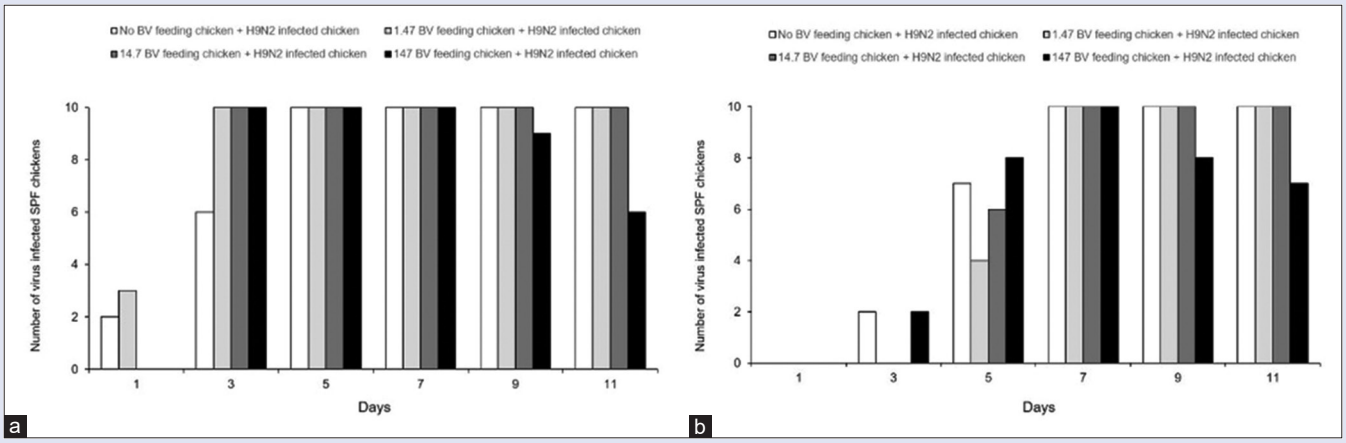

Figure 1: *In vivo* study of prevention of H9N2 infection by bee venom in specific pathogen-free chickens. Number of virus count of oral swab (a) and cloacal swab (b)

RESULTS

50% egg infective dose test

Means of EID_{50} test in the study groups are shown in Table 2. BV was investigated for its effectiveness in the prevention of binding of viruses to the chicken red blood cells (cRBCs). BV binds to the hemagglutinin present in the viruses. As a result, viruses are neutralized; hence, preventing hemagglutinin-mediated binding of viruses with sialic acids present in the surface of cRBCs. No significant differences were seen for EID_{50} test between positive control and BV added groups. Only in negative control without BV, EID_{50} test result was significantly increased. Overall, EID_{50} test was evaluated in different doses of BV for prepared AIV, and there were not any significant differences between treatment groups, including positive control.

In vivo assay of bee venom against H9N2

The real-time RT-PCR, also called quantitative PCR, analysis of oral and cloacal swabs of each chicken was used for the determination of virus load on these chickens. Higher numbers of viruses were detected in both oral and cloacal swab samples than negative control. Overall, we did not notice any reduction in the virus load from BV-treated chickens [Figure 1].

DISCUSSION

The primary objective of the present study was to investigate the possibility of using BV as an anti-influenza agent for animals. According to previous studies, BV has been shown to exert anti-microbial effects against bovine mastitis[6,16] and fish pathogens.[17] While BV decreased the elevated somatic cell count from mastitic quarters,[16] BV also decreased lipopolysaccharide (LPS)-induced inflammatory responses in bovine mammary epithelial cells.[6] In addition, BV inhibited the growth and survival of three bacterial strains.[17]

Bird flu or avian influenza is a viral infection occurring worldwide with subsequent economic and health problems. Due to its high pathogenicity, an influenza pandemic is inevitable and potentially threating humans as well as livestock. Among many types of AIV, H9N2 is known as low pathogenic avian influenza. However, it causes an endemic outbreak in the poultry industry in many Asian countries. The poultry outbreaks of H9N2 are associated with significant economic losses because of low egg production, reduced feed conversion efficiencies, and highly lethal bacterial or viral coinfections.[18] Unfortunately, H9N2 subtype AI virus during 2007 outbreak in Korea was reported to escape vaccine protection due to the excessive use of animal vaccination.[19]

The influenza viruses, typically influenza A and B viruses, possess eight pieces of viral RNA. These viruses contain a host cell-derived envelope membrane carrying the hemagglutination glycoprotein.[4] Hemagglutination as the surface antigen is responsible for most antigenic variations and contains binding site for sialic acid residues on the surface of target cells, mediating the binding of the virus to target cells and the subsequent entry of the viral genome.[20] As such, one of the criteria for a new agent is the inhibition of viral intrusion into host cells through hemagglutination. In this study, we observed the neutralization of H9N2 by BV compared to the virus only group without BV. It is possible that BV possesses a molecule to interrupt the specific-binding affinity of hemagglutination of H9N2 on receptor of cRBCs. According to Pandey *et al.*,[19] receptor specificities the hemagglutination of viruses can be different depending on different viral strains. It implies that antiviral efficacy of BV could be changed against different types of virus strains. Furthermore, BV was applied for the control of H9N2 virus infection in chickens, which resulted in no significant differences. Our preliminary *in vivo* data of BV against H9N2 infection require further extensive study.

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Conflicts of interest

There are no conflicts of interest.

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