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EtNBSe-PDT inhibited proliferation and induced autophagy of HNE-1 cells via downregulating the Wnt/β-catenin signaling pathway

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ABSTRACT

Background: Increasing evidence has suggested that autophagy may play a resistant role during photodynamic therapy (PDT). The Wnt/β-catenin pathway is tightly involved in cell proliferation and autophagy. In this study, we aimed to determine the influence of 5-Ethylamino-9-diethylaminobenzo[*a*]phenoselenazinium (EtNBSe) mediated PDT (EtNBSe-PDT) on autophagy, proliferation and Wnt/β-catenin pathway in human NPC cell line (HNE-1 cells), and further explore the underlying crosstalk between them.

Methods: Cell viability and proliferation was evaluated by MTT assay. Autophagy and Wnt/β-catenin signaling pathway was analyzed by western blotting and immunofluorescence.

Results: It was revealed that EtNBSe-PDT significantly impeded the viability and proliferation of HNE-1 cells. Meanwhile EtNBSe-PDT could notably induce autophagy in HNE-1 cells accompanied with the inhibition of Wnt/β-catenin pathway. The Wnt/β-catenin pathway activator Wnt agonist was found to partially counteract the inhibitory proliferation of HNE-1 cells and suppress the autophagy induced by EtNBSe-PDT. In addition, pretreatment with the autophagy inhibitor 3-methyladenine (3-MA) or Wnt agonist showed the potential in enhancing the cytotoxic effect of EtNBSe-PDT (cell survival from 50.71 ± 4.16% to 24.53 ± 4.27% and from 52.64 ± 3.54% to 35.74 ± 4.27% respectively).

Conclusion: Taken together, this study demonstrated that EtNBSe-PDT suppressed viability and proliferation, and induced autophagy of HNE-1 cells via downregulating the Wnt/β-catenin pathway. The autophagy further constituted the cytoprotective mechanisms involved in HNE-1 cells, which suggested that the combination of EtNBSe-PDT and autophagy inhibitors may be a promising strategy for the treatment of human NPC.

1. Introduction

PDT is an effective, minimally invasive and local targeting clinical approach. It involves the systemic or topical administration of a target localizing photoactivatable compound (photosensitizer) and subsequent irradiation with light of a certain wavelength. When illuminated, the photosensitizer becomes a cytotoxin and generates large amounts of reactive oxygen species, thus inducing complicated biological responses which leads to cell death [1]. Since PDT causes essentially no effect on connective tissues, the anatomical integrity of hollow organs such as the nasopharynx can be preserved in patients undergoing PDT [2]. Therefore, endoscopic PDT with the fiber optic delivery system is especially suitable for NPC with surgical contraindications. In terms of a successful PDT, the damaging stress led by PDT must exceed the cytoprotection threshold. Otherwise, the remaining cells may repopulate the tumor and form a resistance to PDT [3]. Accordingly, in order to improve the therapeutic efficacy of PDT, the mechanisms of PDT-induced cell damage as well as possible cell resistance must be further clarified.

Autophagy is a form of stress adaption response when cells are subjected to environmental stress. In proficient autophagy, cells maintain the intracellular homeostasis by recycling their damaged or degenerated components, hence mediating a cytoprotective effect. And previous studies have reported that blockage of autophagy with pharmacological or genetic interventions accelerated the demise of cells responding to stress [4]. Nevertheless, in a number of pathological settings, the molecular machinery for autophagy contributes to cellular demise directly or favors the engagement of other regulated cell death modalities [5]. Note worthily, our previous research and numerous other studies have revealed the potential involvement of PDT in triggering autophagy [6]. However, the exact role of autophagy in response to PDT is still elusive and the precise molecular mechanisms behind

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PDT-induced autophagy are not well understood. Wnt/β-catenin signaling pathway is well known to be sensitive to intracelluar stress and has been reported to be affected by PDT [7]. Moreover, previous studies reported that certain chemical agents could induce autophagy via Wnt/β-catenin signaling pathway in human tumor cell lines [8,9]. However, the detailed mechanism remains unclear.

EtNBSe is a new synthetic bipolar photosensitizer with many advantages including stable chemical structure, high permeability, rapid absorption, and oncotarget accumulation [10]. Moreover, it has been substantiated that the photoactivity of EtNBSe is 10,000 times as much as that of photofrin and other photosensitizers in the benzophenoxazinium family [10]. Therefore, we explored the effect of EtNBSe-PDT on Wnt/β-catenin pathway, along with its function in NPC cell proliferation and autophagy. In addition, further studies managed to demonstrate autophagic impacts in cell resistance against EtNBSe-PDT of HNE-1 cells.

2. Materials and methods

2.1. Reagents

EtNBSe was synthesized by the chemistry department of Central South University (Changsha, China) using the synthetic method reported in our previous study and its chemical structure is shown in Fig. 1 [10]. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco (Grand Island, NY, USA). Wnt agonist (sc-222416) and 3-methyladenine (3-MA; sc-205596) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 4,6-diamidino-2-phenylindole (DAPI; D8417) and Thiazolyl Blue Tetrazolium Bromide (MTT; M2128) were obtained from Sigma-Aldrich (Danvers, MA, USA). The anti-Cyclin-D(sc-753), anti-β-catenin(sc-7199), anti-c-Myc(sc-40), anti-GSK-3β(sc-7291), anti-Beclin-1(sc-48341), anti-LC3-I(sc-134226), anti-LC3-II(sc-28266), anti-GAPDH (sc-293335) antibodies were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Detailed information about the antibodies is listed and characterized in Table 1. The autophagy activator rapamycin (#9004) was purchased from Cell Signaling Technology (Shanghai, People's Republic of China).

2.2. Cell culture

HNE-1 cells were purchased from Shanghai cell bank at the Chinese Academy of Science (Shanghai, China). All cells were cultured in DMEM medium (Gibco, Grand Island, NY, USA) supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and maintained at 37 °C under a humidified atmosphere of 5% CO_2.

2.3. PDT protocol in vitro

In all EtNBSe-PDT groups, HNE-1 cells were seeded in 6-well culture plates at density of 6×10^5 cells/well and were incubated for 24 h. Then, medium was aspirated from cultures and replaced with PBS containing various concentrations of EtNBSe (100, 200, 400, 600 and 800 nmol/L). The cells were incubated with EtNBSe for 20 min in the dark and then irradiated in the EtNBSe-containing PBS. The irradiation was done using an LED with a wavelength of 635 nm and a laser power density of 50 mW/cm^2. Furthermore, the total energy densities were adjusted by the various irradiation time (0, 30, 60,120 and 240 s; 0, 1.4, 2.8 and 5.6 J/cm^2 light energy density correspondingly). After light irradiation, fresh culture medium was replaced. Then, the treated cells were maintained at 37 °C under an atmosphere of 5% CO_2 in the dark for 24 h and the cell viability and protein levels were evaluated. In the EtNBSe or LED alone group, cells were subjected to only EtNBSe or LED light in the same manner as the EtNBSe-PDT group.

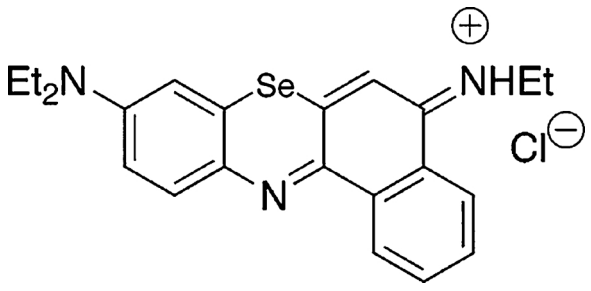

Fig. 1. The chemical structure of EtNBSe.

Table 1
Antibodies used in the present study.

Target	Source	Dilution	Host	Secondary antibody
LC3-I	Santa Cruz Biotechnology	1:500	Rabbit	Antirabbit
LC3-II	Santa Cruz Biotechnology	1:500	Rabbit	Antirabbit
Beclin-1	Santa Cruz Biotechnology	1:500	Rabbit	Antirabbit
β-catenin	Santa Cruz Biotechnology	1:500	Rabbit	Antirabbit
GSK3β	Santa Cruz Biotechnology	1:500	Mouse	Antimouse
c-Myc	Santa Cruz Biotechnology	1:500	Mouse	Antimouse
Cyclin-D	Santa Cruz Biotechnology	1:500	Rabbit	Antirabbit
GAPDH	Santa Cruz Biotechnology	1:500	Rabbit	Antirabbit

2.4. MTT analysis

MTT was used to detect cell viability and proliferation. HNE-1 cells at 5×10^3 cells/well were seeded on 96-well plates. After the attachment, the cells were treated according to the requirement of corresponding groups and incubated for 0, 12, 24, 48 or 72 h at 37℃. 10 μL MTT (5 mg/ml in PBS) was added to each well. Following incubation for 2 h, the supernatants were removed and 150 μl DMSO was added into each well to dissolve the formazan crystals. After oscillated for 10 min, the 96-well plates were then placed in a Microplate reader (Bio-Rad, Hercules, CA, USA) to assess the absorbance at 490 nm.

2.5. Immunofluorescence

HNE-1 cells were grown on coverslips. At 4 h after PDT, HNE-1 cells were washed with PBS for 2 min, fixed with 4% PFA for 10 min and gently rinsed in 0.3% Triton X-100 for 15 min at room temperature. After washing with PBS for 3 min, the cells were blocked with 5% BSA for 1 h and were then incubated with anti-lamp, anti-LC3-II and anti-β-catenin antibodies (dilution 1:500) overnight at 4 °C. Afterwards, Alexa Fluor 577-labeled, Alexa Fluor 647-labeled and Alexa Fluor 647-labeled secondary antibodies (dilution 1:200) were added respectively, to develop the fluorescence staining in a dark chamber. The fluorescence intensity and the co-localization were observed under a fluorescence microscope (BX41 OLYMPOS, Tokyo, Japan) and quantitatively analyzed by Image Pro Plus 6.0.

2.6. Western-blotting analysis

HNE-1 cells after corresponding treatments were washed three times with precooled PBS and harvested in sample buffer containing 20 mmol/L Tris-HCl (pH 8.8), 2.5 mmol/L EDTA, 150 mmol/L NaCl, 10% glycerine, 10% SDS, 1% Triton X-100, 10 mmol/L sodium pyrophosphate and 1 mmol/L PMSF for 30 min. Then the supernatants from cells were centrifugated at 10,000× for 15 min and the sample-loading buffer was added. Equal amount of total protein was loaded and separated by 10% SDS-PAGE and electrophoretically transferred to the polyvinylidene difluoride (PVDF) membrane. The membrane was then blocked for 1 h with 5% bovine serum albumin in Tris-buffered saline and incubated with primary antibodies (listed and characterized in Table 1) overnight at 4 °C. Next the membrane was washed three

rounds with PBST and was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1000) for 2 h at room temperature. Then following another round of washing with TBST, the membrane was developed using electrochemiluminescence, and the signals were detected using Quantity One 1-D Analysis Software (Bio-Rad, Hercules, CA, USA).

2.7. Cell cycle analysis

HNE-1 cells were seeded in the 96-well plates at the density of 5×10^3 cells/well and incubated for 24 h. After corresponding treatments, cells were harvested and fixed with 75% cold ethanol overnight at 4°C. The fixed cells were washed three times with PBS and stained darkly with propidium iodide (PI) for 30 min. The stained cells were analyzed by FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA).

2.8. Statistical analysis

All quantitative data were presented as the mean ± SD from of triplicate independent experiments. The SPSS 19.0 software package was used to perform all statistical analysis. Comparisons between two groups were performed using the Student's-t test, and between multiple groups using ANOVA analysis. P-values of < 0.05 were considered statistically significant.

3. Results

3.1. EtNBSe-PDT decreased cell viability in HNE-1 cells

In order to investigate the antitumor effect of EtNBSe-PDT on NPC cells, human NPC cell line, HNE-1, was cultured with various concentrations of EtNBSe (0, 100, 200, 400, and 600 nmol/L). And then the treated HNE-1 cells were exposed to LED light with increasing light densities, followed by the measurement of cell viability using MTT. As shown in Fig. 2, the viability of HNE-1 cells notably decreased in concentration- and light energy density-dependent manner. And cell viabilities in all EtNBSe-PDT groups treated with 400, 600 and 800 nmol/L EtNBSe and the groups treated with 200 nmol/L EtNBSe combined with 2.8 and 5.6 J/cm^2 light doses were significantly suppressed when compared with the control group (0 nmol/L EtNBSe, 0 J/cm^2). Additionally, the EtNBSe and LED alone groups showed no

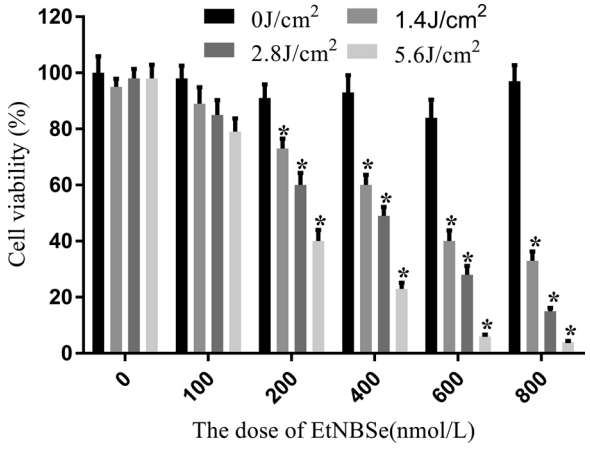

Fig. 2. EtNBSe-PDT decreased HNE-1 cell viability. HNE-1 cells were incubated with different concentrations of EtNBSe (0, 100, 200, 400, 600 and 800 nmol/L), and were irradiated with various light densities (0, 1.4, 2.8 and 5.6 J/cm^2). At 24 h after irradiation, cell viability was determined using the MTT assay. Data is presented as mean ± SD from three independent experiments. *P $<$ 0.05 versus the control group.

significant inhibition of cell viability. Furthermore, at a light dose of 2.8 J/cm^2, the half maximal inhibitory concentration of EtNBSe was 400 ± 23 nmol/L. The inhibition rate in the group that received 400 nmol/L EtNBSe combined with a light dose of 2.8 J/cm^2 was 49.21 ± 4.23%. Thus, all subsequent experiments were performed using 400 nmol/L EtNBSe combined with 2.8 J/cm^2 light energy density.

3.2. EtNBSe-PDT induced autophagy in HNE-1 cells

Previous studies revealed that autophagy could be activated by some photosensitizers mediated PDT. In this study, we investigated whether EtNBSe-PDT could induce autophagy in HNE-1 cells and the well-known autophagy activator Rapamycin was used as the positive control. Firstly, the expression levels of autophagy-related protein LC3-I, LC3-II and Beclin-1 were detected by Western blotting. Upon the induction of autophagy, LC3-I was transformed into LC3-II, which contributed favorably to the formation of autophagosomes. Thus, the LC3-II/LC3-I ratio indicated the level of autophagy. Fig. 3A and B showed that the ratio of LC3-II/LC3-I and expression of Beclin-1 were significantly increased in 200 and 400 nmol/L EtNBSe combined with a light dose of 2.8 J/cm^2 treated groups. In addition, autophagy is characterized by the formation of autophagosomes which then fuse with the lysosome, forming autolysosome. Thus, the relative fluorescence level of LC3-II and Lamp was evaluated to monitor autophagosome and lysosome formation. As shown in Fig. 3C and D, the staining of LC3-II was distributed evenly throughout the cell in the control group, whereas EtNBSe-PDT resulted in more distinctive LC3-II spots and a significant increase in the relative fluorescence intensity of LC3-II in the HNE-2 cells, which was consistent with our western blotting findings. Meanwhile obvious colocalization between the LC3- and Lamp-positive staining was observed 2 h after EtNBSe-PDT, indicating the formation of autolysosome. Taken together, these results indicated that EtNBSe-PDT induced notable autophagy in HNE-1 cells.

3.3. EtNBSe-PDT suppressed the Wnt/β-catenin pathway in HNE-1 cells

Wnt/β-catenin signaling pathway plays an important oncogenic role in many cancers. Moreover, numerous studies have reported that many antitumor therapies could induce cancer cell death by modulating this signaling pathway. Given that, we investigated the effect of EtNBSe-PDT on Wnt/β-catenin pathway in HNE-1 cells. As shown in Fig. 4A and B, 200 and 400 nmol/L EtNBSe combined with a light dose of 2.8 J/cm^2 caused a significant decrease of β-catenin expression. And the expressions of c-Myc and Cyclin-D, well known β-catenin downstream targets were also significantly downregulated by EtNBSe-PDT. Next we explored the impact of EtNBSe-PDT on distribution of β-catenin in HNE-1 cells. And obvious nuclear export of β-catenin was observed in EtNBSe-PDT groups. As shown in Fig. 3C, the RFP-β-catenin shuttled from the nucleus into cytoplasm after administration indicating the inhibition of Wnt/β-catenin pathway. Significantly, β-catenin gradually exhibited an evenly cytoplasmic distribution, which represented the formation of β-catenin destruction complex. Meanwhile, western blotting was used to detect the expression of GSK3β which was the component of destruction complex. We found that the expression of GSK3β increased by nearly two times in EtNBSe-PDT groups compared with the control group. Together, these data indicated that EtNBSe-PDT could inhibit the activation of Wnt/β-catenin pathway in HNE-1 cells.

3.4. EtNBSe-PDT inhibited proliferation via suppressing the Wnt/β-catenin pathway in HNE-1 cells

To further elucidate the cytotoxic impact of EtNBSe-PDT, cells were collected at various time points post EtNBSe-PDT and the proliferation of HNE-1 cells was assessed by MTT assay. It was showed that EtNBSe-PDT significantly inhibited the proliferation of HNE-1 cells compared

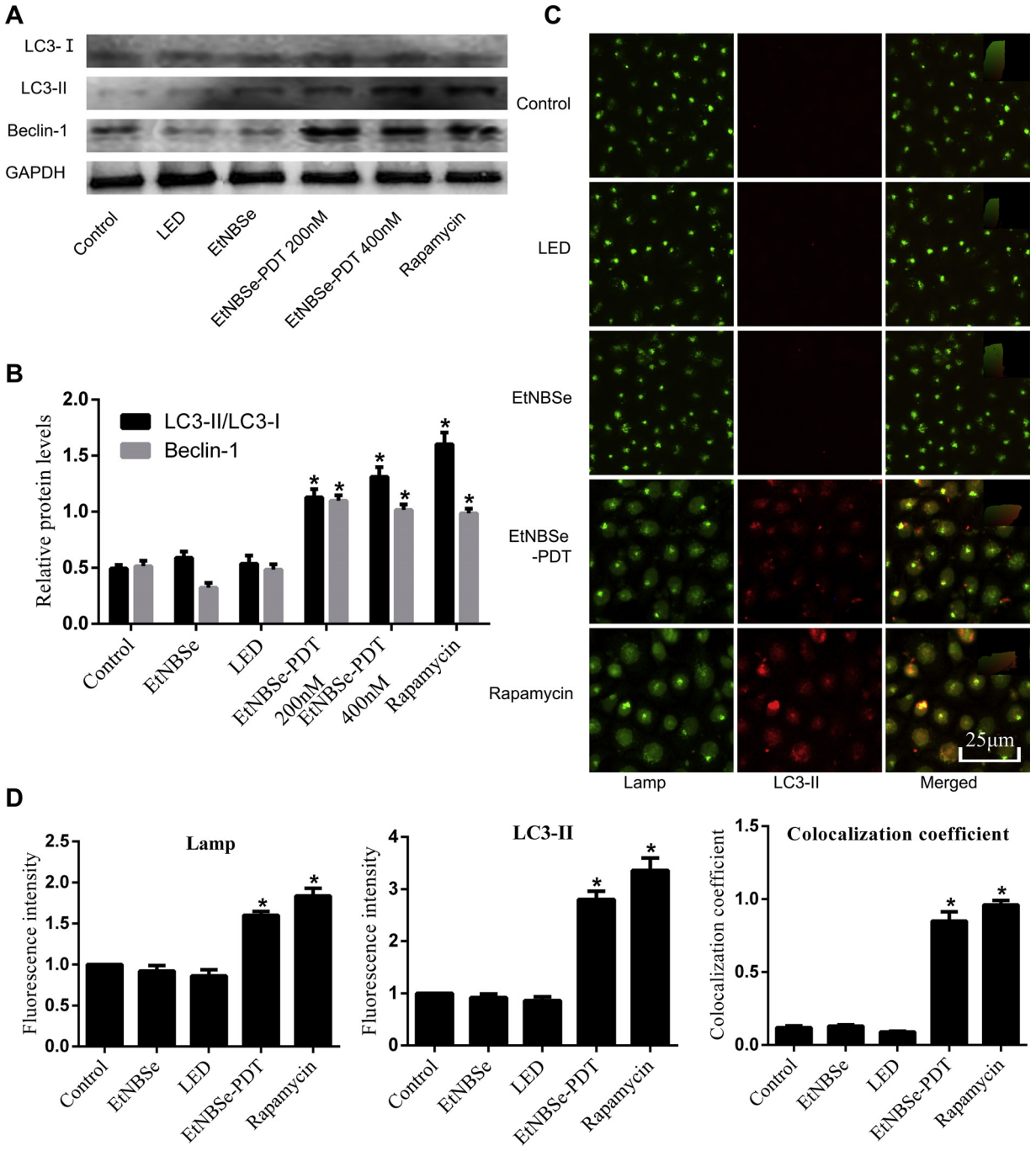

Fig. 3. EtNBSe-PDT induced autophagy in HNE-1 cells. HNE-1 cells were treated with 200 and 400 nmol/L concentrations of EtNBSe and then irradiated with light (635 nm, 2.8 J/cm^2). Some cells were treated with Rapamycin as a positive control. (A): The expression of the autophagy-related proteins LC3-I, LC3-II and Beclin-1 were detected by Western blotting. (B): Statistical analysis of the level of Beclin1 and the rate of LC3-II/LC3-I. The columns represented the mean ± SD of the three independent experiments. *P < 0.05 versus the control group. (C): The effect of EtNBSe-PDT on LC3-II and Lamp co-localization at 2 h after PDT. The scale bar represents 25 μm. (D): Quantitative analysis of relative fluorescence intensity of Lamp and LC3-II, and the co-localization coefficient between them. The columns represented the mean ± SD from three independent experiments. *P < 0.05 versus the control group.

with the control, but the Wnt/β-catenin activator, Wnt agonist partly rescued the suppression in cell proliferation induced by EtNBSe-PDT (Fig. 5A). As reported, the inhibition of proliferation maybe partially attributed to cell cycle arrest. [11] Subsequently, we investigated the cell cycle profile of HNE-1 cells using PI staining and flow cytometry. As shown in Fig. 5B, EtNBSe-PDT caused an obvious accumulation in G1 phase arrest (from 42.57 ± 2.62%–57.19 ± 3.87%) accompanied by a decreased cell population in G2/M phases (from 34.81 ± 2.35%–18.19 ± 1.08%) compared with the control group. The percentage of cell entering the G2 and M phases in the combination of EtNBSe-PDT and Wnt agonist group was 29.16 ± 2.17%, which was greatly more than that of EtNBSe-PDT alone. Meanwhile, data showed that the percentage of EtNBSe-PDT treated cells in the G1 phase was 57.19 ± 3.87%. This value considerably declined to 45.29 ± 3.66% in group co-treatment of EtNBSe-PDT and Wnt agonist. These data indicated that EtNBSe-PDT inhibits proliferation of HNE-1 cells by inducing G1 phase cell cycle arrest via suppressing Wnt/β-catenin.

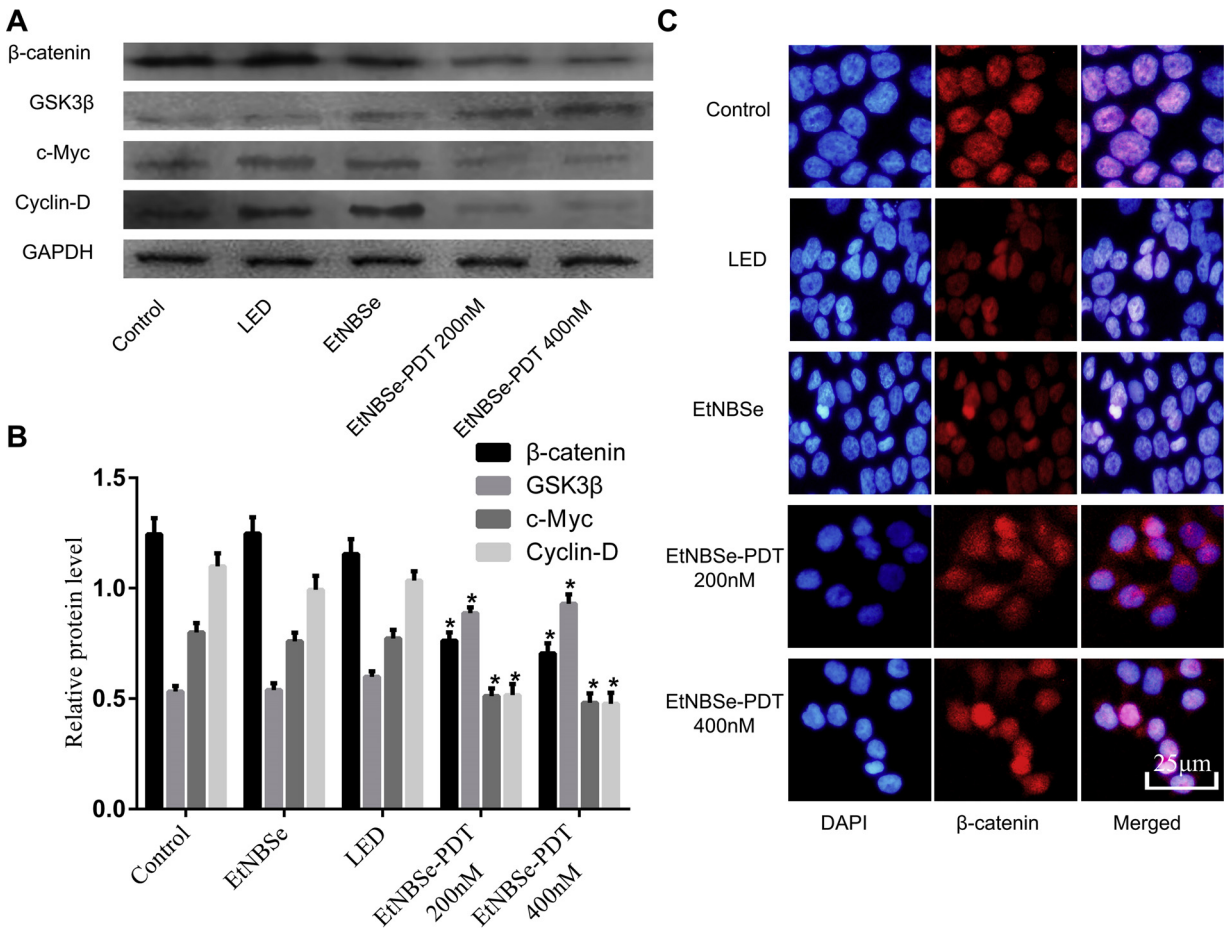

Fig. 4. EtNBSe-PDT suppressed the Wnt/β-catenin pathway in HNE-1 cells. HNE-1 cells were treated with 200 and 400 nmol/L concentrations of EtNBSe and then irradiated with light (635 nm, 2.8 J/cm^2). (A): The expression levels of the Wnt/β-catenin pathway related proteins, including β-catenin, GSK3β, c-Myc, and Cyclin-D, were detected by Western blotting. (B): Quantitative analysis of relative proteins levels. The columns represented the mean ± SD of the three independent experiments. *P < 0.05 versus the control group. (C): Co-localization of β-catenin and nucleus immunostaining in untreated cells or PDT-treated cells. Red fluorescence represents β-catenin immunostaining and blue fluorescence (DAPI) was used for nuclear counterstaining. The scale bar represents 25 μm.

3.5. Wnt/β-catenin pathway was involved in autophagy induced by EtNBSe-PDT in HNE-1 cells

To further confirm the role of cross-talking between autophagy and Wnt/β-catenin pathway in HNE-1 cells, we pretreated some cells with 1 μmol/L Wnt agonist which could activate the Wnt/β-catenin signaling pathway for two hours. Then we performed HNE-1 cells with EtNBSe-PDT (400 nmol/L, 2.8 J/cm^2) and assessed the level of autophagy. Meanwhile, 3-MA, an autophagy inhibitor was set as the negative control. As shown in Fig. 5A and B, the expression of Beclin-1 and the ratio of LC3-II/LC3-I were elevated by EtNBSe-PDT and suppressed by Wnt agonist compared with the control group. As expected, the increased expression of Beclin-1 and the ratio of LC3-II/LC3-I were obviously whittled down by the combination of EtNBSe-PDT and 3-MA compared with the individual role of EtNBSe-PDT (Fig. 6). Moreover, the promoting effect of EtNBSe-PDT on the level of autophagy was significantly counteracted by the cooperation of Wnt agonist. Thus these results above indicated that the inhibition of Wnt/β-catenin signaling pathway was involved in the EtNBSe-PDT induced autophagy (Fig. 6).

3.6. Inhibition of autophagy enhanced the cytotoxic effect of EtNBSe-PDT in HNE-1 cells

To further clarify the relative role of autophagy in HNE-1 cells escape from EtNBSe-PDT induced inhibitory cell viability, autophagy was inhibited by 3-MA and cell viability was measured using MTT. Meanwhile, the combination of PDT and rapamycin group was set as the positive control. As shown in Fig. 7, Similar results were observed when autophagy was suppressed by activating Wnt/β-catenin pathway using Wnt agonist. The MTT results showed that activation of Wnt/β-catenin pathway significantly enabled the enhancement of EtNBSe-PDT induced cell death and decreased the cell viability from 52.64 ± 3.54% to 35.74 ± 4.27%. Furthermore, treatment with the autophagy activator rapamycin decreased cytotoxicity in the HNE-1 cells treated with PDT compared with cells that did not receive rapamycin. However, solely using 3-MA, Wnt agonist or rapamycin did not appear to exert an obvious effect on the A-431 cell survival. Collectively, these data showed that induction of autophagy by EtNBSe-PDT protected HNE-1 cells from PDT-induced cell death, which may make HNE-1 cells resistant to EtNBSe-PDT.

4. Discussion

NPC is one of the most challenging malignancies arising from the epithelial cells lining the nasopharynx [12]. It accounts for over 80% of all head and neck cancers in China. The anatomical position of NPC is adjacent to important nerves and vessels, leading to difficulty in the use of surgery for treatment. Currently, radiotherapy, with or without chemotherapy, has become the primary treatment for NPC. But these therapies produce severe adverse effects and the overall 5 years survival in patients is still only 35%–55% after a standard treatment [13–15].

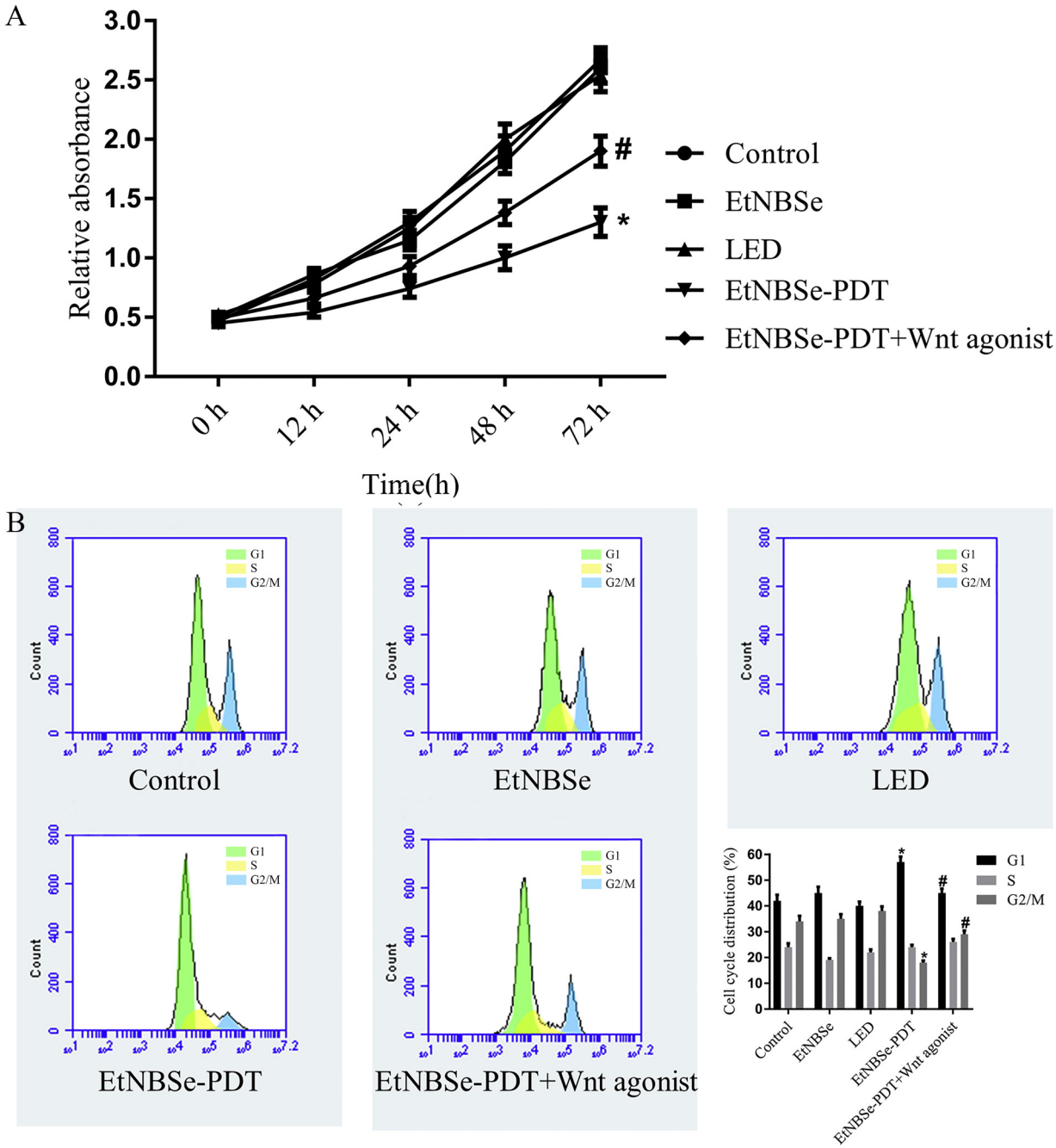

Fig. 5. EtNBSe-PDT inhibited proliferation via suppressing Wnt/β-catenin pathway in HNE-1 cells. Cells were pretreated with or without Wnt agonist (1 μmol/L) for 2 h, and then respectively followed with or without EtNBSe-PDT (400 nmol/L, 2.8 J/cm^2). (A): At 0, 12, 24, 48 and 72 h after irradiation, the cells proliferation was analyzed using MTT assay. (B): PI staining and flow cytometry were performed to estimate cell cycle phase distribution. Quantitative analyzed the count of cells in G1 phase, S phase and G2/M phase. *P $<$ 0.05, compared with the control group. #P $<$ 0.05, compared with the EtNBSe-PDT alone group.

Therefore, it is imperative to develop new antitumor modalities to reduce side effects and to reinforce the effectiveness of therapy in NPC.

PDT has emerged as a minimally invasive cancer treatment based on the creation of extreme stress in tumor cells through the excitation of photosensitizer by light [16,17]. EtNBSe is a novel synthetic bipolar photosensitizer having a heavier atom in place of the centrally located atom, which has the potential to possess much improved photoactivity [18]. Furthermore, our previous studies have shown that EtNBSe-PDT possesses anti-tumor effects on skin squamous cell carcinoma [10,19]. However, its related mechanisms in NPC remain largely unknown.

In this study, our results showed that EtNBSe-PDT significantly suppressed HNE-1 cell viability in a concentration- and light energy density-dependent manner. In contrast, treatment with EtNBSe or light alone showed no obvious inhibitory viability effect on HNE-1 cells. These results indicated that EtNBSe possessed low dark toxicity and high phototoxicity to HNE-1 cells, making it a promising photosensitizer in photodynamic therapy to NPC.

Next, we studied the effects of the combination of 400 nmol/L EtNBSe and a light dose of 2.8 J/cm^2 on HNE-1 cells. The results suggested that EtNBSe-PDT could inhibit cell proliferation, induce cell cycle arrest and autophagy. Concerning autophagy in cancer, this process can be a pro-death as well as a pro-survival pathway and it is largely dependent on the complicated autophagic regulatory machinery. Accordingly, accumulating studies have also suggested the

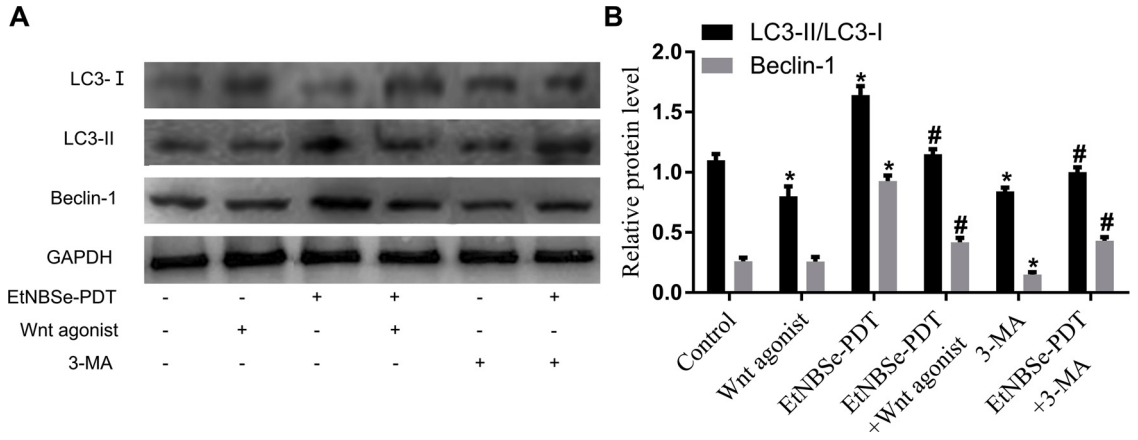

Fig. 6. Wnt/β-catenin pathway was involved in autophagy induced by EtNBSe-PDT in HNE-1 cells. (A): Cells were pretreated with Wnt agonist or 3-MA for 2 h, and then respectively followed with or without EtNBSe-PDT (400 nmol/L, 2.8 J/cm^2). The expression of the autophagy-related proteins LC3-I, LC3-II and Beclin-1 were detected by Western blotting. (B): Statistical analysis of the level of Beclin1 and the rate of LC3-II/LC3-I. Data was presented as mean ± SD from three independent experiments. *P < 0.05, compared with the control group. #P < 0.05, compared with the EtNBSe-PDT alone group.

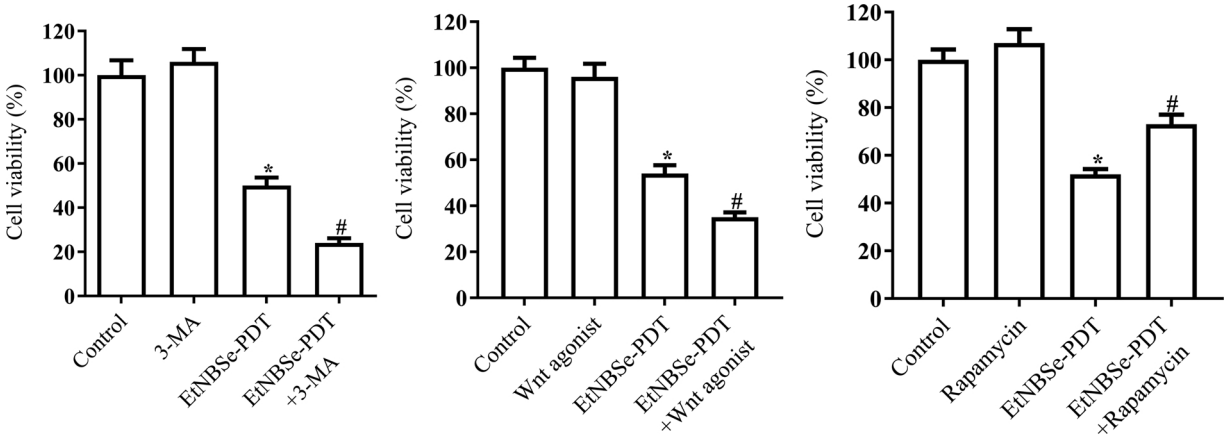

Fig. 7. Inhibition of autophagy enhanced the cytotoxic effect of EtNBSe-PDT in HNE-1 cells. The cells were pretreated with 3-MA, Wnt agonist and Rapamycin for 2 h respectively followed with or without EtNBSe-PDT (400 nmol/L, 2.8 J/cm^2). The effects of EtNBSe-PDT induced cell death were assessed by MTT. Data was presented as mean ± SD from three independent experiments. *P < 0.05, compared with the control group. #P < 0.05, compared with the EtNBSe-PDT alone group.

dual effect of autophagy on PDT [20–22]. Ji et al. found that the death of PC12 and CL1-0 cells induced by 5-aminolevulinic acid mediated PDT could be inhibited by an autophagy inhibitor, indicating that the cell death was autophagic in nature [23]. In contrast, Michael et al. suggested that autophagy might promote cell adaptation and delayed cell death against PDT at low dose [24]. In our experiments, inhibition of autophagy enhanced the cytotoxic effect of EtNBSe-PDT, demonstrating that autophagy contribute to the resistance of HNE-1 cells to EtNBSe-PDT. The detailed mechanisms mediating autophagy were complicated, and may differed with photosensitizer, cellular genotype, and target molecule [25,26]. Further research on the mechanisms underlying autophagy and cytotoxic effect of PDT, will be crucial to provide additional efficient targets for therapeutic intervention in PDT.

Accumulating evidence has demonstrated that the Wnt/β-catenin signaling pathway plays a decisive role in regulating the cellular fate and is overactivated in several human cancers, including NPC [27]. The Wnt/β-catenin pathway is canonical pathway of Wnt families, which is the pleiotropic protein, β-catenin, dependent. It results in the stabilization of a cytoplasmic pool of β-catenin that would otherwise be marked for proteasomal-mediated degradation by a destruction complex, composed of GSK-3β. Upon binding to receptors of the Frizzled family and the co-receptors, extracellular Wnt stimulation transmits a signal to the intracellular adaptor, which inhibits the destruction complex and allows nuclear translocation of stabilized β-catenin to activate gene transcription [28]. In this study, the notable decreased expression of β-catenin and obvious cytoplasmic translocation of β-catenin were observed after the EtNBSe-PDT administration. Consistently, the expression levels of c-Myc and Cyclin-D, the proteins synthetized by target genes of β-catenin, were suppressed. All these results suggested that the Wnt/β-catenin pathway was inhibited by EtNBSe-PDT in HNE-1 cells. However, the role of the Wnt/β-catenin pathway in autophagy is still unclear. Yun et al. activated the Wnt/β-catenin signaling pathway by a tumor suppressor, and found promoted autophagy in human NSCLC cells [29]. Contrary to this finding, here we provided evidence that the pre-treatment of Wnt/β-catenin pathway activator, Wnt Agonist, significantly decreased the autophagy induced by EtNBSe-PDT, indicating that Wnt/β-catenin pathway played a negative role in the induction of autophagy of HNE-1 cells. Similarly, Li et al. also suggested that autophagy could be induced by the triptolide via repressing Wnt/β-catenin signaling in osteosarcoma treatment [30]. Indeed, different tumor mutational status, intracellular microenvironment and complicated crosstalk between signaling pathway may account for these discrepancies. Furthermore, we used pharmacological inhibitor and activating Wnt/β-catenin signaling pathway to down-regulate the level of autophagy under our experimental conditions. It was found that both of the two methods could significantly enhance the sensitivity of HNE-1 cells towards EtNBSe-PDT by a cytotoxicity and proliferation assay.

In conclusion, we demonstrated that EtNBSe-PDT significantly inhibited the viability of HNE-1 cells. In addition, EtNBSe-PDT could

suppress the Wnt/β-catenin signaling pathway, which further promoted autophagy and inhibited proliferation in HNE-1 cells. In particular, inhibition of autophagy enhanced the cytotoxicity of EtNBSe-PDT, which suggests that a combination of EtNBSe-PDT and autophagy inhibitors may be a promising strategy for the treatment of human NPC cell lines.

Conflict of interest

The authors have declared that no conflicts of interest exist.

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