Article

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Role of Endoplasmic Reticulum Stress in Melatonin-induced Apoptosis and Inhibition of Invasion and Migration in Adrenocortical Carcinoma Cells

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Background: Melatonin, a hormone synthesized by the pineal gland and released into the blood, seems to have anti-tumor properties. However, the mechanisms of the anti-cancer effect of melatonin are largely unknown. This study investigated the anti-tumor activity of melatonin in adrenocortical carcinoma (ACC) and analyzed its molecular mechanisms.

Methods: Different concentrations of melatonin were added to ACC cells *in vitro* and *in vivo*. Cell viability was appraised via Cell Counting Kit-8 (CCK-8) assay, cell migration and invasion were appraised via wound healing assay and transwell assay, and cell apoptosis was appraised via flow cytometry. The levels of nuclear factor kappa B (NF-κB)/mitogen-activated protein kinase (MAPK) pathway proteins (c-Jun N-terminal kinase (JNK) and p38) and endoplasmic reticulum stress-related proteins (C/EBP homologous protein (CHOP) and glucose-regulated protein 78 (GRP78)) were appraised via western blot.

Results: Melatonin reduced the proliferation rate, migration rate, and invasion rate of ACC cells, and significantly increased apoptosis of ACC cells in contrast with the Control Check (CK) group. Moreover, melatonin intervention reduced NF-κB/MAPK signal routing (JNK and p38) and endoplasmic reticulum stress (CHOP and GRP78). Treatment with the NF-κB/MAPK pathway inhibitor NF-κB/MAPK-IN-1 (3.48 µM) enhanced the inhibitory effects of melatonin on the activity of ACC cells and increased apoptosis. The subcutaneous tumor model (SW-13) in nude mice further confirmed that melatonin induced apoptosis of ACC cells by reducing endoplasmic reticulum stress, and NF-κB/MAPK signal routing was involved in this effect.

Conclusion: Melatonin induces apoptosis of ACC cells by reducing endoplasmic reticulum stress, and this effects was may be related to the NF-κB/MAPK signal routing. Melatonin may be an effective anti-tumor agent and have great potential as an adjuvant therapy in the future.

Keywords: adrenocortical carcinoma; melatonin; endoplasmic reticulum stress; NF-κB/MAPK; cell apoptosis

Introduction

Adrenocortical carcinoma (ACC), a malignant tumor that occurs in the adrenal cortex, is characterized by high malignancy and poor prognosis [1], the annual incidence of ACC in the population is about 0.7–2.0 cases/million people, accounting for 0.2% of cancer deaths [2]. ACC can occur in any age group, but there are two peak ages of onset, namely, 1–6 years old in childhood and 40–50 years old in middle age. In the early stage of ACC, owing to the lack of specific clinical manifestations and characteristics, about 70% of ACC patients are diagnosed at stages III and IV of the disease [3], in patients with advanced ACC, most cases have local and distant invasion and metastasis; for patients with stage IV ACC, the 5-year survival rate is only 6%–13% [4]. At present, there is no effective treatment for advanced ACC. Surgery, radiotherapy, and chemotherapy have not shown good clinical efficacy. With the emergence of second-generation and third-generation sequencing, genes closely related to the occurrence and development of ACC have been discovered. However, no effective new targets or new drugs for ACC treatment have been found [5]. Therefore, an important focus of research is the development of effective drug therapy for ACC.

Melatonin is a bioactive substance synthesized and secreted by the pineal gland and other cells [6]. The research on melatonin, which has focused on functions including regulation of biological rhythm, stabilization of gonadal function, anti-inflammatory, analgesic, and anti-oxidant activity, and immune regulation, has made great progress [7]. Melatonin's function of regulating biological rhythms can reduce tumor risk and ameliorate the quality of life of cancer patients [8]. It has been shown that melatonin can effectively alleviate breast cancer, prostate cancer, colon cancer,

and other tumors; melatonin can also enhance the efficacy of traditional chemotherapy drugs, reduce their adverse reactions, and prolong the life of patients [9,10]. The result of a recent study indicates that melatonin can also antagonize the effect of chemical poison-induced hepatocellular carcinoma [11]. Although the underlying molecular mechanism of the anti-cancer effect of melatonin in ACC is largely unknown, various studies have reported that the anti-tumor ability of melatonin may be mediated by a variety of mechanisms, including antioxidant activation, inhibition of migration and induction of tumor apoptosis, and reduction of endoplasmic reticulum stress [12,13]. A recent study confirmed the treatment effect of melatonin in ACC patients [14].

The mitogen-activated protein kinase (MAPK) pathway is a key signal transduction network in eukaryotes and plays an important role in cell survival and proliferation [15]. The MAPK family is divided into three categories: p38 MAPK, c-jun N-terminal kinase (JNK), and extracellular signal-regulated kinase 1/2 (ERK1/2) [16]. Recently, a study has shown that p38 MAPK and JNK substrates are involved in cancer cell growth and apoptosis [17], and are regulated by melatonin [18]. Another study has shown that melatonin reduces the activity of human SH-SY5 Y neuroblastoma cells through the MAPK-ERK pathway [19]. However, it is not clear whether melatonin affects the apoptosis of human ACC cells through the MAPK/ERK/p38/JNK signal routing. Therefore, we studied the effects of melatonin on the viability and apoptosis of ACC cells, explored whether melatonin could alleviate endoplasmic reticulum stress to induce apoptosis, and the role of MAPK/ERK/p38/JNK pathway in its effects.

Materials and Methods

Cell Culture

SW-13 (Catalog number: CCL-105) and NCI-H295R (Catalog number: CC-Y1387) cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cell lines were identified using the approved DNA-based method to confirm the origin of the cell line and to check for misrecognition. The cell line was checked against the database of mistakenly identified cell lines maintained by the International Committee for Cell Line Accreditation (ICLAC). The genetic characteristics of cell lines were authenticated by short tandem repeats (STR) profiling, and no cross-contamination was detected. Testing for mycoplasma indicated no contamination. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) culture mediumn (C11995500BT, MilliporeSigma, Darmstadt, Germany) containing 10% FBS, penicillin, and streptomycin at 100 U/L each, and incubated in an incubator (120300, Guansen Biotechnology, Shanghai, China) at 37 °C with 5% CO_2, and then digested and passaged until the cell fusion reached 80%–90%. The logarithmic growth phase cells were taken for experiments. MAPK/ERK/p38/JNK pathway inhibitor nuclear factor kappa B (NF-κB)/MAPK-IN-1 obtained from Beyotime (3.48 µM, HY-147972, Shanghai, China).

IC50 Value Detection of Melatonin

SW-13 and NCI-H295R cells in the logarithmic growth phase were inoculated in 96-well plates at a density of 4000/well. The experimental grouping was conducted with different concentrations of melatonin (0.46875, 0.9375, 1.875 µM). Each concentration was set up with three duplicate wells, and a control grouping without melatonin was set up. After 24 h of drug action, 10 µL of CCK-8 reagent was added to each well and incubated at 37 °C and 5% CO_2 for 4 h. The optical density (OD) value at 450 nm wavelength was appraised by a microplate reader (Varioskan LUX, BioRad, Hercules, CA, USA), and half maximal inhibitory concentration (IC50) was calculated.

CCK-8 was Used to Detect Cell Activity

The cell density was reset to 1×10^4 cells/well, SW-13 and NCI-H295R cells in the logarithmic growth phase were selected. The cells were inoculated into 96-well culture plates in a 37 °C, 5% CO_2 incubator for 24 h with 100 µL per well. The cells were washed with PBS (AM9624, Posino Life Technology, Wuhan, China), and the supernatant in the culture dish was removed. Melatonin was diluted to the corresponding concentration according to the grouping, added to each well plate, and cultured in a 5% CO_2 incubator for 24 h, 48 h, and 72 h at 37 °C, respectively. The cell morphology was observed by microscope (Olympus cx41, MICROSCOPE CENTRAL, Tokyo, Japan) at the end of the culture. Each well was augmented with 10 µL of 5 mg/mL CCK-8 (BS350A, Yisheng Biology, Nanchang, China) and incubated in a 5% CO_2 incubator for 1 h at 37 °C. The OD value was gauged at the same time point at 450 nm wavelength of the microplate reader, and the measured OD value was used to analyze the effect of cell proliferation.

Wound Healing Assay

Firstly, a straight edge was used on the 3.5-cm dish and the lines were marked evenly with a marker stroke, spaced 0.5–1 cm apart for each line. SW-13 and NCI-H295R cells that were in the logarithmic growth phase were selected, and the cell density was reset to 5×10^5 cells/well. Cells were inoculated into 3.5 cm dishes and the cells were cultured overnight at 37 °C and 5% CO_2 saturated humidity. When the cell density reached about 90%, the bottom of the 3.5 cm dish was covered, and the 200 µL gun head was pointed at the ruler, as far as possible perpendicular to the horizontal line scratches behind the back, with the gun head vertical, not inclined. The cells were washed 3 times with PBS, scratched cells were removed, and the cells were observed and photographed under the microscope. Melatonin

was diluted to the corresponding concentration according to the grouping, added to each dish, and cultured in a 5% CO_2 incubator at 37 °C for 72 h. At the end of the culture, cell migration was observed under a microscope. Cell migration ability: migration index (%) = (initial scratch width – scratch width after healing)/initial scratch width × 100%.

Cell Invasion was Appraised by Transwell Assay

The treated SW-13 and NCI-H295R cells were washed with 3 mL PBS, digested with 0.25% trypsin (002PI, China Center for Type Culture Collection, Wuhan, China), and centrifuged at 1000 rpm for 5 min. The supernatant was removed, the cells were washed twice with PBS, and the residual serum was washed away. The cells were resuspended in a serum-free medium and counted using a cell counting plate. The cell concentration was diluted to 3×10^5 cell/mL in a serum-free medium for later use. Matrigel (356234, Corning Incorporated, Corning, NY, USA) was melted a day earlier at 4 °C, and the transwell chamber (3422, Corning Incorporated, Corning, NY, USA), 24-well culture plate, and the tip were precooled overnight at –20 °C. The Matrigel was diluted with serum-free medium to a final concentration of 1 mg/mL and placed on ice. A complete medium containing 800 µL 10% FBS pre-cooled at 4 °C was added to 24-well plates and placed in a transwell chamber. 100 µL Matrigel with a final concentration of 1 mg/mL was added vertically at the bottom of the upper chamber of the transwell chamber, and incubated at 37 °C for 4–5 h to make it dry into a gel. After Matrigel was dried into a gel, a 200 µL cell suspension of each grouping was added to the upper chamber of the transwell chamber, and cultured in an incubator at 37 °C and 5% CO_2 for 24 h. Melatonin was diluted to the corresponding concentration according to the grouping with the basic medium, added to each chamber, and cultured in a 5% CO_2 incubator at 37 °C for 72 h. The transwell was taken out, and the chamber was carefully washed with PBS, and the cells were fixed with 4% paraformaldehyde solution (P0099, Beyotime, Shanghai, China) for 1 h. The cells were stained with 0.5% crystal violet staining solution, placed at room temperature for 20 min, washed with PBS, and wiped clean with a clean cotton ball. Five high-magnification fields were randomly selected from the upper, lower, left, and right sides. The number of cells in the lower chamber was counted as the number of cells penetrating the Matrigel, and the number of cells was used to represent the invasiveness of tumor cells. The number of invasive cell were counted. The experiment was repeated 3 times.

Flow Cytometry

The cell density was reset to 5×10^5 cells/well, and SW-13 and NCI-H295R cells in the logarithmic growth phase were selected. The cells were inoculated in a 6-well culture plate, and 2 mL of complete medium (L310KJ, Yuanpei Biotechnology, Shanghai, China) was added to each well. The cells were cultured in a 5% CO_2 incubator at 37 °C for 24 h. Melatonin was diluted into the corresponding concentration according to the grouping, added to each well plate, and cultured in 37 °C, 5% CO_2 incubator for 72 h. Cells were collected by trypsin digestion without EDTA and washed twice with pre-cooled PBS. The cells were resuspended in 100 µL PBS, augmented with PI (5 µL, 401006, Beibo Biology, Shanghai, China), AV (5 µL), and incubated at 37 °C in the dark for 30 min. PBS was added to wash cells 2 times. The cells were resuspended in 300 µL PBS and appraised by flow cytometry (FACSVerse, BD, Franklin, NJ, USA).

Western Blot

The cell density was reset to 5×10^5 cells/well, and SW-13 and NCI-H295R cells in the logarithmic growth phase were selected. The cells were inoculated in 6 cm dishes, and a 3 mL complete medium was added to each dish. Before culturing cells in a 37 °C, 5% CO_2 incubator for 72 h, melatonin was diluted to concentrations corresponding to each group and added to each well plate. The old culture medium was discarded, and cells were digested with the radioimmunoprecipitation assay (RIPA) buffer (P00138, Beyotime, Shanghai, China), including 1% phenylmethanesulfonyl fluoride (PMSF) lysate and collected by low-speed centrifugation to extract total cell protein. Protein quantification was performed using the Bradford method (BL521A, Biosharp, Beijing, China). The samples were boiled for 5 min, cooled on ice, and centrifuged for 30 s. The supernatant was subjected to polyacrylamide gel electrophoresis (T1010, solarbio, Beijing, China) with a voltage of 100 V for 1 h. Polyvinylidene fluoride (PVDF) membranes (HATF00010, Millipore, Burlington, MA, USA) were sealed with 5% skim milk at room temperature for 1 h, and incubated overnight at 4 °C with primary antibodies: ERK5 (ab40809), JNK (ab307802), ERK1 (ab109282), p38 (ab170099), and glucose-regulated protein 78 (GRP78) (ab213258). Antibodies were obtained from Abcam (1:1000, Shanghai, China). After washing the membrane twice with TBST, the membrane was incubated with fluorescein-labeled secondary antibody (9009-99-0, 1:2000, Abcam, Shanghai, China) at room temperature for 1 h. After washing the membrane three times, the ECL chromogenic agent (ECL-0011, Dingguo Changsheng Biotechnology, Beijing, China) was exposed and imaged with a scanner (EVOS™ M7000, Thermo Fisher, Waltham, MA, USA). The gray value of each band was analyzed using ImageJ software (v.1.37, NIH, Bethesda, MD, USA).

Establishment of Tumor Model in Nude Mice

Specific Pathogen Free (SPF) grade animals of 18–20 g body mass (n = 10, 6-week-old wild-type) were purchased from Sibeifu Biotechnology Co., Ltd. (Beijing, China). Mice were assigned to 2 groups: SW-13 (n = 5, control grouping) and SW-13 + Melatonin (n = 5, drug grouping).

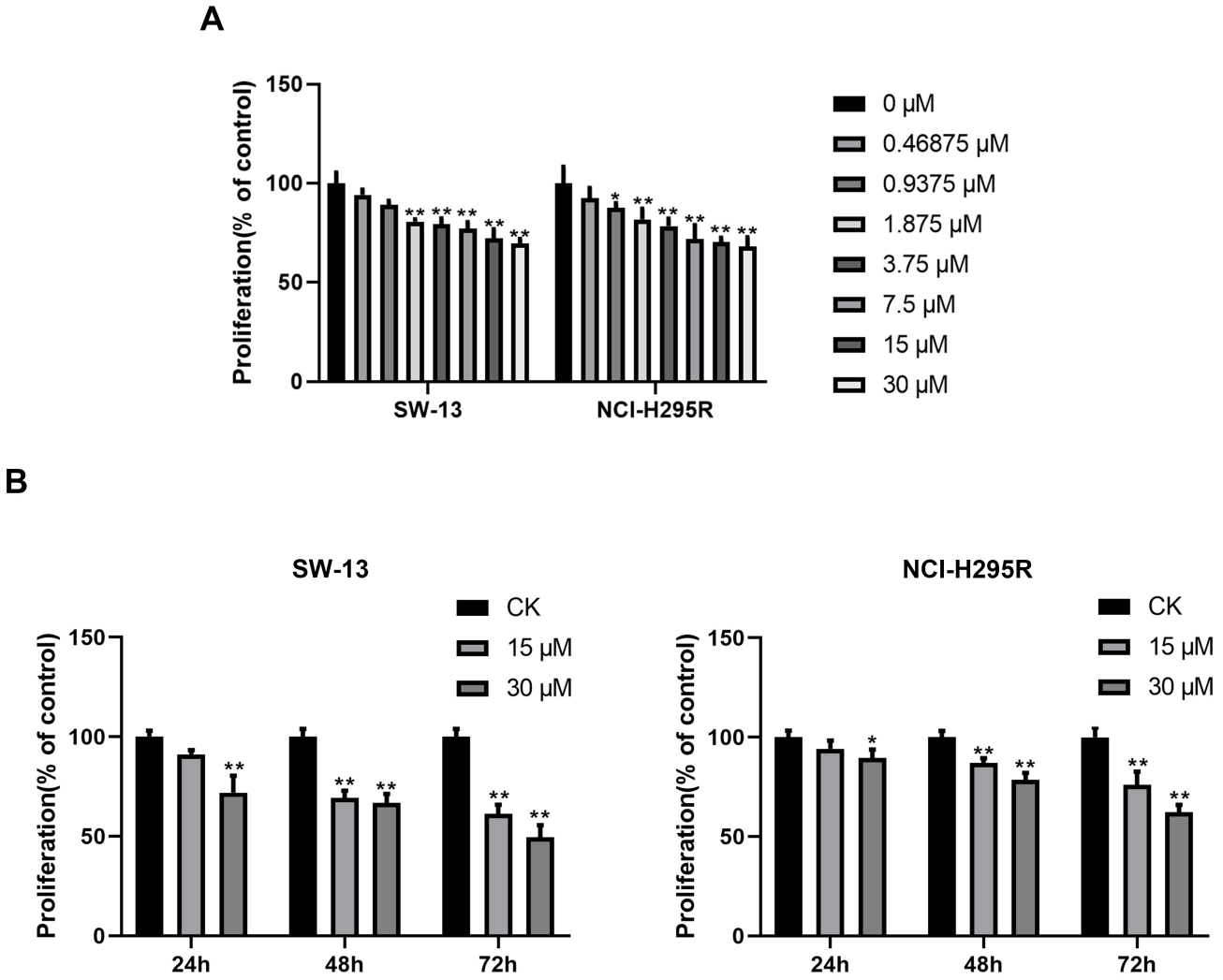

Fig. 1. Melatonin restrained the proliferation rate of adrenocortical carcinoma (ACC) cells. (A) The half maximal inhibitory concentration (IC50) value of Melatonin was measured by Cell Counting Kit-8 (CCK-8) in SW-13 and NCI-H295R cells. Compared with 0 μM grouping, *$p < 0.05$, **$p < 0.01$. (B) CCK-8 was used to detect the proliferation of SW-13 and NCI-H295R cells. Compared with the Control Check (CK) group, *$p < 0.05$, **$p < 0.01$. The data were displayed as the mean "±" standard deviation (n = 3) representing three independent experiments.

The person performing the injection used the left index finger and thumb to pinch the back of the mouse with the abdomen facing them. The left index finger was used to fix the right forelimb, exposing the right armpit of the mouse, and the needle was inserted into the armpit. Each mouse was injected with 1×10^7 cells with an injection volume of 100 μL/mouse. For the SW-13 + Melatonin group, Melatonin (100 mg/kg/d) was injected intraperitoneally every day when the tumor size reached 0.2×0.2 cm^2; the SW-13 group was given an equal amount of saline by gavage. Detection during modeling: The body weight and tumor size of mice were measured every 5 days for 15 days. After 15 days of modeling, the mice were euthanized. After anesthesia (0.3% sodium pentobarbital solution, 40 mg/kg), bled to death, photographed, and the tumor was frozen in liquid nitrogen and stored at –80 °C. All experimental protocols of this study were approved by Zhuoqiang Biotechnology, Co., Ltd. ethics committee (No: ZQZA-2023-021).

Statistical Analysis

Statistical analysis was performed using SPSS 22.0 statistical software (SPSS Inc., Chicago, IL, USA). Normally distributed variables were expressed as mean ± standard deviation ($\bar{x} \pm s$). *T*-tests were used for comparisons between two groups, and analysis of variance was used for comparisons between multiple groups. Post hoc analysis was conducted using the Tukey test. $p < 0.05$ was considered statistically significant.

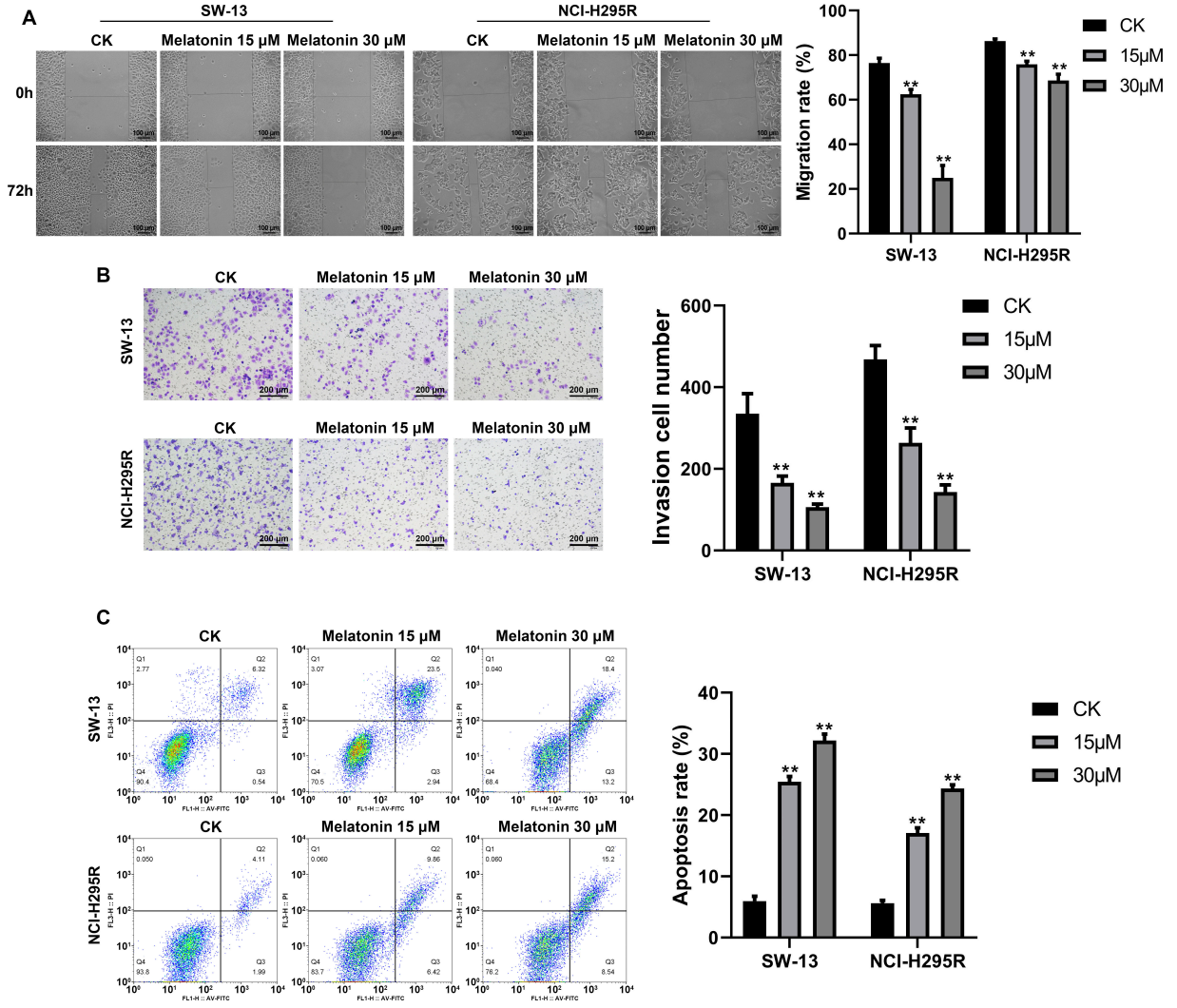

Fig. 2. Melatonin restrained metastasis of ACC cells and induced apoptosis. (A) The wound healing assay was used to evaluate the migration ability of SW-13 and NCI-H295R cells. (B) Transwell assay was used to evaluate the invasion of SW-13 and NCI-H295R cells. (C) Flow cytometry was used to evaluate the apoptosis of SW-13 and NCI-H295R cells. Compared with the CK group, $^{**}p < 0.01$. The data were displayed as the mean "±" standard deviation (n = 3) representing three independent experiments.

Results

Melatonin Restrained the Proliferation Rate of ACC Cells

Cell proliferation rate was substantially attenuated in the melatonin groups, compared with that of the 0 μM group. Cell proliferation rate decreased with increased melatonin concentration, with the lowest rate observed in the 30 μM group. The IC50 value of SW-13 cells was 103.114 μM, and the IC50 value of NCI-H295R cells was 348.781 μM, which was authenticated via CCK-8 assay. Subsequently, 15 μM and 30 μM concentrations were selected for experiments ($p < 0.05$, Fig. 1A). In contrast with the Control Check (CK) group, the cell proliferation rate of the melatonin 15 μM and 30 μM groups decreased substantially, and the cell proliferation rate was the lowest when the melatonin concentration was 30 μM and the treatment time was 72 h, which was validated via CCK-8 assay ($p < 0.05$, Fig. 1B). Subsequently, a Melatonin drug concentration of 30 μM and a treatment time of 72 h were selected for SW-13 cells.

Melatonin Restrained Metastasis of ACC Cells and Induced Apoptosis

Compared with the CK group, migration rates of SW-13 and NCI-H295R cells were substantially reduced after treatment with melatonin at 15 μM and 30 μM for 72 h, which was validated via the wound healing experiment ($p < 0.01$, Fig. 2A). Compared with the CK group, the invasion ability of SW-13 and NCI-H295R cells was substantially reduced after treatment with melatonin at 15 μM and 30 μM for 72 h, which was validated via transwell invasion

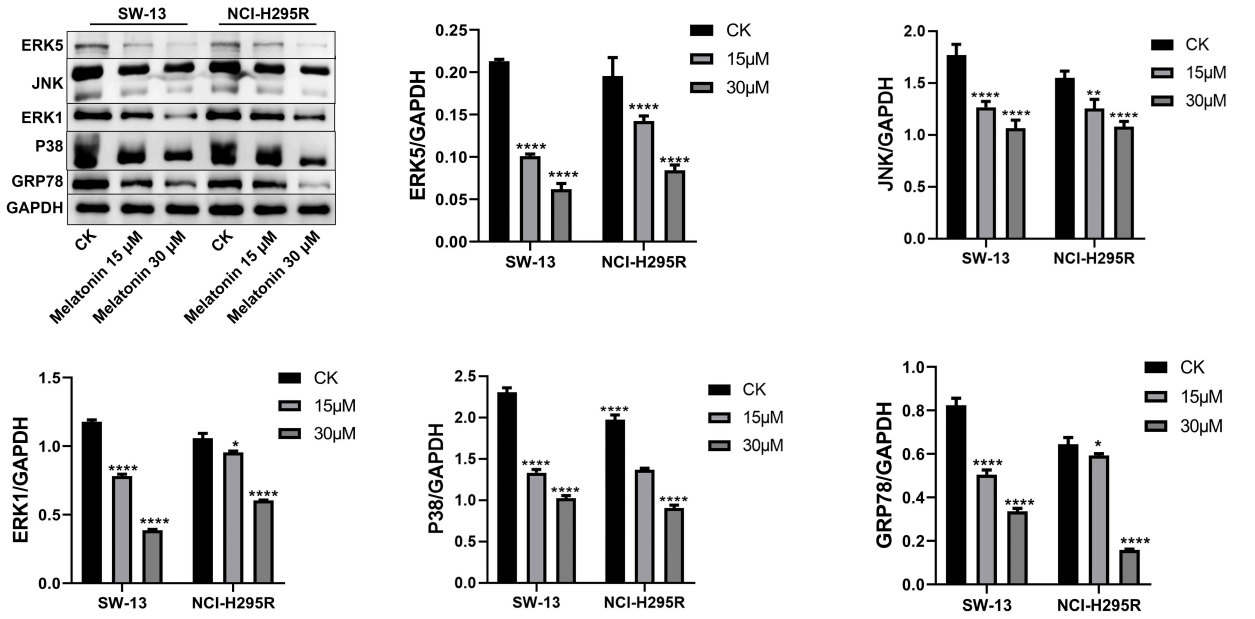

Fig. 3. Melatonin reduced endoplasmic reticulum stress and MAPK/ERK/p38/JNK signal routing in an *in vitro* model. The levels of the extracellular signal-regulated kinase (ERK)/p38/c-Jun N-terminal kinase (JNK) pathway proteins (ERK5, JNK, ERK1, p38) and endoplasmic reticulum stress-related proteins (glucose-regulated protein 78 (GRP78)) were evaluated by WB assay. Compared with CK group, $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$. The data were displayed as the mean "±" standard deviation (n = 3) representing three independent experiments. MAPK, mitogen-activated protein kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

assay ($p < 0.01$, Fig. 2B). Flow cytometry confirmed that, compared with the CK group, the apoptosis rate of SW-13 and NCI-H295R cells was substantially increased in 15 μM and 30 μM melatonin concentration groups after 72 h ($p < 0.01$, Fig. 2C).

Melatonin Reduced Endoplasmic Reticulum Stress and MAPK/ERK/p38/JNK Signal Routing in an in Vitro Model

Compared with the CK group, the expression levels of ERK/p38/JNK pathway proteins (ERK5, JNK, ERK1, p38) and endoplasmic reticulum stress-related proteins (GRP78) in SW-13 and NCI-H295R cells were substantially reduced after treatment with melatonin at 15 μM and 30 μM for 72 h, which was validated via western blot assay ($p < 0.0001$, Fig. 3), suggesting melatonin could reduce endoplasmic reticulum stress and MAPK/ERK/p38/JNK signal routing in an *in vitro* model.

Melatonin Increased Apoptosis of ACC Cells by Reducing Endoplasmic Reticulum Stress and MAPK/ERK/p38/JNK Signal Routing in Vitro

SW-13 cells were treated with the MAPK/ERK/p38/JNK pathway inhibitor NF-κB/MAPK-IN-1 (3.48 μM, HY-147972, Biotech Biotech, Wuhan, China). In contrast with the CK group, the melatonin-treated groups displayed substantially reduced cell proliferation, and the cell proliferation rate was further reduced after NF-κB/MAPK-IN-1 treatment ($p < 0.01$, Fig. 4A). Compared with the CK group, cell migration rate and invasive cell number were substantially abated after treatment with Melatonin and the inhibitor ($p < 0.01$, Fig. 4B,C). Melatonin significantly reduced the cell proliferation rate and increased the apoptosis rate of SW-13 cells compared with the CK group, and these effects were further enhanced after inhibitor treatment ($p < 0.001$, Fig. 4D). Western blot results showed that compared with the CK group, the expression levels of JNK, p38, and GRP78 were significantly reduced after Melatonin treatment, and these reductions were enhanced after inhibitor treatment ($p < 0.001$, Fig. 4E).

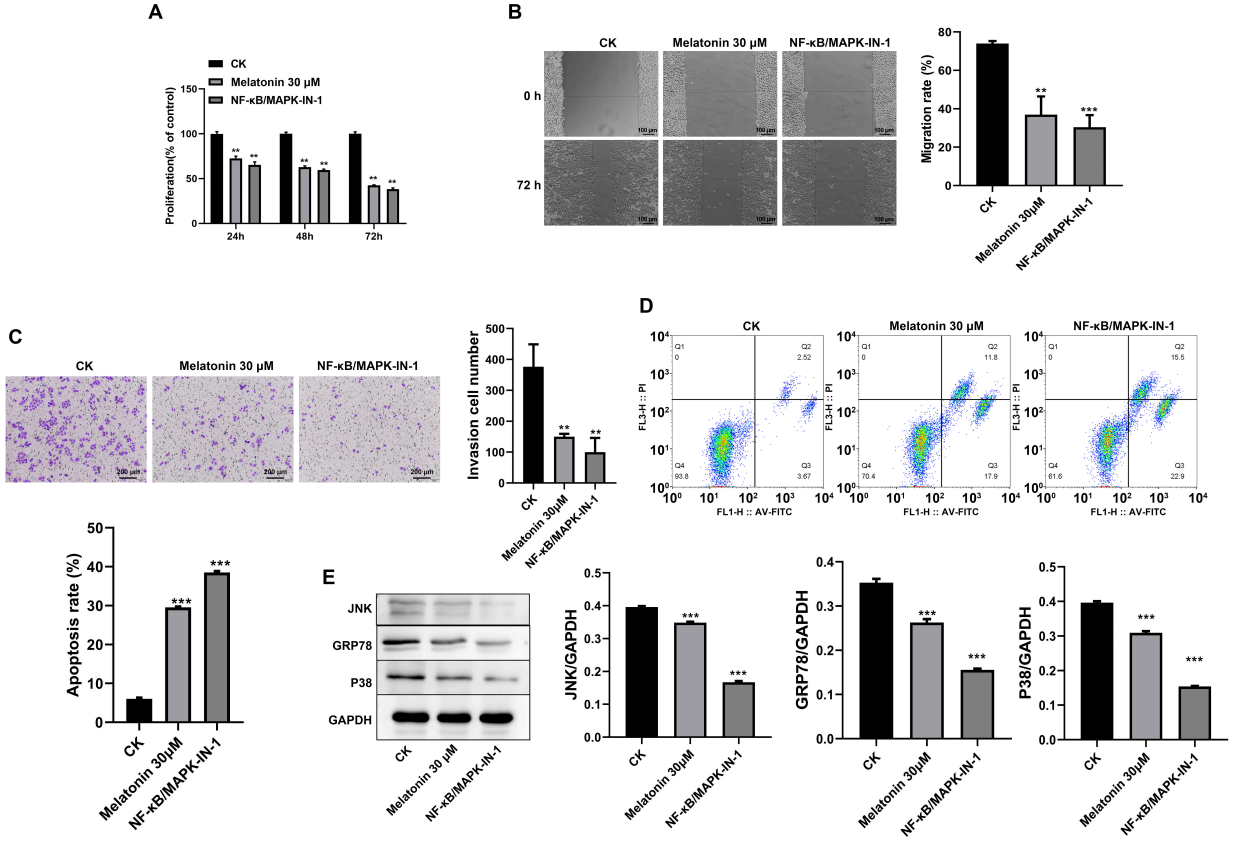

Fig. 4. Melatonin increased apoptosis of ACC cells by reducing endoplasmic reticulum stress and MAPK/ERK/p38/JNK signal routing *in vitro*. SW-13 cells were treated with ERK/p38/JNK pathway inhibitor nuclear factor kappa B (NF-κB)/MAPK-IN-1 (3.48 μM). (A) CCK-8 assay was used to detect the proliferation of SW-13 cells. (B) The wound healing assay was used to evaluate the migration ability of SW-13 cells. (C) Transwell assay was used to evaluate the invasion of SW-13 cells. (D) Apoptosis of SW-13 cells was evaluated by flow cytometry. (E) The levels of JNK, GRP78 and p38 were appraised by western blot. Compared with the CK group, $^{**}p < 0.01$, $^{***}p < 0.001$. The data were displayed as the mean "±" standard deviation (n = 3) representing three independent experiments.

Melatonin Increased Apoptosis of Adrenocortical Carcinoma Cells by Reducing Endoplasmic Reticulum Stress

To evaluate the mechanism of melatonin-induced apoptosis of ACCs *in vivo*, a subcuta neous tumor model in nude mice (SW-13) was constructed. The comparison of body weight of nude mice showed that there was no significant difference in body weight between groups. The comparison of tumor mass in nude mice showed that tumor mass in the melatonin treatment group was substantially reduced compared with the saline group ($p < 0.05$, Fig. 5A). Compared with the saline group, expression of p38 and JNK was significantly reduced, and expression of C/EBP homologous protein (CHOP) increased significantly after Melatonin treatment, which was authenticated via western blot ($p < 0.01$, Fig. 5B).

Discussion

At present, ACC is mainly treated by surgery. The drug treatment of ACC employs an EDP-M (etoposide, adriamycin, cisplatin combined with mitotane) regimen, but the therapeutic effect of this regimen is not satisfactory. The median progression-free survival is only 5 months, and the overall survival rate is only 14.8 months. Most patients cannot tolerate its toxicity and side effects [20]. Traditional Chinese medicine has attracted the attention of scholars both domestically and internationally due to its natural and minimal toxic side effects. With continued research, the anti-tumor effects of traditional Chinese medicine are receiving increasing attention, and some drugs have been approved by USA FDA for clinical phase II trials [21]. Melatonin is a kind of neuroendocrine hormone, which has attracted more and more attention because of its wide distribution, low toxicity, and good histocompatibility [22]. In the current study, we confirmed the anti-tumor effects of melatonin in ACC through basic experimental studies.

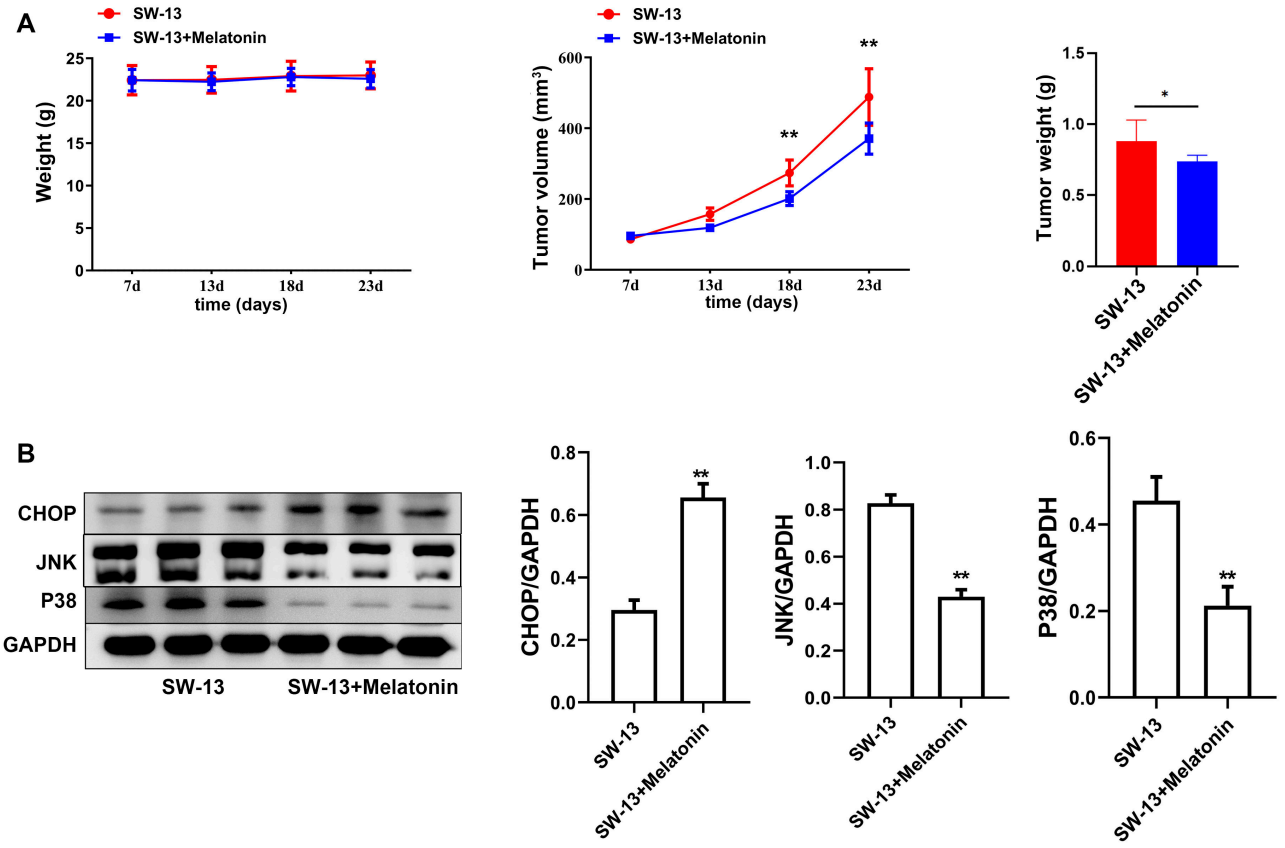

Fig. 5. Melatonin increased apoptosis of ACC cells *in vivo* by reducing endoplasmic reticulum stress and MAPK/ERK/p38/JNK signal routing. The subcutaneous tumor formation model of nude mice (SW-13) was constructed. (A) The body weight and tumor volume of mice were measured. (B) The levels of C/EBP homologous protein (CHOP), JNK and p38 were appraised by western blot. Compared with SW-13 group, $*p < 0.05$, $**p < 0.01$. The data were displayed as the mean "±" standard deviation (n = 3) representing three independent experiments.

At present, it is believed that the mechanisms of melatonin mainly involve anti-tumor cell proliferation, induction of tumor cell apoptosis, regulation of immune function, and inhibition of tumor cell invasion and metastasis [23]. A previous study has shown that melatonin can down-regulate protein kinase B (Akt) and murine doubleminute 2 (MDM2) to promote apoptosis of human gastric cancer cells AGS and MGC803 [24]. Wang *et al*. [25] have shown that melatonin treatment substantially reduced tumor size in a subcutaneous oral cancer xenograft model without obvious systemic side effects, substantially enhanced apoptosis and ferroptosis levels in tumor tissues, and abated autophagy levels. Melatonin-assisted cisplatin alleviates the proliferation of bladder cancer cells by abating PrP-regulated cell stress and cell proliferation signals [26]. Glyphosate and hard water synergistically promote proximal renal tubular epithelial cell aging through PINK1 Parkin-mediated mitochondrial autophagy, whereas melatonin exerts renal protective effects by regulating mitochondrial autophagy [27]. A previous study has also preliminarily confirmed the therapeutic effect of melatonin in ACC [14]. The present study confirmed, through cytology and animal model studies, that, compared to the CK group, melatonin reduced the proliferation, migration, and invasion rates of ACC cells, and substantially induced cell apoptosis.

The expression level of GRP78 is relatively low in mature organs, but it is elevated in tumor tissues. The tumor microenvironment has characteristics such as low sugar, hypoxia, and acidosis, which can cause the aggregation of non-glycosylated and non-folding proteins in the endoplasmic reticulum, thereby initiating endoplasmic reticulum stress [28]. In the present study, melatonin intervention reduced the level of endoplasmic reticulum stress protein GRP78. The MAPK family is an important intracellular signal routing. Under the influence of different stimuli, the MAPK family regulates various physiological functions such as cell proliferation, growth, death, and migration. The generation of these functions is achieved through processes such as activation and inactivation of the MAPK family within cells [29]. The present study evaluated the role of MAPK/ERK/p38/JNK signal routing in melatonin-induced apoptosis of ACC cells and inhibition of endo-

plasmic reticulum stress. The results showed that melatonin intervention could reduce the protein expression related to MAPK/ERK/p38/JNK signal routing (ERK5, JNK, ERK1, p38). MAPK/ERK/p38/JNK pathway inhibitor NF-κB/MAPK-IN-1 (3.48 μM) intervention enhanced the inhibitory effect of melatonin on ACC cell activity and induced cell apoptosis. It was further confirmed that melatonin induces ACC cell apoptosis by reducing endoplasmic reticulum stress, and MAPK/ERK/p38/JNK signal routing was associated with this effects. However, further evaluation of these mechanisms is needed.

Conclusion

In summary, the results of this study confirm that melatonin induces ACC cell apoptosis and alleviates endoplasmic reticulum stress, and this effect was related to the inhibition of the MAPK/ERK/p38/JNK. It provides an experimental basis for the use of melatonin in the clinical treatment of ACC.

Availability of Data and Materials

The data generated or analyzed during this study are included in this published article or obtained from the corresponding author on reasonable request.

Author Contributions

QSL and SS contributed to the concept and designed the research study. SS performed the research. PCH provided help and advice on the research study. QSL analyzed the data. HYY, ZXH, DCL wrote the manuscript and made substantial contributions to the conception and design, acquisition of data and analysis of data. ZJL helped analysis with constructive discussion. All authors contributed to the important editorial changes in the manuscript. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

All experimental protocols of this study were approved by Zhuoqiang Biotechnology, Co., Ltd. ethics committee (No: ZQZA-2023-021).

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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