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Ethyl β-Carboline-3-Carboxylate Increases Cervical Cancer Cell Apoptosis Through ROS-p38 MAPK Signaling Pathway

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Abstract. *Background/Aim: Ethyl β-carboline-3-carboxylate (β-CCE) is one of the effective ingredients of Picrasma quassioides (P. quassioides). As a β-carboline alkaloid, it can antagonize the pharmacological effects of benzodiazepines by regulating neurotransmitter secretion through receptors, thus affecting anxiety and physiology. However, its efficacy in cancer treatment is still unclear. Materials and Methods: We explored the effect of b-CCE on SiHa cells using MTT assay, western blot, flow cytometry, LDH release, T-AOC, SOD, and MDA assays. Results: We investigated the cytotoxicity of β-CCE in SiHa cells and verified that β-CCE could induce cell apoptosis in a time- and concentration-dependent manner. In this process, treatment with β-CCE significantly increased the levels of cytoplasmic and mitochondrial reactive oxygen species (ROS), which disturb the oxidation homeostasis by regulating the total antioxidant capacity (T-AOC), superoxide dismutase (SOD) activity, and malondialdehyde (MDA) production. Notably, the addition of N-acetylcysteine (NAC) (ROS scavenger) effectively alleviated β-CCE-induced apoptosis in SiHa cells. In addition, β-CCE might activate the p38/MAPK signaling pathway, as the pre-treatment with SB203580 (p38 inhibitor) significantly reduced β-CCE-induced apoptosis in SiHa cells. Conclusion: β-CCE has an anti-tumor activity. It activates the p38/MAPK signaling pathway by increasing intracellular ROS levels, which subsequently induce SiHa cell apoptosis. Our results provide a novel therapeutic target for treatment of cervical cancer.*

Cervical cancer is one of the most common female cancers with a high mortality rate worldwide (1). The main causes of cervical cancer include human papillomavirus (HPV) infection, smoking, and prolonged use of oral contraceptives. Cervical cancer has no obvious symptoms, however, as the disease progresses, abnormal vaginal bleeding (2) and vaginal discharge (3), pelvic pain, or pain during sexual intercourse are observed (4). Although cervical cancer can be effectively prevented through vaccination, it is still a growing burden for many developing and developed nations lacking effective screening and treatment methods. Hence, the development of inexpensive and effective drugs against cervical cancer is extremely important.

In recent years, researchers have gradually realized the benefits of using active ingredients extracted from natural herbal medicines, which have very few side effects and are cost-effective. Ethyl β-carboline-3-carboxylate (β-CCE) is a β-carboline alkaloid extracted from *P. quassioides*. Studies have shown that β-carboline alkaloids have many therapeutic effects; for instance, they are used to treat mice pulmonary fibrosis (5), depression (6), bacterial infections (7), inflammatory diseases (8), and cancer (9). The anti-cancer effect of β-carboline alkaloids has been widely studied for the treatment of lung (10), liver (11), gastric (12), colon (13), and ovarian cancer (14). β-CCE is one of the most effective β-carboline alkaloids. However, , the research is usually focused on using β-CCE to stimulate the spontaneous

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discharge in the hippocampus of rats, lower the threshold for inducing convulsions, and reduce the incubation period of convulsions (15-18). However, the anti-cancer effects of β-CCE are less studied. Reactive oxygen species (ROS) regulate cell growth, differentiation, and development under various physiological and pathological conditions. The low concentration of ROS promotes cell proliferation, differentiation, and other physiological activities (19). However, ROS accumulation beyond the normal threshold leads to intracellular oxidation imbalance, resulting in protein and lipid damage, genetic instability, and carcinogenesis (20-22). ROS also play an important role in tumor cells. They promote abnormal cancer cell growth and differentiation, accelerate the metastasis and angiogenesis of cancer cells, and prevent apoptosis (23, 24). The increasing levels of intracellular ROS disturb the antioxidant defense mechanisms that depend on superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPxs), and peroxidase (Prxs) to prevent the formation of excessive ROS and maintain the oxidation-reduction balance by eliminating ROS (25). Excessive ROS inhibit the antioxidant mechanism in cancer cells to promote the development of the disease. However, ROS overproduction, ineffective scavenging of ROS, and lack of antioxidants cause the accumulation of ROS, which induces oxidative stress and damages various organelles in the cell. Thus, they are crucial for the maintenance of cell homeostasis and the normal function of various organelles. At the same time, the existence of a variety of antioxidant enzymes in cells is involved in the regulation of redox homeostasis. It prevents mitochondria-dependent apoptosis and endoplasmic reticulum stress-related signaling pathways that promote cell apoptosis (26, 27). Therefore, regulating the level of ROS production is an effective cancer treatment method. In tumor cells, the mitogen-activated protein kinase (MAPK) is one of the primary oxidative stress-sensitive pathways, which regulates gene expression, survival, apoptosis, and differentiation. Phosphorylation levels of ERK, JNK, and P38 indicate the regulatory effect of intracellular oxidative stress on cells. In this study, we investigated the effects of β-CCE extracted from *P. quassioides* on the apoptosis mechanism in SiHa cervical cancer cells. It is important to develop new drugs for treating cervical cancer and provide a theoretical basis for the development of more effective cervical cancer therapeutics. Simultaneously supplement therapeutic strategies with β-CCE could be effective.

Materials and Methods

Cell culture. Cervical cancer cells (SiHa) and normal liver cells (QSG-7701) were supplied by Stem Cell Therapy and Regenerative Biology Laboratory and cultured in Dulbecco's modified eagle medium (DMEM, Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 1% penicillin (100 U/ml), and streptomycin (100 mg/ml) (P/S) (Solarbio life sciences, Beijing, China). The cells were routinely maintained at 37˚C and 5% CO_2.

MTT assay. The cell viability was detected using the MTT assay. SiHa cells and QSG-7701 cells were seeded at a density of 4,000 cells/well in a 96-well plate (NEST Biotechnology, Wuxi, Jiangsu, PR China) cultured at 37˚C with 5% CO_2 for 12 h. Then the cells were treated with different concentrations (0, 20, 40, 60 μg/ml) of β-CCE for 24 h and incubated with 10μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO, USA) solution for 4 h. Thereafter, the surface fluids were removed and 100 μl DMSO was added to each well at 37˚ and 5% CO_2 for 15 min to solubilize the MTT formazan crystals. Eventually, the absorbance value was measured at the wavelength of 570 nm. All experiments were performed in triplicates.

Detection of intracellular reactive oxygen species. The production of ROS was detected using flow cytometry. SiHa cells were cultured in 6-well plates at 15×10^5 cells/well for 12 h and pre-treated with NAC (reactive oxygen scavenger) for 30 min. Afterward, the cells were treated with β-CCE (40 μg/ml) for 24 h. Subsequently, the cells were recovered in 1.5 ml microcentrifuge tubes and stained with 20 μm DCFH-DA (Solarbio life sciences, Beijing, PR China) by incubating in the dark for 20 min at 37˚C. The samples were analyzed using flow cytometry (FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA). The results were analyzed using the WinMDI (Version 2. 9, BD Biosciences) software.

Detection of mitochondrial ROS. MitoSOX Red (Thermo Fisher Scientific, Waltham, MA, USA) was used to detect the mitochondrial ROS levels using flow cytometry. MitoSOX Red, a derivative of dihydroethidium, can pass through the cell membrane into the mitochondria and emit red fluorescence after being oxidized by superoxide. The cells were seeded at a density of 15×10^5 cells/well in a 6-well plate (NEST Biotechnology) and cultured for 12 h at 37˚C and 5% CO_2. The SiHa cells were pre-treated with NAC for 30 min before treating with various concentrations of β-CCE for 24 h. Next, the cells were harvested, stained with MitoSOX according to the manufacturer's instructions, and analyzed using flow cytometry (FACSCalibur). The results were analyzed using the WinMDI (Version 2. 9) software.

Determination of apoptosis. Apoptotic cells were identified using Annexin-V staining and flow cytometry analysis. SiHa cells subjected to different treatments, and then the different groups of cells were collected in 1.5 ml microcentrifuge tubes. Subsequently, each sample was washed with PBS (Solarbio life sciences, Beijing, China) and resuspended in Annexin-V Binding containing Annexin V-FITC and Propidium Iodide (Solarbio life sciences) for 20 min according to the manufacturer's protocol and analyzed using flow cytometry (FACSCalibur). The results were analyzed using the WinMDI (Version 2.9) software.

Total antioxidant capacity (T-AOC) assay. The total antioxidant capacity (T-AOC) of SiHa cells lysates was measured using the T-AOC Assay Kit (Solarbio life sciences). SiHa cells were seeded in

a 10 cm plate at a density of 5×10^6 cells/well and cultured for 12 h. Different groups of cells were stimulated with β-CCE at different concentrations (0, 20, 40 μg/ml) for 24 h and treated with NAC (10 mM) for 30 min and compared with the cells treated with β-CCE, which were used as control. Subsequently, each group of cells was collected in a beaker with 1 ml extraction solution and T-AOC of each group of cells was detected according to the manufacturer's instructions. Finally, the supernatant was recovered, and the absorbance was read at 593 nm and 600 nm using a microplate reader. The experiments were performed in triplicates for each group.

Malondialdehyde (MDA) assay. The Malondialdehyde (MDA) content in cells was measured using Micro Malondialdehyde (MDA) Assay Kit (Solarbio life sciences). SiHa cells were cultured in a 10 cm cell culture plate at a density of 5×10^6 cells/well for 12 h, and then pre-treated with NAC. Subsequently, cells were treated with various concentrations of β-CCE (0, 20, 40 μg/ml) for 24 h. The cells were harvested in a breaker with 1 ml extraction solution, then centrifuged at 8,000×g at 4˚C for 10 min. Finally, the supernatant was recovered, and absorbance was read at 532 nm and 600 nm using a microplate reader. The content of MDA was calculated according to the manufacturer's instructions. The experiments were performed in triplicates for each group.

Superoxide dismutase (SOD) assay. The intracellular levels of superoxide dismutase (SOD) were measured using the SOD Assay kit (Solarbio life sciences). The cells were plated in 10 cm culture plates at a density of 5×10^6 cells/well and incubated for 12 h. Then, the cells were subjected to different treatments, including β-CCE concentration (0, 20, 40 μg/ml) treatment group and NAC pretreatment control group. SOD activity of all groups of cells was measured according to the guidelines of the manufacturer. The experiments were performed in triplicates for each group.

Lactate dehydrogenase (LDH) assay. The content of LDH in the media determines the cell membrane integrity and was detected using the LDH activity detection kit (Solarbio life sciences). SiHa cells were seeded in a 10 cm culture plate at a density of 5×10^6 cells/well for 12 h. Then, the cells were subjected to various treatments, according to experimental groups, for 24 h. The cell supernatants were analyzed for LDH activity using the detection kit according to the manufacturer's instructions. The experiment was performed in triplicates for each group.

Western blot assay. Briefly, the cells were subjected to various treatments, according to experimental groups, and were collected in 1.5 ml microcentrifuge tubes. Cells were lysed on ice and centrifuged, and the supernatants were collected. Then, the total protein concentration was determined. Samples were loaded and separated using 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were blotted with primary antibodies against caspase9 (Cas9; Cell Signaling Technology, Beverly, MA, USA, 9505), caspase7 (Cas7; Cell Signaling Technology, 9491), Bax (Abcam Cambridge, MA, USA, 32503), Bcl-xL (Santa Cruz Biotechnology, Dallas, TX, USA, sc-8392), ERK (Santa Cruz Biotechnology, sc-135900), p-ERK (Santa Cruz Biotechnology, sc-7383), p38 (AbFrontier, Seoul, Republic of Korea, LF-MA0126), p-p38 (Santa Cruz Biotechnology, sc-7973), JNK (Santa Cruz Biotechnology, sc-7345), p-JNK (Santa Cruz Biotechnology, sc-6254), α-tubulin (Abcam, ab7291), and β-actin (Abcam, ab7291) (dilution, 1:5,000) at 4˚C overnight. The membranes were washed five times with tris buffered saline containing Tween-20 (TBST) [10 mM Tris HCl (pH 7.5), 150 mM NaCl, and 0.2% Tween-20] and were subsequently incubated with horseradish peroxidase conjugated goat anti-rabbit IgG or anti-mouse IgG for 1 h at room temperature (RT). After removing excess antibodies by washing with TBST, specific protein bands were detected, using a chemiluminescence detection system (GE Healthcare Life Sciences, Chalfont, UK) according to the manufacturer's protocol.

Statistical analysis. Data are expressed as the mean±SD. All data were analyzed for statistical significance using two-way ANOVA or Student's *t*-test, by using SPSS. $p<0.05$ was considered significant.

Results

Ethyl β-carboline-3-carboxylate reduces the viability of SiHa cervical cancer cells by inducing apoptosis. The cytotoxic effect of β-CCE on SiHa cells was explored. Figure 1A shows the molecular structure of β-CCE. Cell viability was analyzed using the MTT assay following treatment of SiHa cervical cancer cells and QSG-7701 normal liver cancer cells with different concentrations of β-CCE (0, 20, 40, 60 μg/ml) for 24 h. As shown in Figure 1B, the viability of SiHa cells was significantly reduced, and weak cytotoxicity was observed in the QSG-7701 cells (Figure 1C). The IC_{50} value of β-CCE in SiHa cells was found to be 33.06 μg/ml. In order to investigate the apoptotic effect of β-CCE on SiHa cells, the cells were treated with β-CCE at a concentration of 40 μg/ml for 0, 1, 3, 6, 12, 24 h, and the expression of apoptosis related proteins was analyzed using western blotting. The results showed that the expression of pro-apoptotic protein Bax, cleaved-Cas 9 (c-Cas9), and 7 proteins was significantly increased, and the expression of anti-apoptotic Bcl-xL protein was significantly reduced in a time-dependent manner (Figure 1D). Figure 1E, F, and G shows the quantitative analysis of data shown in Figure 1D which were analyzed using the SPSS software. These results indicate that β-CCE induced apoptosis in SiHa cells in a concentration- and time-dependent manner.

Ethyl β-carboline-3-carboxylate induces ROS production and decreases the antioxidant capacity in SiHa cells. To investigate whether the β-CCE-induced apoptosis in SiHa cells was related to the intracellular ROS levels, the SiHa cells were pre-treated with NAC (ROS scavenger) for 30 min and then treated with 40 μg/ml of β-CCE for 24 h. The intracellular ROS production was examined using flow cytometry and DCFH-DA staining. The results showed that β-CCE treatment causes ROS accumulation in SiHa cells, and NAC reduces the levels of ROS increased by the β-CCE treatment in SiHa cells (Figure 2A). At the same time, SiHa cells were treated with various concentrations of β-CCE (0,

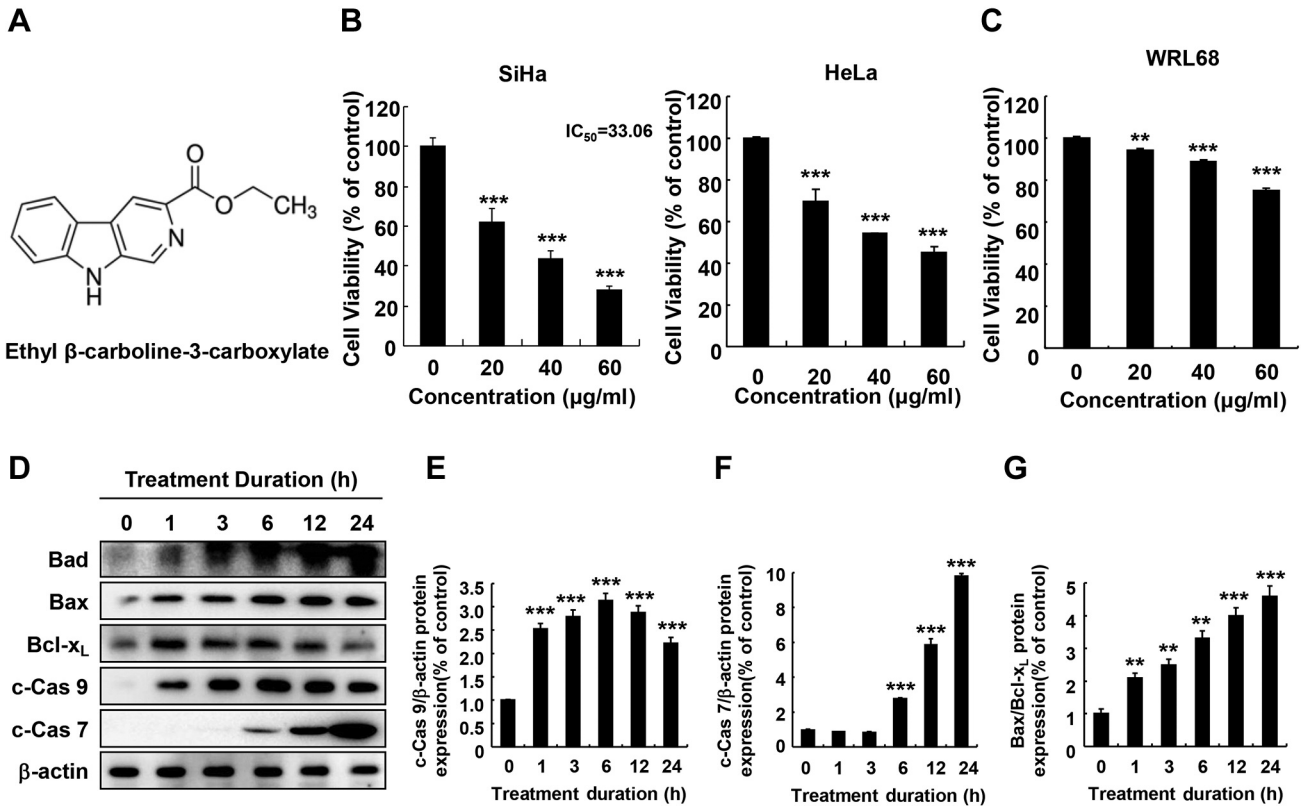

Figure 1. *Effect of β-CCE on cell viability and apoptosis in SiHa cells. (A) The chemical structure of ethyl β-carboline-3-carboxylate (β-CCE). (B) SiHa and Hela cells were treated with different concentrations of β-CCE (0, 20, 40, 60 μg/ml) for 24 h. Cell viability was measured using the 3-(4,5-dimethyldiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Each value represents the mean (±SEM) from at least three independent experiments (***p<0.001). (C). WRL68 cells were treated with different concentrations of β-CCE (0, 20, 40, 60 μg/ml) for 24 h. Cell viability was measured using the MTT assay. Each value represents the mean (±SEM) from at least three independent experiments (**p<0.01, ***p<0.001) (C). The protein levels of c-Cas7, c-Cas9, Bad, Bax, and Bcl-xL were detected using western blotting analysis in SiHa cells after treatment with 40 μg/ml β-CCE for different time intervals (0, 1, 3, 6, 12, and 24 h) and the related protein expression levels are represented as the mean±SD. Figure 1E, F, and G show the quantitative analysis of data in Figure 1D (**p<0.01, ***p<0.001).*

20, 40 μg/ml), and the NAC pre-treatment group was incubated with 40 μg/ml β-CCE for 24 h. We also examined the production of intracellular ROS and mitochondrial ROS by flow cytometry using dihydroethidium (DHE) and MitoSOX staining, respectively, to clarify the cause of the β-CCE-induced apoptosis in SiHa cells. The results indicated that intracellular ROS and mitochondria ROS were significantly increased, while ROS levels were reduced in the NAC pre-treatment groups (Figure 2B and C). Antioxidant ability plays an important role in cells, and ROS accumulation can cause oxidative stress, reducing the antioxidant capacity. We next examined the total-antioxidant capacity, SOD activity, and MDA content in SiHa cells. After the cells were pre-treated with NAC for 30 min, they were treated with β-CCE (0, 20, 40 μg/ml) for 24 h. The results showed that the total antioxidant capacity and SOD activity of β-CCE-treated cells was significantly reduced in a concentration-dependent manner, while NAC significantly increased the intracellular total-antioxidant capacity and SOD activity in SiHa cells. Simultaneously, the MDA content in SiHa cells was significantly increased after β-CCE treatment (Figure 2D and E). However, compared with the β-CCE treatment group, the MDA content of the NAC pre-treatment group was significantly reduced (Figure 2F). This result indicated that β-CCE could induce oxidative stress in SiHa cells.

Ethyl β-carboline-3-carboxylate induces cell apoptosis through mitochondrial dysfunction, and NAC could alleviate this damage. Mitochondria-mediated intrinsic apoptotic pathway occurs in response to various stimuli, including oxidative stress, and ROS play an important role in redox homeostasis. To investigate the damage caused by β-CCE to the mitochondria during apoptosis, SiHa cells were pre-treated with NAC and then treated with 0, 20, and 40 μg/ml β-CCE for 24 h. Finally, the LDH release from the cells was measured. The results showed that the release of LDH was

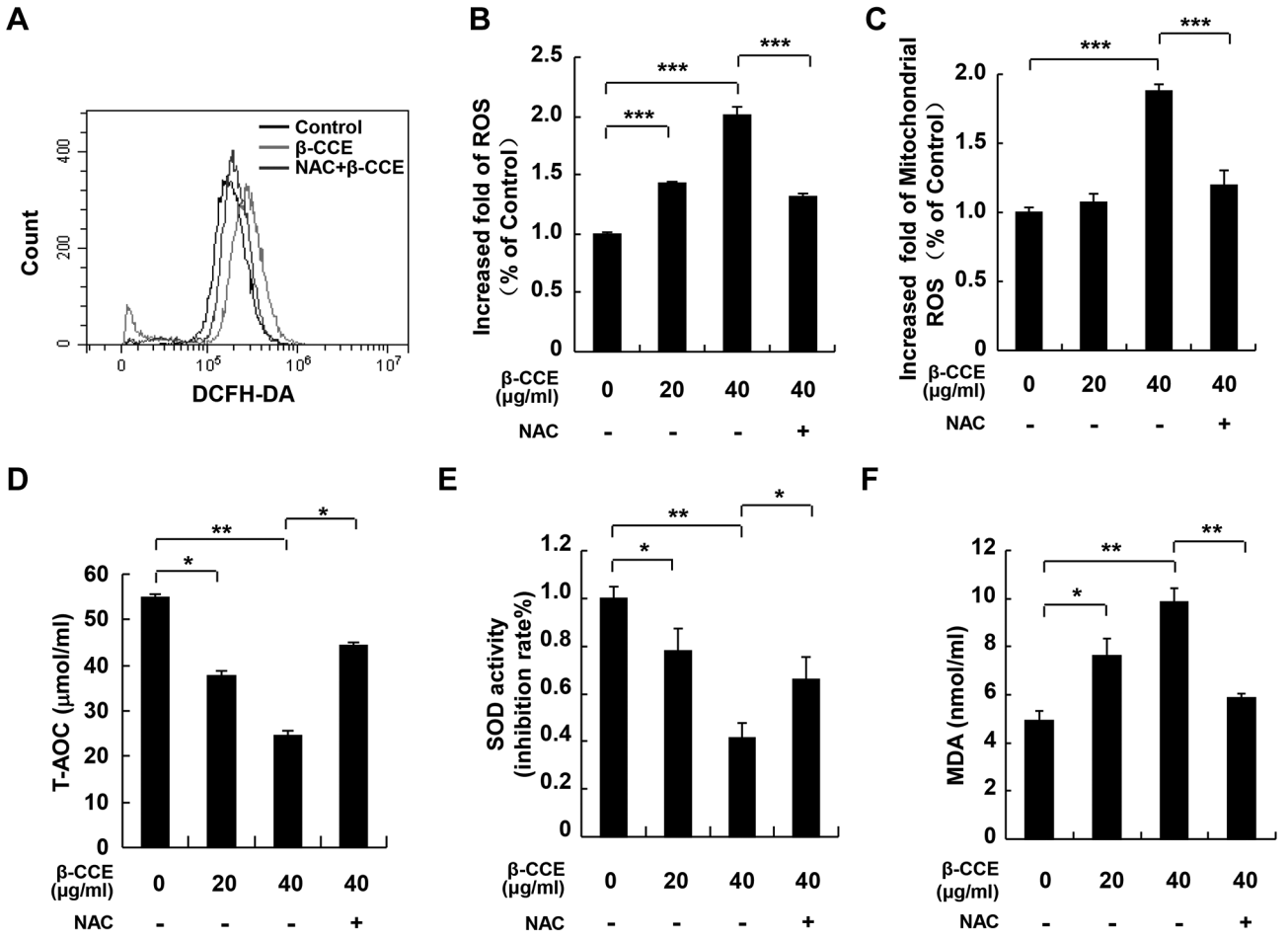

Figure 2. *β-CCE-induced reactive oxygen species (ROS) accumulation and antioxidant capacity changes in SiHa cells. SiHa cells were pre-treated with NAC for 30 min, and then treated with different concentrations of β-CCE (20, 40 μg/ml) for 24 h. (A). The levels of intracellular ROS in SiHa cells were analyzed using flow cytometry and staining with DCFH-DA (black represents the control, red represents the β-CCE treatment group and blue represents the NAC pre-treatment group). (B) Quantitative analysis of data in Figure 2A. Data are presented as mean±SD (***p<0.001). (C) The levels of mitochondrial ROS in SiHa cells were analyzed using flow cytometry stained with MitoSOX (red). Data are represented as the mean±SEM from at least three independent experiments (***p<0.001). (D) Cell Total Antioxidant Capacity (T-AOC) was detected using the T-AOC assay kit. Data are represented as the mean±SEM from at least three independent experiments (*p<0.05, **p<0.01). (E) Cell superoxide dismutase (SOD) activity was detected using the SOD assay kit. Data are represented as the mean±SEM from at least three independent experiments (*p<0.05, **p<0.01). (F) The content of MDA in SiHa cells was detected using the Malondialdehyde (MDA) assay kit. Data are represented as the mean±SEM from at least three independent experiments (*p<0.05, **p<0.01).*

significantly increased after treatment of cells with β-CCE. However, NAC could significantly reduce the release of LDH (Figure 3A). To investigate whether the β-CCE-induced release of LDH was related to the apoptosis in SiHa cells, we set up similar experimental groups and detected apoptosis using flow cytometry. As shown in Figure 3C, we found that the apoptosis in β-CCE-treated cells increased in a concentration-dependent manner and NAC reduced the apoptosis in SiHa cells. Based on the effect of β-CCE on apoptosis, we examined the expression levels of pro-apoptotic protein Bax, c-Cas 9, and 7 proteins and the expression of anti-apoptotic Bcl-xL using western blotting. The results showed that that the expression levels of Bax, c-Cas 9, and 7 proteins were significantly increased, whereas that of Bcl-xL was significantly reduced in the β-CCE treatment group. Notably, the expression levels of Bax, c-Cas 9 and 7 proteins were significantly decreased, and those of Bcl-xL were significantly increased in the NAC pre-treatment group compared to the β-CCE treatment group (Figure 3C). Figure 3D, E, and F shows the quantitative analysis data of the data presented in Figure 3C.

Effect of Ethyl β-carboline-3-carboxylate treatment on MAPK signaling in SiHa cervical cancer cells. MAPK signaling

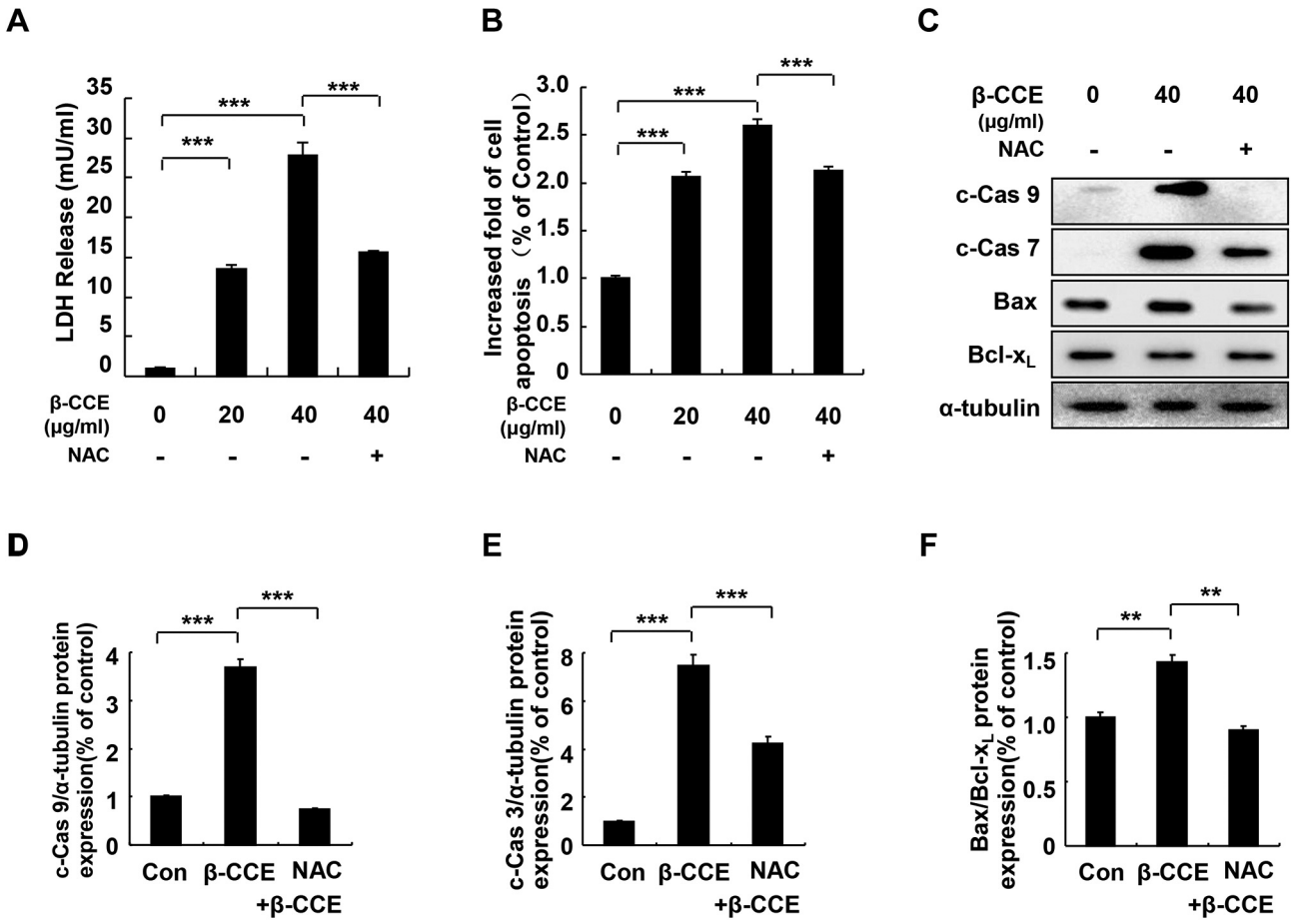

Figure 3. *Effect of β-CCE on mitochondrial damage and apoptosis in SiHa cells. SiHa cells were pre-treated with NAC for 30 min and then treated with different concentrations of β-CCE (20, 40 μg/ml) for 24 h. (A) Intracellular mitochondrial damage was detected using the Lactate dehydrogenase (LDH) assay kit. Data are represented as the mean±SEM from at least three independent experiments (***p<0.001). (B) The apoptosis in SiHa cells was analyzed using flow cytometry and staining with Annexin V/PI. Data are presented as the mean±SEM from at least three independent experiments (***p<0.01). (C) Expression of related apoptotic proteins c-Cas7, c-Cas9, Bax, Bcl-xL were detected using western blotting analysis in SiHa cells. Figure 3D, E, and F show the quantitative analysis of data in Figure 3D (**p<0.01, ***p<0.001).*

pathway regulates physiological functions, such as cell proliferation and apoptosis. In order to detect the effect of β-CCE on the MAPK signaling pathway, SiHa cells were treated with β-CCE (40 μg/ml) for different time intervals (0, 1, 3, 6, 12, 24 h), then phosphorylation levels of ERK, JNK, and p38 proteins were detected using western blotting. The results showed that the phosphorylation levels of ERK proteins were significantly decreased, and those of JNK and p38 proteins were significantly increased in SiHa cells after β-CCE treatment in a time-dependent manner (Figure 4A). To explore the relationship between the activation of MAPK signaling pathway and cell apoptosis, we pre-treated SiHa cells with ERK, JNK, and p38 proteins phosphorylation inhibitors for 24 h prior to β-CCE treatment (40 μg/ml), then apoptosis was detected using Flow Cytometry. The results showed that the addition of SB203580 (p-p38 inhibitor) scientifically reduced apoptosis in SiHa cells (Figure 4B). Figure 4C shows the quantitative analysis of the data presented in Figure 4B. It has been known that MAPKs participate in the regulation of many cellular processes, including cell growth, survival, and apoptosis. At the same time, our result showed that β-CCE-induced apoptosis of SiHa cells stimulated changes in the MAPK signaling pathway. In order to further understand the influence of p38 on apoptosis, the SiHa cells were pretreated with SB203580 (p38 inhibitors) for 30 min before the β-CCE treatment. The changes in expression of apoptosis-related proteins were detected using western blotting. As shown in the Figure 4D, the expression of c-Cas7 and Bax proteins was significantly reduced and that of Bcl-xL was significantly increased (Figure 4D). To further explore whether ROS cause the activation of the MAPK signaling, SiHa cells were pre-treated

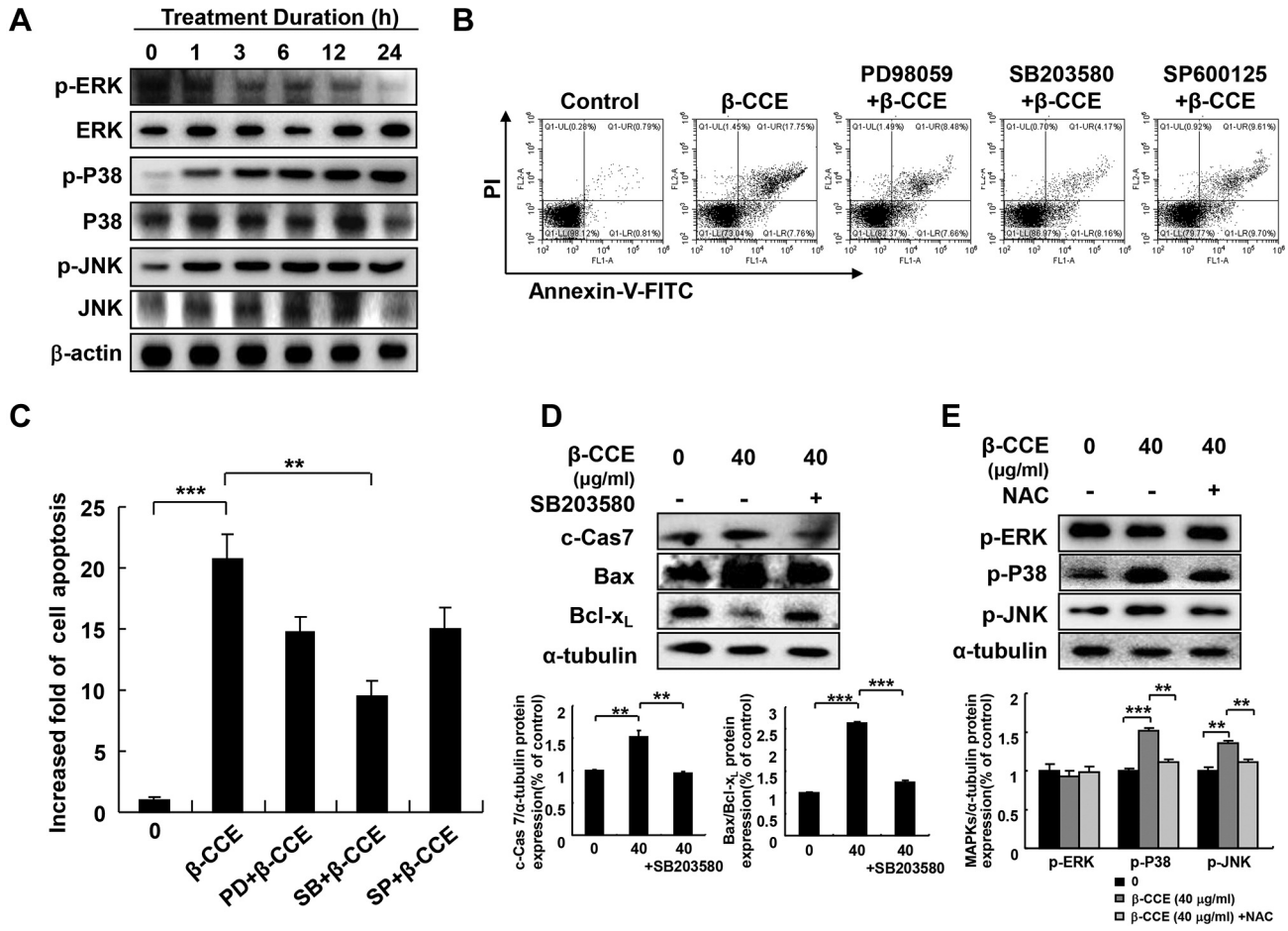

Figure 4. *Effect of β-CCE on MAPK signaling pathways in SiHa cells. (A) Protein expression level of p-ERK, ERK, p-p38, p38, p-JNK, JNK were detected by western blotting analysis in SiHa cells after treatment with 40 μg/ml β-CCE for different time intervals (0, 1, 3, 6, 12, and 24 h). (B) The cellular apoptosis was measured using Annexin-V/PI staining observed with flow cytometry after the treatment with β-CCE and MAPK inhibitors (SB203580 for p38, SP600125 for JNK, and PD98059 for ERK). (C) Quantitative analysis of data in Figure C. (D) SiHa cells were pretreated with SB203580 (p38/MAPK inhibitors) for 30 min, and then treated with β-CCE (40 μg/ml) for 24 h. Protein expression levels of c-caspase7, Bax, and Bcl-xL were detected using western blotting. (E) SiHa cells were pretreated with NAC for 30 min, then, treated with β-CCE (40 μg/ml) for 24 h. Protein expression levels of p-ERK, p-P38, and p-JNK were detected using western blotting.*

with NAC for 30 min and β-CCE (40 μg/ml) for 24 h, then the phosphorylation levels of ERK, JNK, and p38 proteins were detected using western blotting. The results showed that the removal of ROS reduced the P38 phosphorylation levels (Figure 4E), indicating that β-CEE can increase oxidative stress, activate the MAPK signaling pathway and induce apoptosis in SiHa cells.

Discussion

As an effective biological ingredient, β-CCE is widely present in *P. quassioides*, human urine, and the brain (28, 29). The beneficial effects of β-CCE on panic disorders, anxiety, and other social behaviors were recognized through neuro-psychopharmacological studies in the 1880s (30). β-CCE as a benzodiazepine-derived antagonist is often compared with nordiazepam, which has a certain regulatory effect on human neurological diseases (31). However, the effects of β-CCE on cancer are not yet known. We found that the β-CCE could damage the mitochondria and increase ROS levels to induce apoptosis in SiHa cells. In addition, we also revealed that apoptosis in SiHa cells is regulated by ROS-p38/MAPK signaling. Therefore, this study primarily evaluated the toxicity of β-CCE on cervical cancer SiHa cells.

ROS as second messengers can affect proteins, lipids, and various physiological and pathological functions in cells (32, 33). In this study, we found that β-CCE can cause cell apoptosis (Figure 1) and explored the mechanism of apoptosis. The research showed that apoptosis was caused by β-CCE-induced ROS accumulation in SiHa cells.

Mitochondria are energy producers and the primary source of ROS in cells (34). However, excessive production of ROS can induce DNA, lipid, and protein damage and changes in mitochondrial membrane permeability, resulting in mitochondrial dysfunction (Figure 2A-C). To maintain cell homeostasis, diverse antioxidant enzymes in the mitochondria regulate the redox balance (35, 36). By detecting the content of superoxide dismutase (SOD), oxide reductase, total antioxidant content, and MDA levels, we showed that β-CCE can significantly reduce the intracellular levels of SOD and increase cellular MDA production (Figure 2E and F).

During this process, the BH3-only proteins (BAX and BAK) are activated and inserted into the outer mitochondrial membrane, releasing cytochrome-C, apoptosis-inducing factors, endonuclease G, and other mitochondrial proteins. Cytochrome-C interacts with apoptotic protease activator 1 to form an apoptotic complex that initiates the activation of Cas9, which ultimately leads to cell damage (37). At the same time, changes in ATP production and LDH levels also promote apoptosis (Figure 3).

Oxidative stress-sensitive kinases include extracellular signal-regulated kinases ½ (ERK1/2), c-Jun amino (N)-terminal kinases ½/3 (JNK1/2/3), p38 isoforms (α, β, γ and δ), and ERK5 (38). When SiHa cells were stimulated with β-CCE, the intracellular ROS levels increased significantly and activated the MAPK signaling pathway. At the same time, the p38 is more sensitive to oxidative stress stimuli compared to other kinases (39). The p38 phosphorylation inhibitor and NAC (ROS scavenger) significantly reduced the occurrence of apoptosis compared to ERK and JNK inhibitors (Figure 4). It has been reported that *P. quassioides* promotes apoptosis in SiHa cervical cancer cells by activating the p38/MAPK signaling pathway, which provides an effective theoretical basis for the β-CCE (monomer of *P. quassioides*) inducing SiHa cell apoptosis (40). However, this study has some limitations. The direct evidence to show the molecular interaction between p38 MAPK and ROS and mitochondrial damage in β-CCE-induced SiHa cell apoptosis was not shown. These interactions should be further explored at molecular level, and the mechanism should be clarified in detail.

In summary, our results indicate that β-CCE-induced apoptosis in SiHa cervical cancer cells is caused by the accumulation of cytoplasmic and mitochondrial ROS levels. At the same time, p38/MAPK and mitochondrial-dependent apoptosis signaling pathways are activated in this process. Our research will broaden the application of β-CCE in research and provide new options for cervical cancer treatment.

Conflicts of Interest

The Authors declare that there are no conflicts of interest in regard to this study.

Authors' Contributions

H.N.S., D.P.X., Y.D.C., and T.K. performed the experiments and wrote the article. Y.H.J., C.X.R., X.Y.G., H.N.Z., W.Q.X., Y.H.H., and Y.H.H. performed the data analysis. H.N.S., Y.D.C., and T.K. made substantial contributions to conception and design. All Authors read and approved the final article.

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