JoP Journal of Pharmacopuncture
Journal of Pharmacopuncture 2022;25(4):390-395
pISSN: 2093-6966 • eISSN: 2234-6856
https://doi.org/10.3831/KPI.2022.25.4.390
Original Article
Check for updates

Dual Cytotoxic Responses Induced by Treatment of A549 Human Lung Cancer Cells with Sweet Bee Venom in a Dose-Dependent Manner

Yu-Na Hwang[1], In-Seo Kwon[1], Han-Heom Na[1,2], Jin-Sung Park[3], Keun-Cheol Kim[1]*

[1]Department of Biological Sciences, College of Natural Sciences, Kangwon National University, Chuncheon, Republic of Korea
[2]Kangwon Center for System Imaging, Kangwon National University, Chuncheon, Republic of Korea
[3]Korean Pharmacopuncture Institute, Seoul, Republic of Korea

Received October 25, 2022
Reviewed November 29, 2022
Accepted November 30, 2022

***Corresponding Author**
Keun-Cheol Kim
Department of Biological Sciences, College of Natural Sciences, Kangwon National University, 1 Kangwondaehak Rd, Chuncheon 24341, Republic of Korea
Tel: +82-33-250-8532
E-mail: kckim@kangwon.ac.kr

Objectives: Sweet bee venom (sBV) is purified from *Apis mellifera*, containing a high level of melittin—its main component. It has been used as a therapeutic agent for pain relief and anti-inflammation, as well as for treating neuronal abnormalities. Recently, there have been studies on the therapeutic application of sBV for anticancer treatment. In the present study, we investigated the pharmacological effect of sBV treatment in A549 human lung cancer cells.
Methods: We used microscopic analysis to observe the morphological changes in A549 cells after sBV treatment. The MTT assay was used to examine the cytotoxic effect after dose-dependent sBV treatment. Molecular changes in sBV were evaluated by the expression of apoptosis marker proteins using western blot analysis.
Results: Microscopic analysis suggested that the growth inhibitory effect occurred in a dose-dependent manner; however, cell lysis occurred at a concentration over 20 μg/mL of sBV. The MTT assay indicated that sBV treatment exhibited a growth inhibitory effect at a concentration over 5 μg/mL. On fluorescence activated cell sorting analysis, G0 dead cells were observed after G1 arrest at treatment concentrations up to 10 μg/mL. However, rapid cell rupture was observed at a concentration of 20 μg/mL. Western blot analysis demonstrated that sBV treatment modulated the expression of multiple cell death-related proteins, including cleaved-PARP, cleaved-caspase 9, p53, Bcl2, and Bax.
Conclusion: sBV induced cell death in A549 human lung cancer cells at a pharmacological concentration, albeit causing hemolytic cell death at a high concentration.

Keywords: sweet bee venom, cell death, hemolysis, a549 cells, cell cycle

INTRODUCTION

Bee venom is extracted from the Vespidae family, such as honeybee (*Apis mellifera*), Bumble bee (*Bombus pascuorum*), and the wasp (*yellow hornet*, *Vespa crabro*). The extracted bee venom is a mixture of various organic substances, including phospholipases, melittin, apamin, amines, and amino acids. Bee venom has potent anti-inflammatory properties, and has a therapeutic effect on certain medical conditions, such as rheumatoid arthritis and multiple sclerosis [1, 2]. However, a hypersensitivity reaction to bee venom causes itching, swelling, and pain, although it varies from one individual to another [3]. The most severe side effect of bee venom is fatal anaphylaxis [4]. It has been reported that these side effects are caused by enzyme components in bee venom, like Phospholipase A2, Hyaluronidase, Phospholipase B, and Phosphatase [5].

Sweet bee venom (sBV) has been developed by purifying the main component and removing the harmful substances of bee venom [6, 7]. Melittin is the main component of sBV, accounting for more than 50% of the constitution of bee venom, and it exerts the principal pharmacological activity of bee venom. Melittin is a polypeptide comprising 26 amino acids, with a

molecular weight of 2.8 kDa [8, 9]. Previous studies have suggested that melittin has multiple biological effects such as antiviral, antibacterial, antifungal, anti-parasitic, and anti-tumor effects [10]. The mechanism of action of melittin, as a non-selective cytolytic peptide, involves physical and chemical disruption of all prokaryotic and eukaryotic cell membranes [11]. Recent studies have suggested that melittin can induce cell cycle arrest, cell growth inhibition, and apoptosis in various cancer cell types [12-14]. Moreover, there was a change in the expression of cell death-related proteins after combinatory treatment of melittin and sorafenib in HepG2 hepatocellular carcinoma cells, suggesting that melittin has a synergistic anticancer effect with sorafenib. This may represent a novel enhancing strategy for hepatocellular carcinoma treatment [15]. Our recent study on THP-1 monocytic leukemia cells suggested that sBV exhibits both pharmacological and toxic effects depending on its concentration [16]. Moreover, the pharmacological action of sBV is associated with cell death induction and inflammatory cytokine expression. Although sBV has shown promising pharmacological efficacy in an inflammatory response, further studies are required to clarify the molecular mechanism of sBV administration in the other types of cells during cancer treatment. Therefore, in this study, we examined the pharmacological effects of sBV in A549 human lung cancer cells.

MATERIALS AND METHODS

1. Cell culture and reagents

We purchased an A549 human lung cancer cell line from the ATCC (USA). These cells were cultured in an RPMI-1640 medium containing 10% fetal bovine serum and 1X penicillin/streptomycin in a humidified CO2 incubator. sBV was a purified product of bee venom extracted from *Apis mellifera* and was generously obtained from the Korean Pharmacopuncture Institute. The drugs were dissolved in appropriate solvents as per the manufacturer's protocol.

2. Microscopic examination and cell viability assay

sBV was used to treat cultured A549 cells in a dose-dependent manner for 24 h. The change in cell morphology was observed with an optical microscope (Nikon Ts100, Japan). We seeded 5×10^3 cells in 96-well plates for the MTT assay. sBV was administered in each well in triplicate and incubated for up to 72 h. Cell viability was determined by incubation with 0.5 mg/mL MTT solution (Sigma Aldrich, USA) for 4 h. The amount of MTT-formazan was determined at an absorbance of 570 nm. All the reagents were prepared as recommended by the manufacturer's protocol.

3. Fluorescence activated cell sorting (FACS) (flow cytometry) analysis

A549 cells were cultured after treatment with various concentrations of sBV. The cells were harvested, washed twice in fetal bovine serum, and fixed in 70% ethanol for 24 h. Cells were stained with propidium iodide (50 mg/mL) for 30 min. Cell cycle distribution was analyzed with flow cytometric analysis according to the Becton Dickinson's protocols.

4. Western blot

Proteins were extracted using RIPA lysis buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) and supplemented with proteasome inhibitors. Protein concentrations were measured using Bradford reagents (Thermo Fisher, USA). A total of 50 μg of protein was separated by SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes.

The membranes were incubated with specific primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, USA). Finally, the proteins were detected using an enhanced chemiluminescence protein detection kit (Amersham Inc., UK). Primary antibodies for caspase 9 and RIP1 were purchased from CST (USA). PARP1, BAX, P53, and β-actin were obtained from Santa Cruz (USA). Bcl2 was purchased from Abcam (USA).

RESULTS AND DISCUSSION

We investigated the cell death of cancer cells during sBV treatment in a concentration-dependent manner. For this purpose, we treated A549 human lung cancer cells with various concentrations of sBV, and observed the morphological changes in the cells with light microscopy after 24 h. There were no morphological changes at an sBV concentration of 5 μg/mL; however, the cell morphology was damaged at an sBV concentration of 10 μg/mL, suggesting that cell death occurred from this concentration onwards. Interestingly, acute cell rupture was

observed at an sBV concentration of 20 μg/mL, and complete cell death occurred at this concentration of sBV (Fig. 1).

Our previous study also suggested that distinct pharmacological efficacies for cell death patterns in sBV-treated THP-1 cells were concentration-dependent [16]. This distinct pharmacological activity might be found in only sBV treatment, and not in other anticancer medication. Low sBV concentrations cause growth inhibition in many cell types, including cancer cells and inflammatory cells; however, high sBV concentrations can cause significant cell death [16]. This distinct effect of sBV is presumed to occur due to the difference in pharmacological effects around 20 μg/mL in a cell-based experiment. The main component of sBV is melittin [17], which exerts a variety of pharmacological and toxicological effects, including strong surface activity on cell lipid membranes, hemolytic activity, and potential anti-tumor properties [18]. Based on this property, melittin can be modified using nanotechnology as a non-selective cytolytic peptide to enhance cellular uptake and endosomal/lysosomal escape [19].

We also confirmed the effect on cellular proliferation by examining the time-dependent and concentration-dependent behavior after sBV treatment at various concentrations using the MTT assay (Fig. 2A). Treatment with sBV at a concentration of 5 μg or less had no significant pharmacological effect in A549 cells, whereas at a concentration of 10 μg or more, cell growth inhibitory effect was exhibited, consistent with the morphological changes in A549 cells. In particular, cell growth was completely inhibited at an sBV concentration of 20 μg. Therefore, we suggest that sBV inhibits cell growth in a concentration-dependent manner, albeit showing strong cell growth inhibition at a concentration of 20 μg or more. We also performed FACS analysis to examine cell cycle changes in the treatment condition with various sBV concentrations (Fig. 2B). In the 5 μg treatment condition, the G1 population of A549 cells was slightly increased from 12 h of treatment onwards. However, 10 μg of sBV treatment showed G1 arrest from 6 h onwards. Subsequently, G0 dead cells were observed, suggesting that sBV can induce cell death in A549 cancer cells through G1 arrest. However, microscopic observation showed that treatment with 20 μg sBV caused a rapid rupture of cells. Cell growth inhibition is closely related to various types of cell death. Therefore, to examine the molecular mechanism of cell death induction by sBV, we observed the expression of apoptosis marker proteins, such as PARP-1, Caspase 9, p53, Bcl-2, and Bax, using Western blot analysis (Fig. 3). A549 cells were treated with sBV in a concentration-dependent manner for 24 h. We found that treatment with sBV increased the cleaved form of PARP-1 and caspase 9, as well as the expression of p53 and Bax. However, sBV treatment decreased the expression of the Bcl-2 protein, which is important for maintaining the function of mitochondrial membrane and cell growth. These results confirmed that sBV treatment increased apoptosis. We also investigated whether sBV treatment regulates proteins for necroptosis, but no significant changes were observed in the RIP1 protein expression. On the other hand, the expression of β-actin was decreased at 20 μg/mL sBV treatment, suggesting that that A549 cells were completely damaged at this concentration, as shown in the FACS analysis. Previous reports have suggested that bee venom induces apoptotic cell death in A549 and NCI-H460 lung cancer cells through the enhancement of death

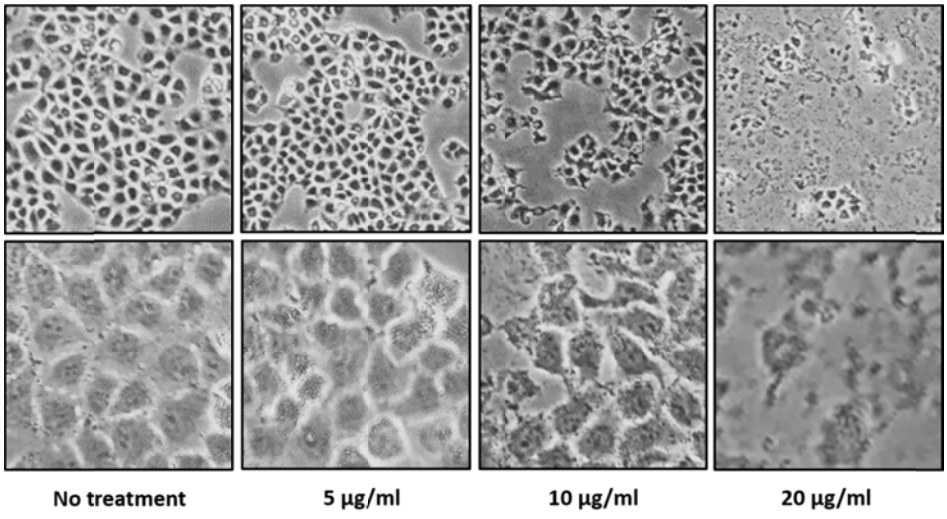

Figure 1. Morphological changes in sBV-treated A549 cells. A549 cells were treated with sBV at 5 μg/mL, 10 μg/mL, and 20 μg/mL concentrations for 24 hr. Morphological changes were observed with a phase contrast microscope. Acute cell rupture was observed with 20 μg sBV treatment.

 https://doi.org/10.3831/KPI.2022.25.4.390

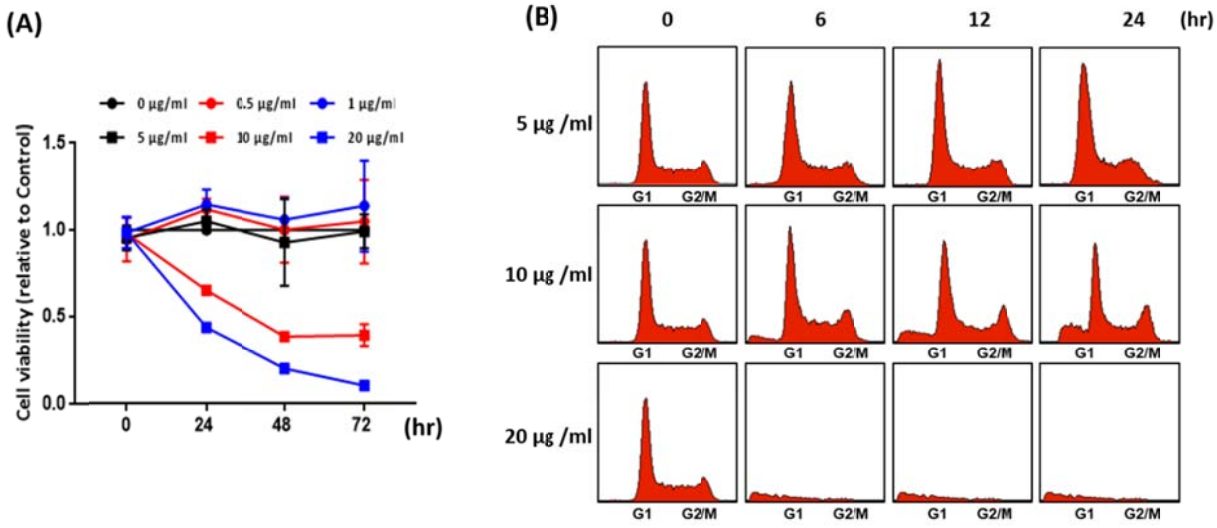

Figure 2. Growth inhibitory effect and FACS analysis in sBV-treated A549 cells. (A) A549 cells were treated with 5 μg/mL, 10 μg/mL, and 20 μg/mL of sBV for 72 hr. MTT assay was performed to examine cell proliferation. (B) FACS analysis was performed after sBV treatment at various concentrations. Cell death was observed after G1 arrest at a pharmacological concentration, but acute cell death was observed with 20 μg sBV treatment.

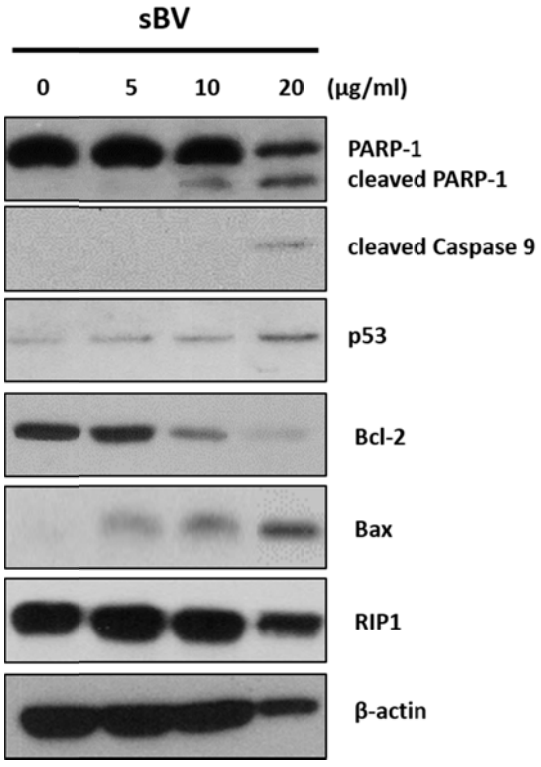

Figure 3. Expression of cell death-related proteins in sBV-treated A549 cells. Western blot analysis was performed after sBV treatment in A549 cells. Cleaved forms of PARP-1 and Caspase 9 were increased, and p53 or Bax was also upregulated with sBV treatment in a dose-dependent manner.

receptor 3 (DR3) expression and inhibition of the NF-κB pathway [20]. Moreover, combination treatment of TNF-like weak inducers of apoptosis, docetaxel and cisplatin, with bee venom synergistically inhibited both A549 and NCI-H460 lung cancer cell growth with further downregulation of NF-κB activity [21]. Accumulated data clearly suggest that melittin, the principal component of sBV or bee venom, has potential for cancer treatment [22]. The pharmacological effect—cell death induction—of melittin seems to be superior to that caused by any other anticancer drug [23, 24]. However, our current study indicates that selecting an appropriate concentration of sBV might be an important consideration in cancer treatment.

CONCLUSION

sBV induced cell death in A549 human lung cancer cells at a pharmacological concentration, albeit causing hemolytic cell death at high concentrations. These data suggest that sBV might exert strong pharmacological activity of cell death induction after growth inhibition or acute cell rupture according to the concentration. However, it determining the appropriate concentration of sBV in cancer treatment might be an important consideration.

ACKNOWLEDGMENT

This work was supported by a fund (2016R1D1A3B0200 6754) and "Regional Innovation Strategy (2022RIS-005)" from the National Research Foundation funded by the Ministry of Education (MOE). In part, this work was also supported by a fund (2021R1I1A1A01047397).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ORCID

Yu-Na Hwang, https://orcid.org/0000-0002-5250-6375
In-Seo Kwon, https://orcid.org/0000-0002-8019-3169
Han-Heom Na, https://orcid.org/0000-0003-3753-3129
Jin-Sung Park, https://orcid.org/0000-0002-1963-3938
Keun-Cheol Kim, https://orcid.org/0000-0003-3047-0380

REFERENCES

1. Abdela N, Jilo K. Bee venom and its therapeutic values: a review. Adv Life Sci Technol. 2016;44:18-22.
2. Ali M. Studies on bee venom and its medical uses. Int J Adv Res Technol. 2012;1(2):69-83.
3. Golden DB. Insect sting allergy and venom immunotherapy: a model and a mystery. J Allergy Clin Immunol. 2005;115(3):439-47; quiz 448. Erratum in: J Allergy Clin Immunol. 2005;115(6): 1128.
4. Lieberman P, Simons FE. Anaphylaxis and cardiovascular disease: therapeutic dilemmas. Clin Exp Allergy. 2015;45(8):1288-95. Erratum in: Clin Exp Allergy. 2015;45(12):1914.
5. Hossen MS, Shapla UM, Gan SH, Khalil MI. Impact of bee venom enzymes on diseases and immune responses. Molecules. 2016;22(1):25.
6. Jo N, Roh J. Systemic immediate hypersensitive reactions after treatment with sweet bee venom: a case report. J Pharmacopuncture. 2015;18(4):59-62.
7. Park JW, Jeon JH, Yoon J, Jung TY, Kwon KR, Cho CK, et al. Effects of sweet bee venom pharmacopuncture treatment for chemotherapy-induced peripheral neuropathy: a case series. Integr Cancer Ther. 2012;11(2):166-71.
8. Jeong YJ, Cho HJ, Whang K, Lee IS, Park KK, Choe JY, et al. Melittin has an inhibitory effect on TNF-α-induced migration of human aortic smooth muscle cells by blocking the MMP-9 expression. Food Chem Toxicol. 2012;50(11):3996-4002.
9. Tusiimire J, Wallace J, Woods N, Dufton MJ, Parkinson JA, Abbott G, et al. Effect of bee venom and its fractions on the release of pro-inflammatory cytokines in PMA-differentiated U937 cells co-stimulated with LPS. Vaccines (Basel). 2016;4(2):11.
10. Lyu C, Fang F, Li B. Anti-tumor effects of melittin and its potential applications in clinic. Curr Protein Pept Sci. 2019;20(3):240-50.
11. Rady I, Siddiqui IA, Rady M, Mukhtar H. Melittin, a major peptide component of bee venom, and its conjugates in cancer therapy. Cancer Lett. 2017;402:16-31.
12. Jo M, Park MH, Kollipara PS, An BJ, Song HS, Han SB, et al. Anti-cancer effect of bee venom toxin and melittin in ovarian cancer cells through induction of death receptors and inhibition of JAK2/STAT3 pathway. Toxicol Appl Pharmacol. 2012;258(1):72-81.
13. Liu CC, Hao DJ, Zhang Q, An J, Zhao JJ, Chen B, et al. Application of bee venom and its main constituent melittin for cancer treatment. Cancer Chemother Pharmacol. 2016;78(6):1113-30.
14. Zhou C, Ma J, Lu Y, Zhao W, Xu B, Lin J, et al. TERT promoter regulating melittin expression induces apoptosis and G_0/G_1 cell cycle arrest in esophageal carcinoma cells. Oncol Lett. 2021; 21(1):16.
15. Mansour GH, El-Magd MA, Mahfouz DH, Abdelhamid IA, Mohamed MF, Ibrahim NS, et al. Bee venom and its active component Melittin synergistically potentiate the anticancer effect of Sorafenib against HepG2 cells. Bioorg Chem. 2021;116:105329.
16. Ryu JM, Na HH, Park YJ, Park JS, Ahn BS, Kim KC. Sweet bee venom triggers multiple cell death pathways or spurs acute cell rupture according to its concentration in THP-1 monocytic leukemia cells. Genes (Basel). 2022;13(2):223.
17. Kim H, Park SY, Lee G. Potential therapeutic applications of bee venom on skin disease and its mechanisms: a literature review. Toxins (Basel). 2019;11(7):374.
18. Yavari M, Salesi Z, Derakhti A, Azimzadeh S, Varpaei HA, Esmaeili H, et al. Melittin and breast cancer: a brief review of the evidence. J Nur Patient Saf. 2020;1:1-7.
19. Wang A, Zheng Y, Zhu W, Yang L, Yang Y, Peng J. Melittin-based nano-delivery systems for cancer therapy. Biomolecules. 2022; 12(1):118.
20. Choi KE, Hwang CJ, Gu SM, Park MH, Kim JH, Park JH, et al. Cancer cell growth inhibitory effect of bee venom via increase of death receptor 3 expression and inactivation of NF-kappa B in NSCLC cells. Toxins (Basel). 2014;6(8):2210-28.
21. Wen T, Song L, Hua S. Perspectives and controversies regarding the use of natural products for the treatment of lung cancer. Cancer Med. 2021;10(7):2396-422.
22. Lin TY, Hsieh CL. Clinical applications of bee venom acupoint injection. Toxins (Basel). 2020;12(10):618.

23. Duffy C, Sorolla A, Wang E, Golden E, Woodward E, Davern K, et al. Honeybee venom and melittin suppress growth factor receptor activation in HER2-enriched and triple-negative breast cancer. NPJ Precis Oncol. 2020;4:24.
24. Oršolić N. Bee venom in cancer therapy. Cancer Metastasis Rev. 2012;31(1-2):173-94.