

Research Article

Bee Venom Triggers Autophagy-Induced Apoptosis in Human Lung Cancer Cells via the mTOR Signaling Pathway

Ji Eun Yu , Yuri Kim, Da Eun Hong, Dong Won Lee, Ju Young Chang, Seung Sik Yoo, Min Ji Kim, Dong Ju Son, Jaesuk Yun, Sang-Bae Han, and Jin Tae Hong 

College of Pharmacy and Medical Research Center, Chungbuk National University, 194-31, Osongsaengmyeong, 1-ro, Osong-eup, Cheongju-si, Chungbuk 28160, Republic of Korea

Correspondence should be addressed to Jin Tae Hong; jinthong@chungbuk.ac.kr

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In oriental medicine, bee venom has long been used as a therapeutic agent against inflammatory diseases. Several studies have reported that isolated and purified bee venom components are effective in treating dementia, arthritis, inflammation, bacterial infections, and cancer. In previous studies, we reported that bee venom inhibits cell growth and induces apoptotic cell death in lung cancer cells. In the present study, we assessed whether bee venom affects autophagy and thereby induces apoptosis. Bee venom treatment increased the levels of autophagy-related proteins (Atg5, Beclin-1, and LC3-II) and the accumulation of LC3 puncta. We found that bee venom could induce autophagy by inhibiting the mTOR signaling pathway. In addition, we found that hydroxychloroquine (HCQ)- or si-ATG5-induced autophagy inhibition further demoted bee venom-induced apoptosis. Bee venom-induced autophagy promotes apoptosis in lung cancer cells and may become a new approach to cancer treatment.

1. Introduction

Bee venom is a type of poison in the venom sac of honeybees, and many studies have been conducted on the use of apitherapy in oriental medicine [1]. Recently, bee venom has been registered as an alternative medicine and is used as an additive for food and medicine. Accordingly, studies on the ingredients, pharmacological activities, and toxicity of bee venom are actively being conducted. Bee venom is mainly composed of peptide components such as melittin, apamin, and adolapin; enzymes, such as phospholipase A2 (PLA2); and monoamines, such as dopamine, histamine, norepinephrine, and serotonin (known as neurotransmitters) [1–7]. These components may exhibit anti-inflammatory, analgesic, antipyretic, anticonvulsant, cytotoxic, and anticancer effects [8–15]. In recent years, several *in vitro* and *in vivo* studies have reported the anticancer effects of bee venom on many cancer cells [8, 9, 16–19]. We previously found that bee venom increases apoptosis, inhibiting the growth of lung cancer cells [19]. Apoptosis and autophagy concomitantly regulate cell fate [20, 21]. Autophagy and

apoptosis occur in cells through various stress-related pathways.

Autophagy is a protein degradation process that essentially occurs in cells to maintain homeostasis against various cellular stresses [22–24]. Autophagy occurs all the time in most tissues and is involved in the generation and differentiation of organelles or in tissue remodeling by maintaining the balance between the synthesis and degradation of cell organs and components [25–29]. Autophagy is induced by changes in external environments such as disordered protein- or misfolded proteins-induced stress, nutrient deficiency, growth factor reduction, ER stress, or pathogen infection [30–35]. Autophagy is known to serve as an intermediate mediator in cancer, aging, and inflammatory responses [36–47].

The role of autophagy in cancer is very complex and remains unclear. Autophagy has two conflicting functions in cancer. It can suppress cancer by maintaining genetic stability through the removal of the substrates of carcinogens or the degradation of unfolded proteins and damaged cell organelles [48, 49]. On the other hand, it can promote the

growth of cancer cells by providing the necessary substances for growth [46, 49, 50]. However, our study revealed the mechanism by which autophagy induces apoptosis in lung cancer cells. In fact, recent studies have focused on autophagy in human lung cancer cells [51]. These studies have reported the development of lung cancer treatments that could induce autophagy and eventually lead to apoptosis in lung cancer cells [52, 53]. In the present study, we therefore investigated whether bee venom affects autophagy in lung cancer cells and thus contributes to inducing apoptosis.

2. Methods

2.1. Cell Culture and Treatment. NCI-H460 lung cancer cells, A549 lung cancer cells, A172 glioblastoma cells, MDA-MB-231 breast cancer cells, and Hep3B liver cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). NCI-H460 and A549 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 $\mu\text{g}/\text{ml}$ penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. A172, MDA-MB-231, and Hep3B cells were cultured in DMEM supplemented with 10% FBS, 100 $\mu\text{g}/\text{ml}$ penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin. The cells were cultured in an incubator with a humidified atmosphere of 5% CO_2 at 37°C.

Bee venom powers were provided by Chung Jin Biotech Co. (Ansan, Korea). The composition of the bee venom was as follows: 40–50% melittin, 2–3% apamin, 2–3% MCD-peptide 401, 0.5–1% adolapin, 0.1–0.8% protease inhibitor, 0.5–2% secapin, 1–2% procamine A and B, 1% hyaluronidase, 10–13% PLA2, 0.5–2% histamine, and 0.2–1% dopamine.

2.2. Cell Lysates Preparation and Western Blotting. Cell lysis and western blotting were performed as previously described [54]. Cells were lysed with lysis buffer (20 mM Tris-HCl pH 7.8, 0.1% NP-40, 200 mM NaCl, 2 mM EDTA, 5 mM EGTA with protease inhibitor, and protease inhibitor) and centrifuged at 13,000 $\times g$ for 20 min at 4°C. The proteins were detected using an ECL substrate ($\#$ WBKLS0500, Millipore, Billerica, MA) and visualized using the Fusion Solo S chemiluminescence detection system (Vilber Lourmat, Collégien, France). The primary antibodies were as follows: anti-LC3 ($\#$ 2775, CST, Beverly, MA); anti-ATG5 ($\#$ 12994, CST); anti-p62 ($\#$ 8025, CST); anti-Beclin-1 ($\#$ 3738, CST); anti-phospho-mTOR ($\#$ 2971, CST); anti-mTOR ($\#$ 2972, CST); anti-phospho-4E-BP1 ($\#$ 2855, CST); anti-4E-BP1 ($\#$ 9644, CST); anti-phospho-p70 S6 kinase ($\#$ 9205, CST); anti-S6 kinase ($\#$ 9202, CST); anti-phospho-ULK ($\#$ A90736, Abclonal, Wuhan, China); anti-ULK ($\#$ A8529, Abclonal); anti-cleavedcaspase-3 ($\#$ 9661, CST); anti-caspase-9 ($\#$ 9502, CST); anti-Bcl-2 ($\#$ sc-7382, Santa Cruz, Dallas, TX); anti-Bax ($\#$ sc-7480, Santa Cruz); and anti- β -actin (sc-517582, Santa Cruz).

2.3. Immunocytochemistry. An immunocytochemistry assay was performed as described previously [55]. The fixed NCI-H460 cells were incubated with primary antibodies overnight at 4°C, and Alexa Fluor 488 ($\#$ A32723, $\#$ A32731,

Invitrogen, Carlsbad, CA) or Texas Red ($\#$ T-862, $\#$ T-2767, Invitrogen)-conjugated secondary antibodies were incubated for 1 h at room temperature. The cells were then incubated with 1 $\mu\text{g}/\text{ml}$ DAPI ($\#$ D9542, Sigma-Aldrich, St. Louis, MO) for 5 min at room temperature and mounted with Fluoromount-G Mounting Medium ($\#$ 0100-01, Southern Biotech, Birmingham, AL). The cells were visualized using the Zeiss Axio Observer fluorescence microscope system (Carl Zeiss, Oberkochen, Germany).

2.4. Autophagy Flux Assay. The autophagy flux assay was performed as previously described [55]. The autophagic flux was monitored using the CYTO-ID[®] Autophagy Detection Kit (Enzo Life Sciences Inc., Farmingdale, NY), according to the manufacturer's protocol. Cells were stained with CYTO-ID[®] Green Detection Reagent for 1 h at 37°C in the dark. The cells were visualized using the Zeiss Axio Observer fluorescence microscope system (Carl Zeiss). Digital images were analyzed using ZEN 2.1 software (Carl Zeiss).

2.5. RT-qPCR Assay. The RT-qPCR assay was performed as previously described [54]. Total RNA was isolated using RiboEx Total RNA ($\#$ 301-001, GeneAll Biotechnology Co., Seoul, Korea) and reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit ($\#$ 4368813, Applied Biosystems, Foster City, CA), according to the manufacturer's protocol. Real-time PCR was performed using SYBR Green PCR Master Mix ($\#$ 4344463, Applied Biosystems) and analyzed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). All primers used for RT-qPCR were designed using Primer3 on the website and purchased from Bioneer Corp. (Daejeon, Korea).

2.6. Cell Viability Assay. The cell viability assay was performed as previously described [55]. Cells were plated onto 96-well plates and treated with 0, 0.1, 0.5, and 1 $\mu\text{g}/\text{ml}$ bee venom for 24 h. After 24 h, cell viability was assessed using the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay ($\#$ M5655, Sigma Aldrich), according to the manufacturer's protocol. In brief, a 5 $\mu\text{g}/\text{ml}$ MTT solution was added and incubated at 37°C for 4 h, following which 100 μl DMSO was added to each well. The absorbance of each sample was read using a microplate reader at a wavelength of 540 nm.

2.7. Flow Cytometry (FACS). Cells were washed with PBS and harvested using trypsinization. The cells were stained with propidium iodide (PI) and annexin V for 5 min at dark room temperature. The samples were performed using a BD FACS Calibur[™] FlowCytometer (BD Biosciences, San Diego, CA), and the data were analyzed through the CellQuest Pro v6.0 software (BD Biosciences).

2.8. Statistical Analysis. Statistical analyses were performed using GraphPad Prism 5 software. All error bars reported are the standard deviations (SDs), unless indicated otherwise.

Pairwise comparisons were performed using Student's *t*-test. Multiple comparisons were performed using a one-way analysis of variance followed by Tukey's tests. Differences between groups were considered significant at *P* values < 0.05.

3. Results

3.1. Bee Venom Induces Autophagy in Human Cancer Cells. There are many reports on the relationship between apoptosis and autophagy. Apoptosis and autophagy induce cell death in a cooperative manner [20, 21, 56]. We previously found that bee venom inhibits cell growth and induces apoptotic cell death in human lung cancer cells [19]. In the present study, we assessed whether bee venom affects autophagy in lung cancer cells. To examine the effect of bee venom on the viability of A549 and NCI-H460 cells, we analyzed the cytotoxic activity using the MTT assay. We found that bee venom inhibited the growth of lung cancer cells in a concentration-dependent manner. The IC₅₀ values after 24 h of bee venom treatment were 2.9 and 3.18 μg/ml for NCI-H460 and A549 cells, respectively (Supplementary Figure S1). Bee venom inhibited the growth of both of these lung cancer cells; however, it more effectively inhibited the growth of NCI-H460 lung cancer cells. Therefore, we selected NCI-H460 cells for further analyses. To examine whether bee venom affects autophagy, we assessed the level of LC3. After the cleavage of the C-terminal region of LC3 with ATG4, LC3-I is generated. LC3-I is converted into LC3-II by phosphatidylethanolamine (PE) [57]. LC3-II is widely used as an autophagy marker because it is attached to the autophagosome membrane. A western blot analysis revealed that the level of LC3-II increased in a concentration-dependent manner following bee venom treatment (Figure 1(a)). Moreover, bee venom treatment increased the level of LC3-II in a time-dependent manner (Figure 1(b)). To confirm whether the autophagosome markers LC3 puncta could accumulate in cells by bee venom treatment, fluoresce microscopic analysis was performed. The bee venom treatment resulted in significant LC3 puncta accumulation in a concentration-dependent manner (Figure 1(c)). Moreover, bee venom treatment increased LC3 puncta accumulation in a time-dependent manner (Figure 1(d)). To determine whether autophagy can be induced by bee venom in other cancer cell lines, we confirmed the efficacy of bee venom against A172 glioblastoma, MDA-MB-231 breast cancer, and Hep3B liver cancer cell lines. Bee venom treatment increased the LC3-II band and LC3 puncta in various cancer cell lines (Supplementary Figures S2a and S2b). We also assessed the levels of other autophagy-regulating proteins in cells treated with bee venom. In bee venom-treated cells, the level of p62 decreased, while the levels of ATG5 and Beclin-1 increased in concentration- and time-dependent manners (Figures 1(e) and 1(f)). Similarly, the mRNA expression levels of ATG5, Beclin-1, and LC3-II increased in bee venom-treated cells (Supplementary Figure S3). These results suggest that bee venom induces autophagy in cancer cells, particularly lung cancer cells.

3.2. Bee Venom Enhances Autophagic Flux. Autophagy induction or autophagic flux blockade can increase the level of LC3-II and autophagosome formation. To evaluate whether bee venom affects autophagic flux, cells were treated with bee venom with or without the autophagy inhibitor hydroxychloroquine (HCQ). HCQ is known to increase the level of LC3-II by inhibiting the fusion of autophagosomes with lysosomes to block the degradation of autophagosomes. Therefore, the increase in the level of LC3-II in the presence of HCQ is indicative of enhanced autophagic flux. The level of LC3-II increased after treatment with bee venom or HCQ alone. Treatment with bee venom in the presence of HCQ further increased the level of LC3-II (Figure 2(a)). In addition, LC3 puncta increased even in the presence of HCQ (Figure 2(b)). The change in autophagy levels caused by bee venom was assessed using the CYTO-ID Autophagy Detection Kit, another method for measuring autophagic flux. The CYTO-ID Autophagy Detection Kit selectively stains autophagic vacuoles. No staining was noted in control cells. However, staining was observed in bee venom-treated cells (Figure 2(c)). These results indicate that bee venom can enhance autophagy.

3.3. Bee Venom Induces Autophagy by Inhibiting the mTOR Pathway. To unravel the mechanism by which bee venom induces autophagy, we confirmed the involvement of mTOR, a well-known suppressor of autophagy. The expression of phosphorylated mTOR decreased in bee venom-treated cells. The downstream factors of mTOR are p70 S6 kinase 1 (S6K1) and eIF4E-binding protein 1 (4E-BP1). mTOR phosphorylates S6K1, and activated S6K1 promotes translation initiation through the activation of S6 ribosomal protein [58]. mTOR phosphorylates 4E-BP1 and inactivates it, thereby promoting translation [59]. We observed that bee venom treatment reduced S6K1 phosphorylation and 4E-BP1 phosphorylation in a dose-dependent manner (Figure 3(a)). UNC-51-like autophagy activating kinase 1 (ULK1) is a key regulator required for the nucleation of the autophagosome and the formation of the autophagosome membrane. Activated mTORC1 inhibits autophagy by direct phosphorylation of ULK1 at S757 [60, 61]. This phosphorylation inhibits ULK1 kinase activity and subsequent autophagosome formation. As shown in Figure 3(b), mTORC1 phosphorylation of ULK1 at S757 was inhibited after bee venom treatment. Rapamycin, an mTOR inhibitor, was used to validate the role of mTOR in bee venom-induced autophagy. Rapamycin alone significantly increased the level of LC3-II; however, after pretreatment with rapamycin, bee venom did not increase the level of LC3-II (Figure 3(c)). These results suggest that bee venom induces autophagy through the mTOR pathway.

3.4. Bee Venom Mediates Autophagy-Induced Apoptosis. Compound-induced autophagy may be either pro-survival or pro-death in cancer therapy. We previously found that bee venom treatment could increase apoptotic cell death in a concentration-dependent manner [19]. To assess the role of bee venom-induced autophagy in bee venom-induced

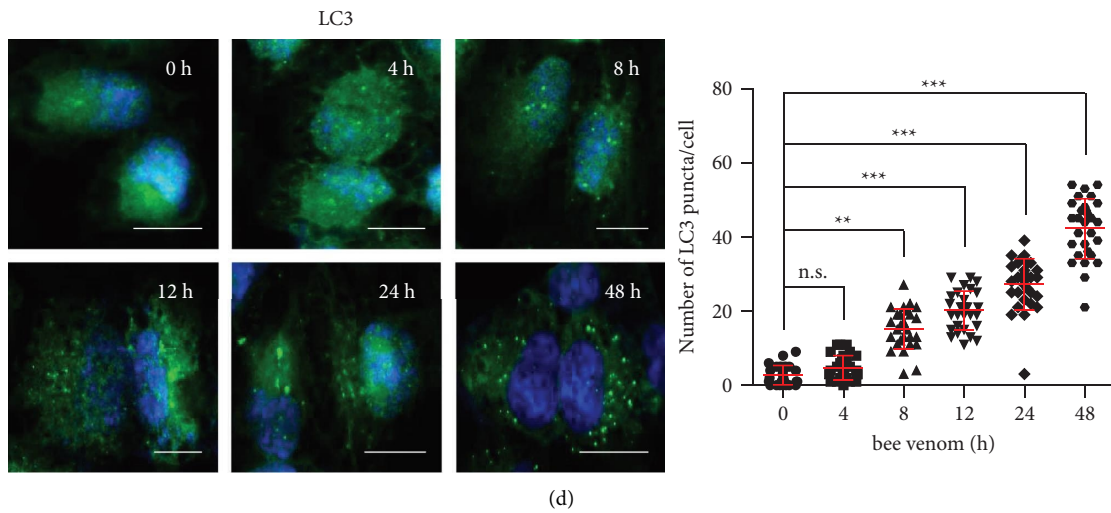
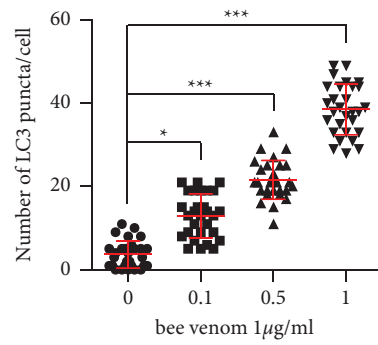
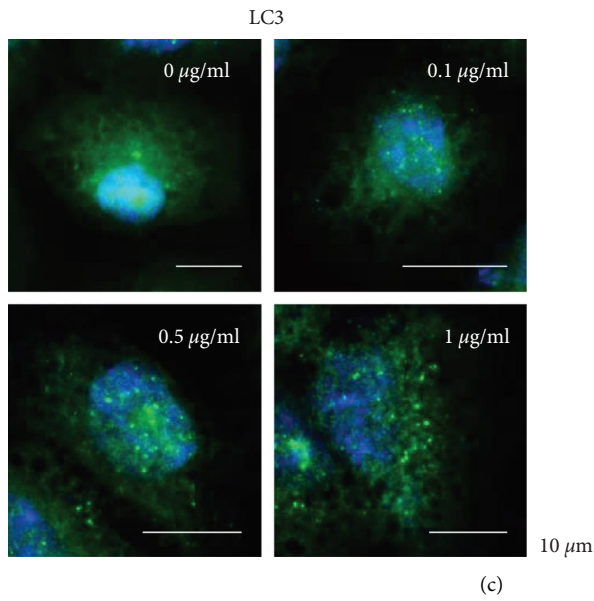
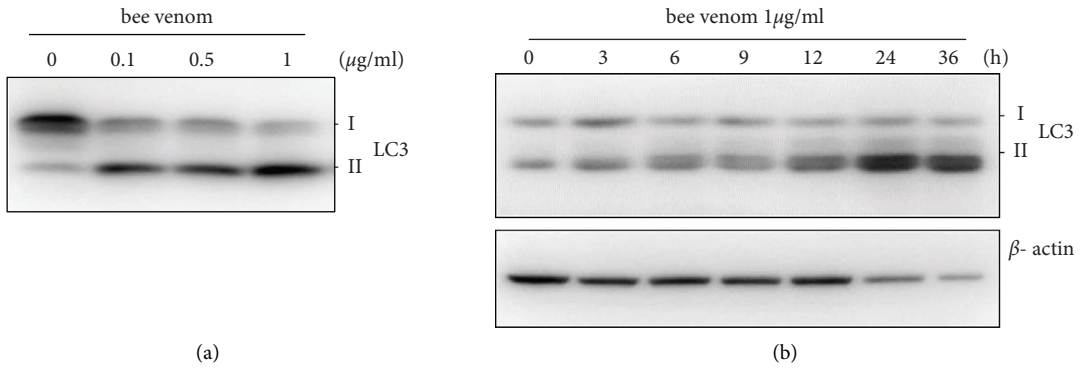


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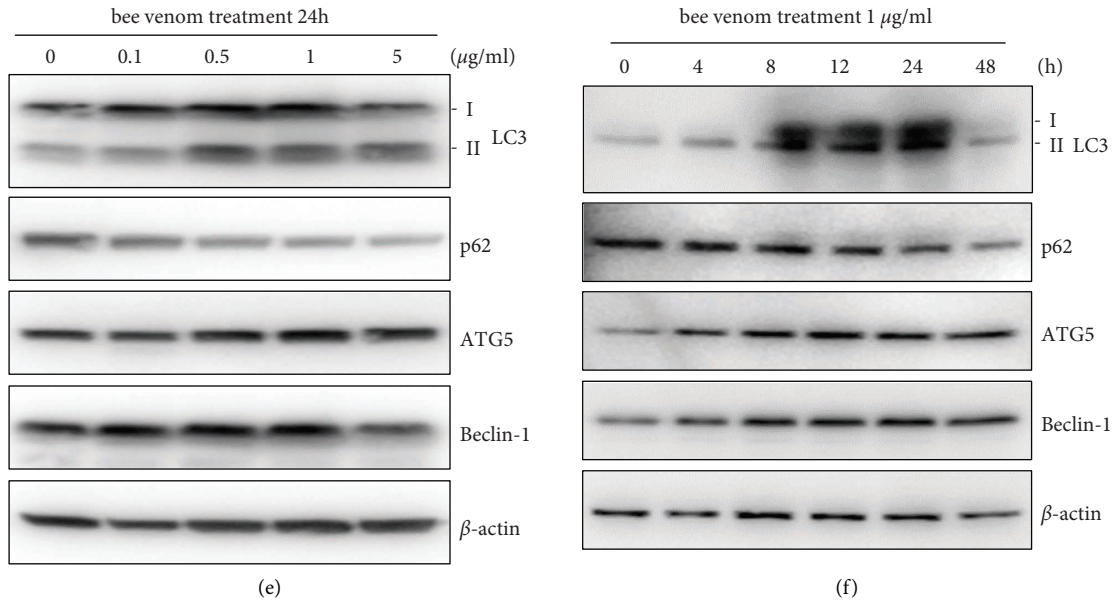


FIGURE 1: Bee venom induces autophagy in a dose- and time-dependent manner. (a) NCI-H460 cells were treated with bee venom (0.1, 0.5, and 1 $\mu\text{g/ml}$) for 24 h. LC3 levels were evaluated by immunoblotting. (b) NCI-H460 cells were treated with 1 $\mu\text{g/ml}$ bee venom for the indicated time. LC3 levels were evaluated by immunoblotting. (c) NCI-H460 cells were treated with bee venom (0.1, 0.5, and 1 $\mu\text{g/ml}$) for 24 h. The formation of LC3 puncta was detected after bee venom treatment under a fluorescence microscope. The percentage of cells along with the number of LC3 puncta was calculated. Scale bar, 10 μm . (d) NCI-H460 cells were treated with 1 $\mu\text{g/ml}$ bee venom for the indicated time. The formation of LC3 puncta was detected after bee venom treatment under a fluorescence microscope. The percentage of cells along with the number of LC3 puncta was calculated. Scale bar, 10 μm . (e) NCI-H460 cells were treated with bee venom (0.1, 0.5, 1, and 5 $\mu\text{g/ml}$) for 24 h. The indicated protein levels were evaluated by immunoblotting. (f) NCI-H460 cells were treated with 1 $\mu\text{g/ml}$ bee venom for the indicated time. The indicated protein levels were evaluated by immunoblotting.

apoptosis, we used the autophagy-specific inhibitor HCQ. Results of the MTT assay revealed that, compared with bee venom treatment alone, cell viability significantly increased after bee venom treatment in the presence of HCQ (Figure 4(a)). In addition, western blot analysis revealed that HCQ treatment significantly reduced the bee venom-induced levels of cleaved caspase-3, cleaved caspase-9, and Bax but increased the level of Bcl-2 (Figure 4(b)). Furthermore, compared with bee venom treatment alone, bee venom treatment in the presence of HCQ significantly reduced the percentage of apoptotic cells (Figure 4(c)). We also observed the cell cycle using FACS analysis. As a result, it was confirmed that the number of subG1 cells increased in the bee venom-treated cells. However, bee venom and HCQ-cotreated cells showed a similar cell population to control cells (Figure 4(d)). Apoptotic cells were once again confirmed by annexin V staining, and the number of annexin V-positive cells increased by bee venom treatment (Figure 4(e)). Compared with bee venom treatment alone, bee venom and HCQ-cotreated cells reduced the percentage of annexin V-positive cells.

ATG5 is a key autophagy regulator and an integral part of the ATG5-ATG12-ATG16L1 complex that catalyzes ATG8 lipidation, which is essential for autophagosome formation and expansion. ATG5 is also indispensable for the fusion of autophagosomes with lysosomes in canonical autophagy and noncanonical autophagy [62, 63]. We further investigated

whether bee venom-mediated autophagy affects apoptosis by transfecting ATG5 siRNA (Figure 5(a)). Bee venom treatment with control siRNA increased the levels of the apoptosis-related proteins cleaved caspase-3, cleaved caspase-9, and Bax but decreased the level of Bcl-2. However, the depletion of ATG5 with bee venom treatment reduced the levels of apoptosis-related proteins (Figure 5(b)). In addition, the depletion of ATG5 reduced bee venom-induced apoptosis (Figure 5(c)). These results were confirmed once again by FACS analysis. Bee venom significantly increased the number of subG1 and annexin V-positive cells. The depletion of ATG5 with bee venom treatment reduced the subG1 and annexin V-positive cells (Figures 5(d) and 5(e)). These data indicate that autophagy is necessary for bee venom-induced apoptosis.

4. Discussion

In the present study, we found that bee venom induces autophagy in lung cancer cells. Our results provide evidence that bee venom induces autophagy. We also proved that the mTOR pathway is associated with bee venom-induced autophagy. Moreover, our findings suggested that bee venom-induced autophagy could be important for bee venom-induced apoptotic cell death in lung cancer cells.

Bee venom is used as an effective treatment option against various musculoskeletal pains, inflammatory diseases, neuroparalytic diseases, and immune-related diseases

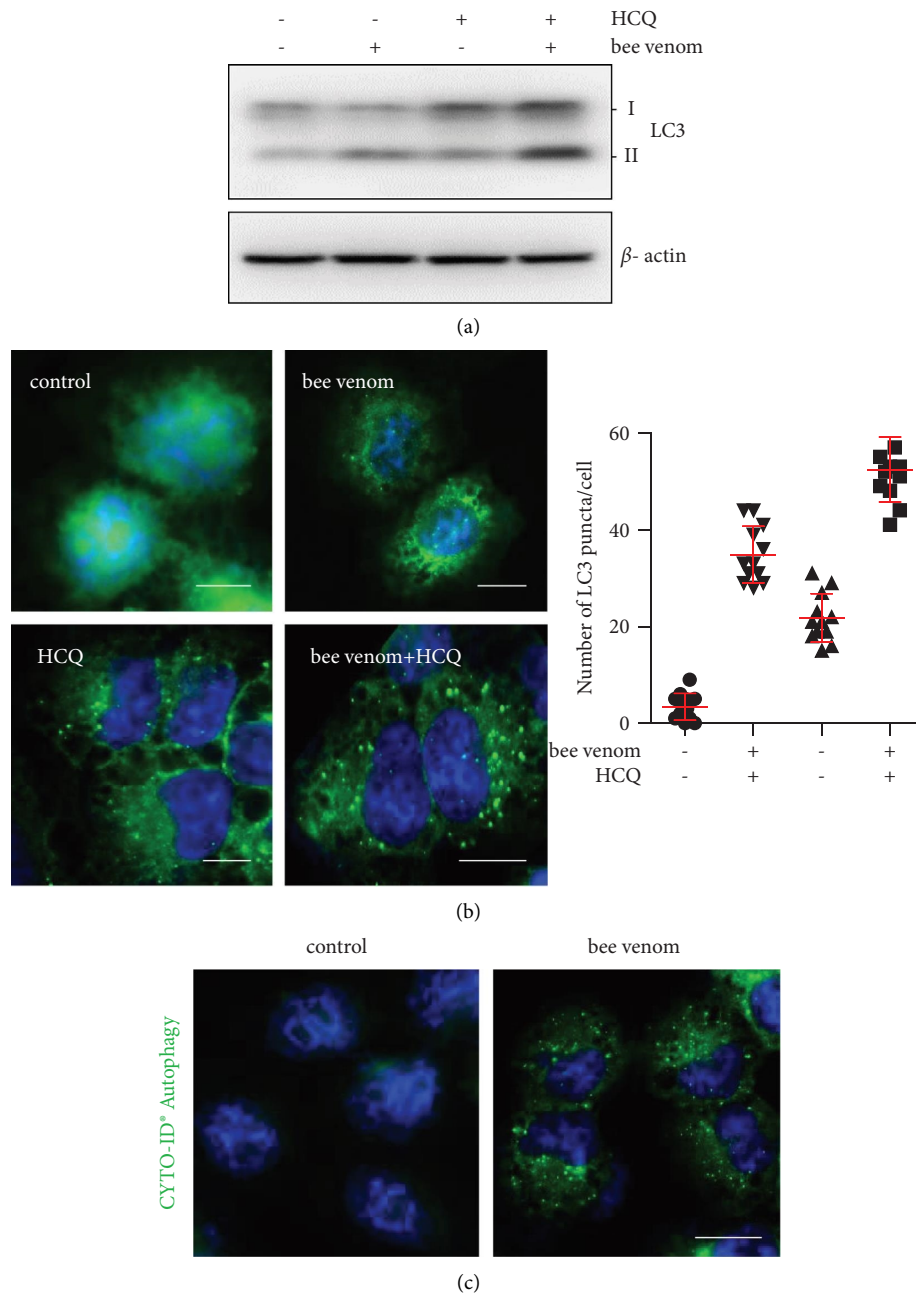


FIGURE 2: Bee venom induces autophagic flux in the lung cancer cell. (a and b) NCI-H460 cells were treated with 1 μ g/ml bee venom for 24 h after pretreatment with or without the autophagy inhibitor HCQ (25 μ M) for 1 h LC3 levels were evaluated by immunoblotting (a) or immunocytochemistry (b). The nuclei were counterstained with DAPI. Scale bar, 10 μ m. (c) NCI-H460 cells were treated with 1 μ g/ml bee venom and then stained with CYTO-ID for 1 h at 37°C in the dark. The nuclei were counterstained with DAPI. Scale bar, 5 μ m.

[4, 10, 12, 13, 15]. Many studies have also reported that bee venom has growth-inhibitory effects on various cancer cells, such as colon cancer, cervical cancer, ovarian cancer, lung cancer, and prostate cancer cells [8–10, 64, 65]. In addition, several studies have been conducted on the pain control and preventative effects of bee venom on malignant osteoporosis and peripheral neuropathy, as well as on the intractable symptoms related to cancer and chemotherapy [1]. Several studies have revealed that under stress conditions, cells first attempt to repair damaged cells with autophagy and survive;

however, in the event of failure, autophagy-mediated programmed cell death, apoptosis, and necrosis/necroptosis progress damaged cells into cancer cells [20, 21, 50, 56]. Thus, autophagy has emerged as a powerful mediator of programmed cell death [20, 21, 56]. In our previous study, we showed that bee venom induces apoptosis in lung cancer cells through apoptotic pathways [19]. Although several studies have revealed that bee venom acts against cancer by inducing apoptosis, the mechanisms underlying bee venom-induced apoptosis remain unclear.

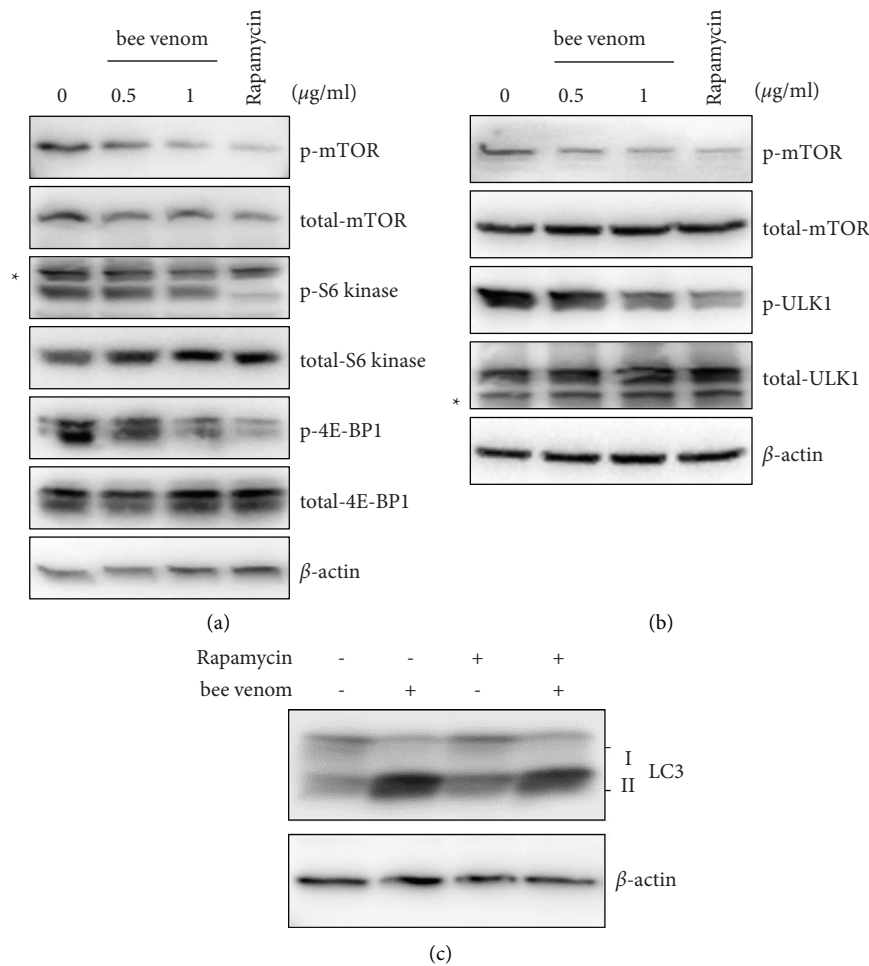


FIGURE 3: The mTOR pathway participated in bee venom-induced autophagy. (a and b) NCI-H460 cells were treated with bee venom (0.5 and 1 $\mu\text{g/ml}$) or 0.5 μM rapamycin for 24 h. The indicated protein levels were evaluated by immunoblotting. * indicates a nonspecific band. (c) NCI-H460 cells were treated with 1 $\mu\text{g/ml}$ bee venom for 24 h in the absence or presence of rapamycin (0.5 μM). LC3 levels were evaluated by immunoblotting.

In the present study, bee venom-induced autophagy was evidenced by the conversion of the autophagosomal marker LC3 and the formation of LC3 punctate structures. We also found that bee venom-induced autophagy occurs in human glioblastoma (A172), colon cancer (SW480), breast cancer (MDA-MB-231), and liver cancer cell lines (Hep3B). We examined whether the increased level of LC3-II following bee venom treatment induces protein degradation in cell organelles by autophagosome-lysosome fusion. The levels of LC3-II and LC3 puncta increased in bee venom-treated cells in the presence of the autophagosome-lysosome fusion inhibitor HCQ. Collectively, these results indicate that bee venom can induce autophagic flux in lung cancer cells.

We further showed that bee venom induces autophagy through the mTOR signaling pathway. mTOR regulates cell fate through protein synthesis and cell cycle control in response to several signals, such as energy status, nutritional status, insulin level, and growth factors [58]. The most important protein in autophagy is mTOR, and the mTOR pathway has been reported to be involved in autophagy in various cancers. The well-known substrates of mTOR are

4E-BP1 and S6K1, which regulate the protein synthesis process [58]. Some genes involved in the infiltration of cancer cells are regulated by mTOR through 4E-BP1 translation inhibition factors. mTOR inhibitors, such as sirolimus, everolimus, and temsirolimus, which are approved or in the clinical stages for cancer treatment, can inhibit the mTOR signaling pathway and inhibit the development of cancer cells into invasive types of cancer [66]. We found that the phosphorylation of 4E-BP1 and S6K1 was reduced in bee venom-treated cells. It is also well known that ULK1 is a major regulator of autophagy initiation and is regulated by mTOR. In the present study, we found that the phosphorylation of ULK1 has been reduced in bee venom-treated cells. These data suggest that bee venom-induced autophagy is dependent on the mTOR signaling pathway.

Autophagy plays a role in both cancer cell survival and death in cancer therapy [20, 56]. Therefore, we confirmed whether bee venom-induced autophagy affects apoptosis. We found that HCQ, an inhibitor of autophagy, may potentially attenuate the cytotoxic effects of bee venom on NCI-H460 cells. Moreover, we found that HCQ treatment

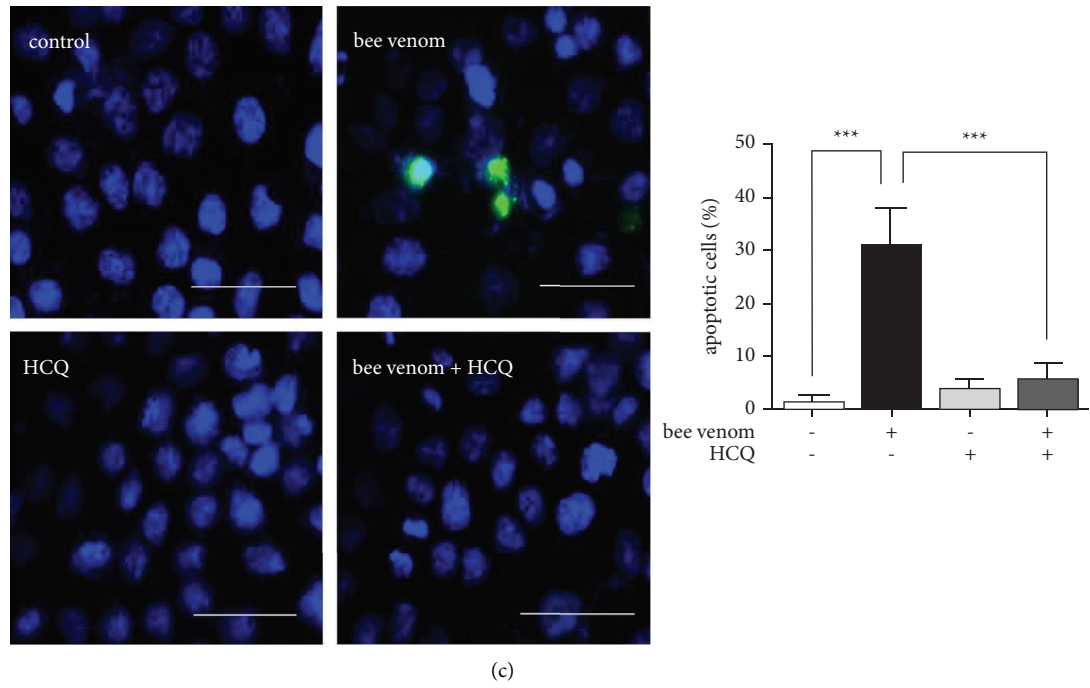
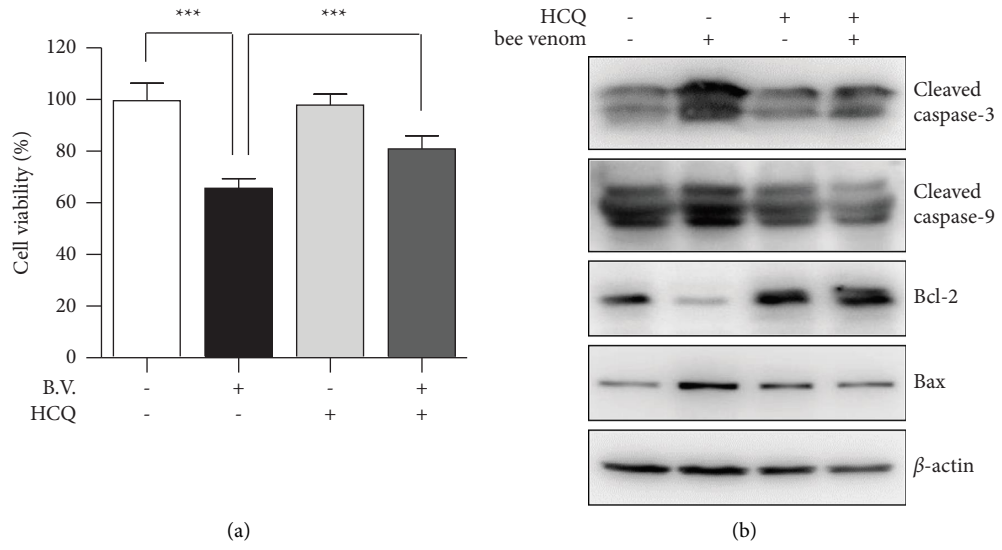


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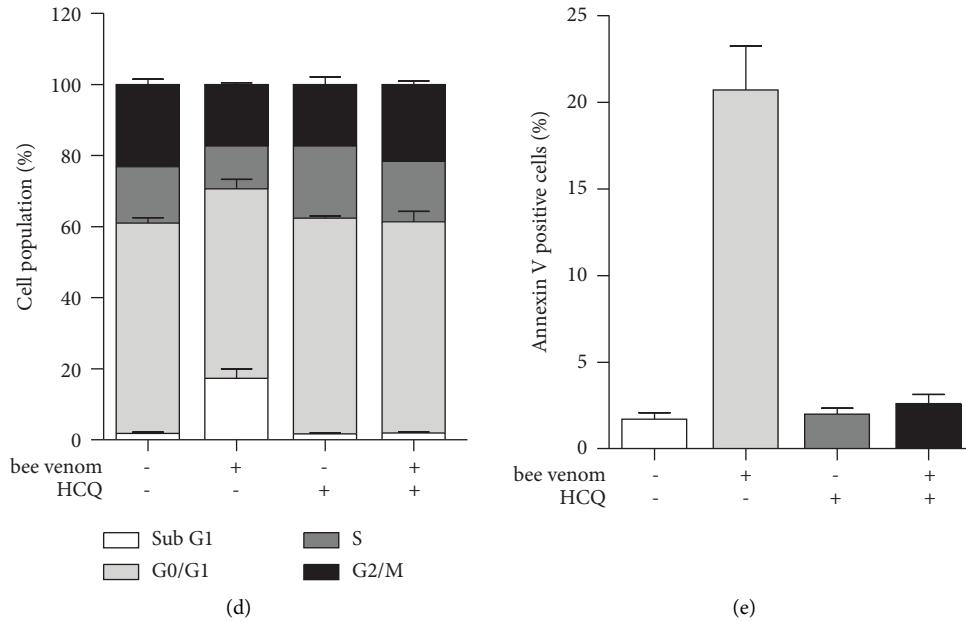


FIGURE 4: Inhibition of autophagy reduces bee venom-induced apoptotic cell death. (a) NCI-H460 cells were treated with 1 $\mu\text{g}/\text{ml}$ bee venom for 24 h in the absence or presence of the autophagy inhibitor HCQ. The cell viability of bee venom-treated cells was measured using the MTT assay. The data are presented as the mean \pm SD of three independent experiments. $***P < 0.05$. (b) NCI-H460 cells were treated with 1 $\mu\text{g}/\text{ml}$ bee venom for 24 h in the absence or presence of HCQ (5 μM). The indicated protein levels were evaluated by immunoblotting. (c) Representative fluorescence microscopic images showing DAPI (blue) and TUNEL (green) nuclear staining in cells treated with 1 $\mu\text{g}/\text{ml}$ bee venom for 24 h in the absence or presence of the autophagy inhibitor HCQ (5 μM). Scale bar, 50 μm . The number of positively stained cells was counted in three different fields and averaged. The data are presented as the mean \pm SD of three independent experiments. $***P < 0.05$. (d) NCI-H460 cells were treated with 1 $\mu\text{g}/\text{ml}$ bee venom for 24 h in the absence or presence of the autophagy inhibitor HCQ. The cell population of bee venom-treated cells was measured using FACS analysis. The data are presented as the mean \pm SD of three independent experiments. (e) NCI-H460 cells were treated with 1 $\mu\text{g}/\text{ml}$ bee venom for 24 h in the absence or presence of the autophagy inhibitor HCQ. The apoptosis of bee venom-treated cells was measured using FACS analysis. The data are presented as the mean \pm SD of three independent experiments.

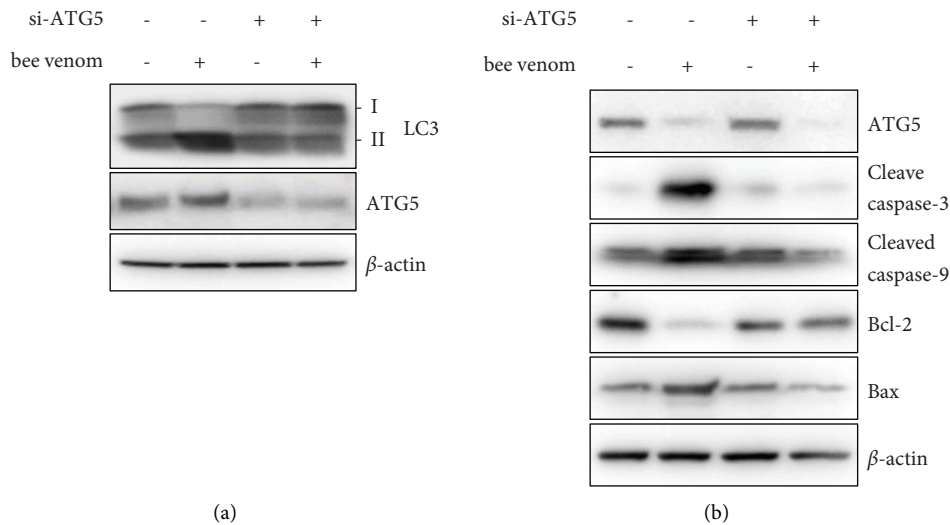


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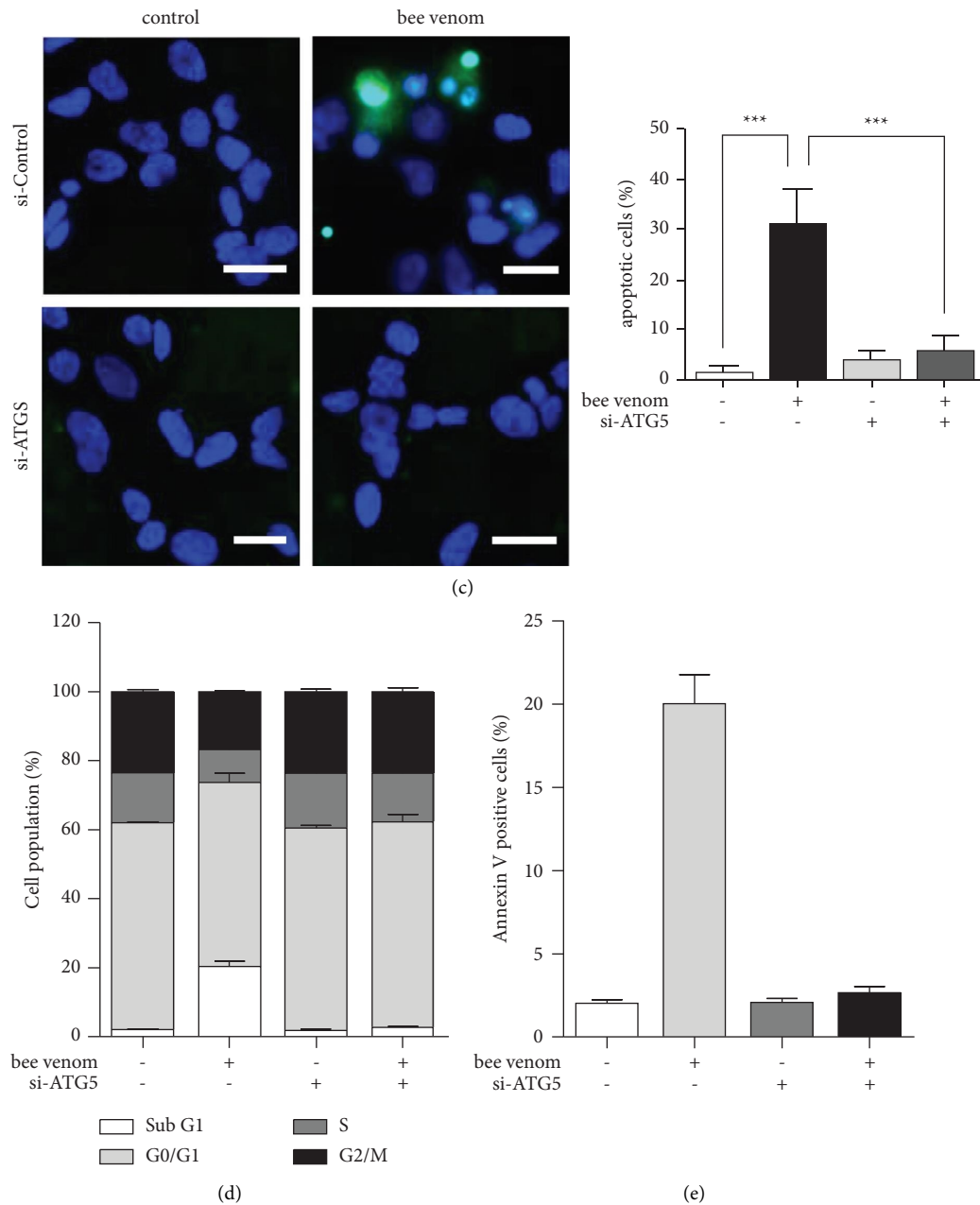


FIGURE 5: Bee venom-induced autophagy enhances apoptosis and triggers cell death in NCI-H460 cells. (a–c) NCI-H460 cells were transfected with si-ATG5 and then treated with 1 $\mu\text{g}/\text{ml}$ bee venom for 24 h. A western blot analysis was then performed to analyze the level of autophagy-related (a) and apoptosis-related (b) proteins. (c) Representative fluorescence microscopic images showing DAPI (blue) and TUNEL (green) nuclear staining. Scale bar, 50 μm . The number of positively stained cells was counted in three different fields and averaged. The data are presented as the mean \pm SD of three independent experiments. $***P < 0.05$. (d) NCI-H460 cells were transfected with si-ATG5 and then treated with 1 $\mu\text{g}/\text{ml}$ bee venom for 24 h. The cell population of bee venom-treated cells was measured using FACS analysis. The data are presented as the mean \pm SD of three independent experiments. (e) NCI-H460 cells were transfected with si-ATG5 and then treated with 1 $\mu\text{g}/\text{ml}$ bee venom for 24 h. The apoptosis of bee venom-treated cells was measured using FACS analysis. The data are presented as the mean \pm SD of three independent experiments.

could significantly reduce bee venom-induced apoptosis in NCI-H460 cells. On blocking autophagosome formation with the depletion of Atg5 using siRNA, bee venom-induced cytotoxicity and apoptosis were attenuated; these findings were similar to those of the HCQ combination treatment. The blocking of autophagy can reduce apoptosis with bee venom treatment. Many studies have revealed that small

molecules such as tyrosine kinase inhibitors, mTOR inhibitors, or HDAC inhibitors trigger autophagy-induced apoptosis in *in vivo* and *in vitro* lung cancer cells [52, 53]. These findings indicate that bee venom could induce autophagy in various cancer cells, and the induction of autophagy could play a significant role in bee venom-induced apoptosis.

5. Conclusions

In conclusion, we demonstrated that bee venom can induce autophagy in many types of cancer cells. Our data indicated that the mTOR signaling pathway is the critical regulator of bee venom-induced autophagy. Moreover, we found that HCQ- or si-ATG5-induced autophagy inhibition further demoted bee venom-induced apoptosis. These data indicate that bee venom-mediated autophagy could be important for bee venom-induced apoptosis, and the mTOR pathway may play a role in the underlying mechanism.

Data Availability

All the data generated or analyzed during this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Ji Eun Yu and Yuri Kim contributed equally to this work.

Acknowledgments

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Supplementary Materials

Supplementary Figure S1. Bee venom affects the viability of A549 and NCI-H460 cells. Supplementary Figure S2. Bee venom induces autophagy in various cancer cell lines. Supplementary Figure S3. The expression level of autophagy-related mRNA increases in bee venom-treated cells. (*Supplementary Materials*)

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