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Photodynamic therapy combined with temozolomide inhibits C6 glioma migration and invasion and promotes mitochondrial-associated apoptosis by inhibiting sodium-hydrogen exchanger isoform 1

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ABSTRACT

Objective: As a targeted therapeutic technique for glioma inhibition, photodynamic therapy (PDT) has gradually become a focus of basic research related to glioma treatment. The capacity of PDT to kill glioma cells involves varieties of pathways. In glioma cells, activated sodium-hydrogen exchanger isoform 1 (NHE1) can inhibit the cytotoxic effect of temozolomide (TMZ), promote cell migration and invasion, and inhibit cell apoptosis by changing the acid-base equilibrium. The purpose of our study was to explore if PDT combined with TMZ can effectively inhibit glioma cells by influencing NHE1 in vitro.

Methods: We analyzed the expression levels of proteins such as NHE1, ezrin, vimentin, Bcl-2, and Bax by Western blot analysis, we assessed the migration and invasion of rat C6 glioma cells by Transwell assay, and we evaluated C6 cell apoptosis in vitro by flow cytometry.

Results: Western blot results indicated that NHE1, ezrin and vimentin were downregulated after cotreatment of C6 cells, and intracellular acidification was detected by a fluorometric intracellular pH assay. The migration and invasion capacities of C6 cells were significantly hindered after cotreatment, as shown by the Transwell assay. Experimental data also revealed a significant increase in cell apoptosis after cotreatment, as detected by flow cytometry; corresponding proapoptotic changes in Bcl-2, Bax and caspase-3 were also observed in vitro.

Conclusion: These results demonstrate that PDT combined with TMZ can inhibit C6 cell migration and invasion and promote mitochondrial-associated apoptosis by inhibiting NHE1. Therefore, this study provides supporting evidence for a potential method for the treatment of glioma.

1. Introduction

Photodynamic therapy (PDT) involves the use of a suitable light source to stimulate the corresponding photosensitizer to kill tumor cells through the activation of reactive oxygen species (ROS), injury of the tumor vasculature, as well as induction of the antitumor immune response [1–3]. As a tumor treatment method, PDT is widely used to treat malignant tumors of the skin, digestive tract and lung, but it is a new method for brain tumors. In neurosurgery, fluorescence imaging of photosensitizers has guided PDT for adjuvant treatment of primary glioma, recurrent glioma, and brain metastatic tumors. Previous studies have confirmed that PDT for gliomas is safe, sensitive, and selective, and leads to significant prolongation of good quality survival, tumor relapse, and reduction in further interventions; the incidence of clinical complications due to thromboembolism, cerebral edema, and skin photosensitivity were very low, i.e., 2%, 1.3%, and 1–3%, respectively [4]. However, due to the strong migration and invasion characteristics of glioma, some patients with glioma who are treated with PDT still have recurrence, and the efficacy of this approach should be improved by combination with other therapies (such as chemotherapy) to control migration and invasion.

NHE1 is a membrane transporter that can activate membrane matrix metalloproteinases and increase the invasiveness of cells by increasing the activity of the ERK1/2 and p38 MAPK signaling pathways [5]. Studies have shown that NHE1 also plays an important role in cell migration by regulating cell volume, stabilizing the cytoskeletal structure, promoting cell membrane anchoring. Therefore, NHE1 plays a particularly important role in tumor cell migration and invasion. In

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addition, NHE1 maintains the intracellular pH (pH_i) balance; the destruction of NHE1 by some factors can reduce the pH_i, and intracellular acidification is a cell death-inducing factor [6]. Therefore, NHE1 also plays an important role in glioma cell death [7]. TMZ is currently internationally recognized as an antiglioma chemotherapy drug, but in clinical practice, the effect of chemotherapy is not obvious, mainly due to drug tolerance [8]; tolerance to TMZ is related to the activation of the NHE1 protein [9]. To date, no studies have reported the effect of PDT or PDT combined with TMZ on the NHE1 protein or on the migration and invasion of glioma cells, and no study has evaluated whether NHE1 elicits apoptotic changes in glioma cells.

In this study, we found that PDT or PDT combined with TMZ can reduce the expression of NHE1 in C6 cells. PDT combined with TMZ can not only inhibit migration and invasion but also promote apoptosis in C6 cells by reducing NHE1. Moreover, TMZ resistance resulting from the activation of NHE1 in response to TMZ might be caused by a low concentration of TMZ in glioma cells, and the inhibitory effect of NHE1 became increasingly obvious as the concentration of TMZ increased. Taken together, these results demonstrate that PDT combined with TMZ provides a potential method to treat glioma by influencing the expression of NHE1.

2. Materials and methods

2.1. Chemicals

Hematoporphyrin monomethyl ether (HMME), a second-generation photosensitizer, was purchased from Yuanye Bio-Technology Co., Ltd. (Shanghai, China). HMME was stored at 2–8 °C, avoiding exposure to light and moisture. TMZ, a first-line chemotherapy drug for glioma, was purchased from Sigma (St. Louis, MO). TMZ was stored and used according to the manufacturer's instructions.

2.2. Cell culture

The C6 cell line was obtained from Baili Biotechnology Co., Ltd. (Shanghai, China), and the STR genotype was detected. C6 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (HyClone, Thermo) supplemented with 10% fetal bovine serum (BI) in a humidified incubator (Thermo, USA) at 37 °C with 5% CO_2. C6 cells in the logarithmic phase were used to carry out the following experiments in our study.

2.3. PDT treatment in vitro

Hematoporphyrin monomethyl ether (HMME) was dissolved in dimethyl sulfoxide (DMSO) before use. The final concentration of HMME in the 24-well plates after dilution with medium was 20 μg/ml. C6 cells in the logarithmic phase were placed in 24-well plates (500 μl of medium/well) at a density of approximately 5×10^4 cells/well. For the experiments, the C6 cells were divided into six groups: control (Con), HMME (20 μg/ml), pure laser therapy (LT 0.75 J/cm^2), PDT (HMME 20 μg/ml + LT 0.75 J/cm^2), TMZ (200 μmol/L), and PT (PDT + TMZ). After 24 h of culture, 20 μg/ml HMME was added to the medium in the HMME, PDT, and PT groups. The plates were then wrapped in aluminum foil and incubated for 4 h. The C6 cells were washed three times with 1x phosphate-buffered saline (PBS), and medium with 10% fetal bovine serum was added before laser irradiation. The cells were evenly irradiated by red light at a wavelength of 632.8 nm with a high-power helium neon laser (Melles Griot, America). The final energy was kept stable at 0.75 J/cm^2, and the values of the photosensitizer and laser energy were selected according to the previous literature and a previous experimental study [10]. All experiments were performed in the dark.

2.4. Intracellular pH measurement

The Fluorometric Intracellular pH Assay Kit (Sigma, America) utilizes a proprietary cell-permeable fluorescent indicator, BCFL-AM, to measure relative pH_i changes. The pH_i values in different groups of C6 cells were measured at different time points (0, 2, 4, 8, 16, 24, 48 h) after cell treatment. C6 cells were cultured overnight in growth medium at 40,000-80,000 cells/well/100 μl in a 96-well plate. Assay Buffer (1x) and Dye Loading Solution were prepared before adding 100 μl/well of Dye Loading Solution to each group, and the cells were cultured, protected from light, in a 5% CO_2, 37 °C incubator for 30 min, followed by incubation at room temperature for an additional 30 min. Finally, the pH assay was performed by measuring the fluorescence at λ_{ex} = 490/ λ_{em} = 535 nm for ratio measurements.

2.5. Measurement of cell migration and invasion by Transwell assays

C6 cells (1×10^5) were distributed evenly into 100 μl of serum-free medium after different treatment regimens and were seeded on top of a polycarbonate Transwell membrane (8.0 μm pore size, Corning). Then, 500 μl of medium containing 10% fetal bovine serum was added to the lower chamber. After incubation at 37 °C for 24 h, the membranes were rinsed with PBS, and cells were removed from the upper chamber with cotton swabs. The migrated cells on the bottom of the membrane were fixed in 4% paraformaldehyde for 20 min and then stained with 0.1% crystal violet for 30 min. Migrated cells were observed under a microscope (Olympus) with a 20x objective lens. Similarly, a 1:500 dilution of Matrigel matrix (Corning) was added to the upper chamber before adding cells (2×10^5 cells/100 μl) for the cell invasion assay.

2.6. Western blot assay

Proteins were extracted from the cells using urea buffer (2 M thiourea, 4% CHAPS, 40 mm Tris-base, 40 mm DTT, and 2% carrier ampholytes). Protein signals were visualized by ECL, and the membranes were imaged with the Image studio system (ECL, LI-COR, Lincoln, Georgia, USA). Finally, protein quantification was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.7. Immunofluorescence assay

After three washes with PBS, C6 cells on cover slips cultured in 24-well plates were fixed for 20 min with 4% paraformaldehyde, and the expression of the NHE1 protein was detected according to the classic immunofluorescence assay. Images of the different groups were captured by a Zeiss LSM510 confocal microscope (Carl Zeiss, Heidelberg, Germany).

2.8. Flow cytometric apoptosis assay

C6 cell apoptosis under different treatment conditions was measured with the Annexin V-FITC Apoptosis Kit. After digestion with trypsin, C6 cells were washed with PBS and resuspended in 100 μl of binding buffer. Then, 5 μl of annexin V-FITC was added for 10 min, and 5 μl of propidium iodide (PI) was added for 5 min in the dark after uniform shock absorption at room temperature. After completion, 400 μl of binding buffer was added to each sample. The cells were analyzed using a Becton Dickinson Biosciences FACSCalibur (BD Biosciences, CA).

2.9. Determination of caspase-3 activity

The Caspase-Glo 3/7 Assay (Promega, America) was used to detect the activity of caspase-3 in C6 cells 24 h after treatment. The cells were cultured and treated in white-walled multiwell plates, and the activity

of intracellular caspase-3 was detected as described previously [11].

2.10. ROS detection

C6 cells were treated with 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (Beyotime) for 30 min and washed with PBS. The fluorescence signals were then analyzed by flow cytometry (BD Biosciences, CA).

2.11. Statistical analysis

Data are expressed as the mean ± the standard error of the mean (S.E.M.) or standard deviation (SD). Statistical analysis was performed using GraphPad software, version 7. Student's *t*-test was used to assess statistical significance. Each experiment was repeated at least three times, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Combined PDT and TMZ treatment decreased the expression of the NHE1 protein

The confocal laser assay clearly showed that NHE1 expression was decreased after PDT and TMZ treatment, and separate treatment with the HMME or the LT yielded no change compared with the control group. Interestingly, NHE1 expression was significantly decreased after PT treatment (Fig. 1a). Moreover, Western blotting also indicated that NHE1 expression in C6 cells was significantly decreased after PT treatment (Fig. 1b and c). This evidence strongly indicated that PT treatment is better than TMZ or PDT treatment in terms of the change in NHE1 protein expression in C6 cells.

3.2. TMZ has dose-dependent effects on NHE1 protein expression

NHE1 is a transmembrane protein that is mainly located in the membrane and is highly expressed in glioma cells. Interestingly, with the increase in TMZ concentration, the NHE1 protein expression level was initially increased; however, it began to decrease when TMZ reached a certain concentration, and Western blotting results confirmed these changes (Fig. 2a and b). In conclusion, TMZ has dose-dependent effects on NHE1 expression in C6 cells.

3.3. The effect of combined treatment on NHE1 results in decreased pH_i

Among many factors, NHE1 plays a major role in maintaining the pH_i, and the survival of cells requires a stable acid-base balance. We further examined whether PDT or PT changed the pH_i in C6 cells by affecting NHE1, thereby disrupting the acid-base balance in the cell and causing cell death. The ratio measurement showed that compared with the control group, there were no significant changes in the groups treated with the HMME and the LT at different time points. The ratio of PDT decreased gradually over time, reaching its lowest point at 24 h, and the ratio at 48 h showed a recovery. The ratio of TMZ decreased

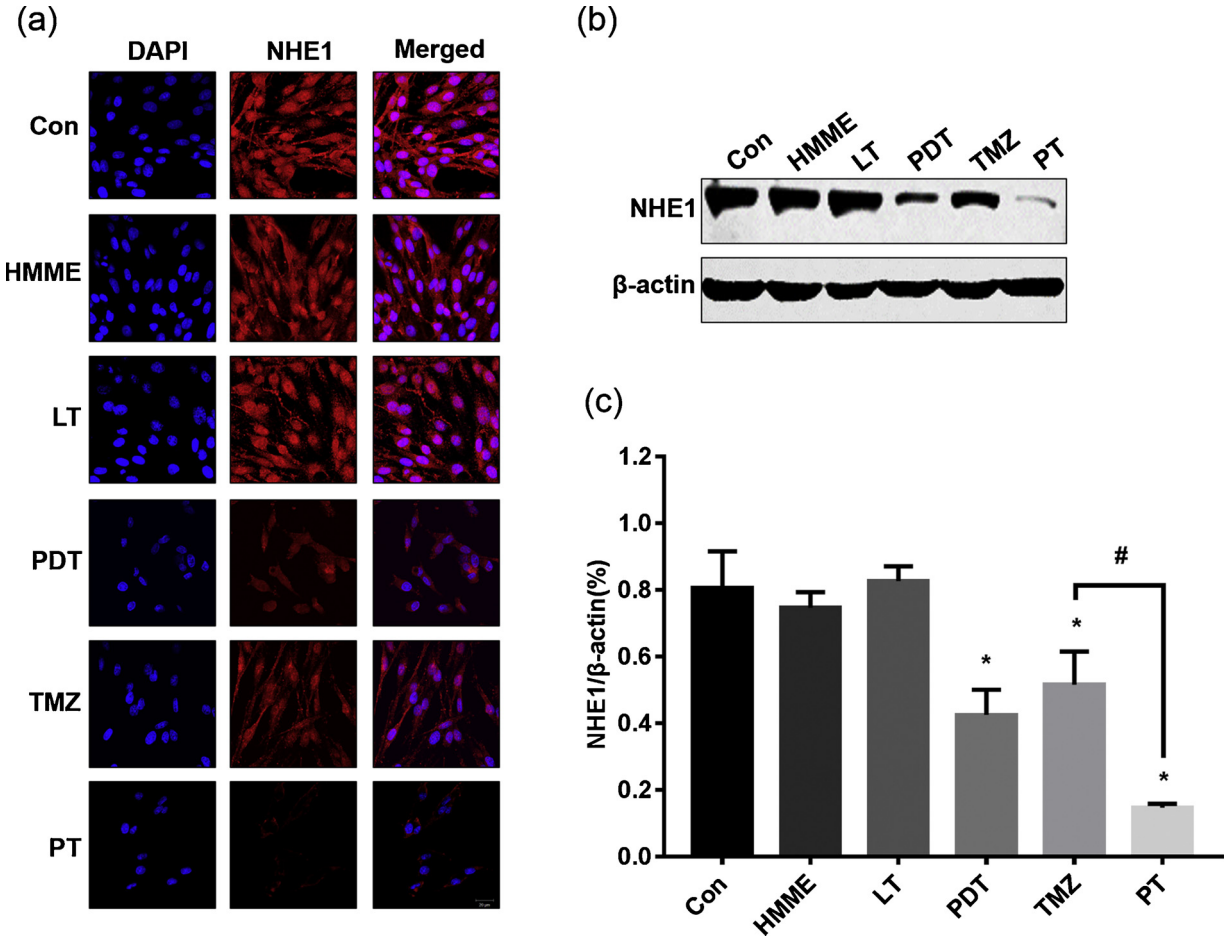

Fig. 1. NHE1 protein expression in C6 cells. (a) Immunofluorescence images showing the expression of NHE1 protein (red) and the position of the cell nucleus (blue) in C6 cells under six different conditions. Cells were treated with control (Con), HMME (20 g/ml), pure laser therapy (LT 0.75 J/cm2), PDT (HMME 20 g/ml + LT 0.75 J/cm2), TMZ (200 μmol/L), and PT (HMME 20 g/ml + LT 0.75 J/cm2 + TMZ 200 μmol/L). NHE1 immunosignals are located at the cell membrane (merged images). (b, c) Representative immunoblots for expression of NHE1 in C6 cells after exposure to different treatment conditions for 24 h; β-Actin was used as a loading control. Data are the means ± S.E.M. (n = 3). *P < 0.05 versus Con; #P < 0.05 versus TMZ.

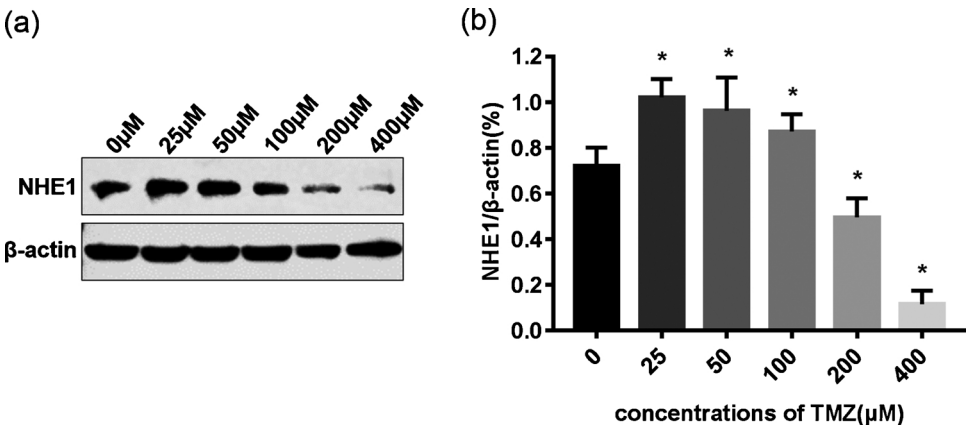

Fig. 2. (a) Western blot analysis of NHE1 under TMZ in a gradient of different concentrations (0, 25, 50, 100, 200, 400 μmol/L) for 24 h; β-Actin was used as a loading control. (b) NHE1 expression gradually increases as the concentration increases below 100 μmol/L, and then decreases gradually above concentrations of 100 μmol/L. Data are the means ± S.E.M. (n = 4). *P < 0.05 versus Con.

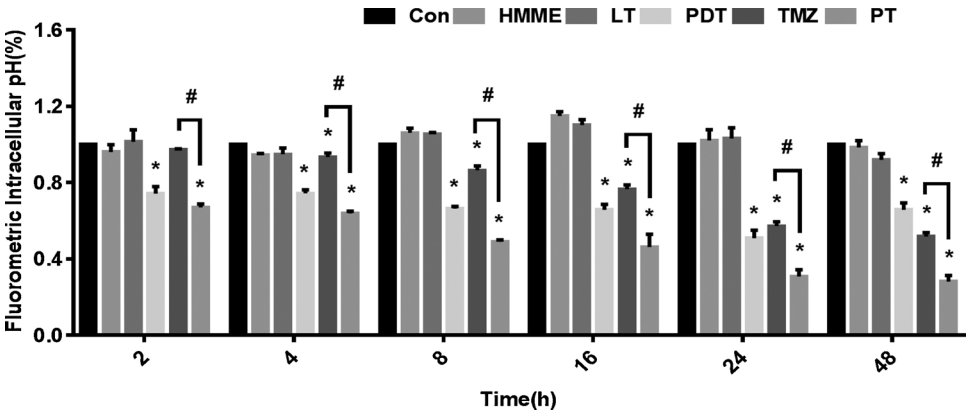

Fig. 3. BCFL-AM-loaded C6 cells show changes in pH_i under different groups. Cells were exposed to Con, HMME (20 g/ml), LT (0.75 J/cm2), PDT (HMME 20 g/ml + LT 0.75 J/cm2), TMZ (200 μmol/L), and PT (HMME 20 g/ml + LT 0.75 J/cm2 + TMZ 200 μmol/L) over time (0, 2, 4, 8, 16, 24, 48 h). Summary data are shown; all data were analyzed using GraphPad Prism 7 software. Data are the means ± S.E.M. (n = 3). *P < 0.05 versus Con; #P < 0.05 versus TMZ.

gradually with time after 4 h. Compared with the ratio after PDT and TMZ treatments, the ratio was significantly reduced at 2 h after PT treatment. Interestingly, the ratio did not decline at 2 h after TMZ treatment, but lower values were found after PT treatment than after PDT treatment (Fig. 3). The results also indicated that compared with the pH_i after PDT and TMZ treatments, the pH_i decreased significantly after PT treatment.

3.4. Combined PDT and TMZ treatment inhibited C6 cell migration and invasion

A Transwell assay showed that the migration capacity of C6 cells was significantly reduced compared with other groups after PT treatment (Fig. 4a). In addition, the invasion capacity was consistent with migration (Fig. 4b). The activation of ezrin and vimentin can promote the migration and invasion of glioma [12]. Western blotting results showed that ezrin and vimentin levels in C6 cells were decreased after TMZ and PDT treatment, and the effect of PT treatment was more obvious (Fig. 4c and d).

3.5. Combined treatment with PDT and TMZ induced C6 cell apoptosis

PDT and TMZ can promote C6 glioma cell apoptosis, as indicated by flow cytometry. Apoptosis was more obvious after PT treatment, but no distinct apoptosis occurred in the Con, HMME and LT groups (Fig. 5a). The activity of caspase-3 showed the greatest increase in the PT group compared with others (Fig. 5b). We also found that PDT and TMZ can reduce the expression of the apoptosis-related protein Bcl-2 and can increase the expression of Bax, and these effects were stronger after PT treatment (Fig. 5c). In conclusion, PT can significantly promote apoptosis in C6 cells by activating caspase-3 and affecting apoptotic proteins.

3.6. ROS release after combined treatment with PDT and TMZ

The release of intracellular ROS was detected by flow cytometry. The results showed that there was no significant difference in fluorescence intensity between HMME and LT compared with the control. The fluorescence intensities of the PDT and TMZ groups were increased, and the level of increase was more obvious in the PT group than in the control group (Fig. 5d). Therefore, it can be concluded that PT can significantly increase the release of ROS and participate in mitochondria-associated apoptosis.

4. Discussion

NHE1 is widely present in the cell membrane and exchanges intracellular H^+ with extracellular Na^+ at a 1:1 ratio to maintain the pH balance inside and outside the cell. Previous studies have reported that the regulation of the pH_i via the NHE1 system constitutes a limiting step for growth factor-stimulated ribosomal protein S6 phosphorylation [13], and growth factor can stimulate dormant cells to enter the cell cycle [14]. The expression of NHE1 in tumor cells can lead to the acidification of the microenvironment around the tumor [15], which can promote tumor metastasis through the degradation of extracellular matrix [16] and suppress the immune surveillance by inhibiting natural killer (NK) cell activation [17] and the cytolytic activity of cytotoxic T lymphocytes [18]. As the main regulator of pH_i, suppressed NHE1 would generate a decline in ATP formation as a consequence of lactic acid accumulation, which leads to metabolic disorders and cell death [19]. Therefore, NHE1 activation can promote cell growth, migration, invasion and inhibit apoptosis [20]; this effect also occurs in glioma cells [21]. In the present study, we verified that in C6 cells, PDT can induce the downregulation of NHE1, which was more obvious after combined treatment with TMZ. Interestingly, lower doses of TMZ

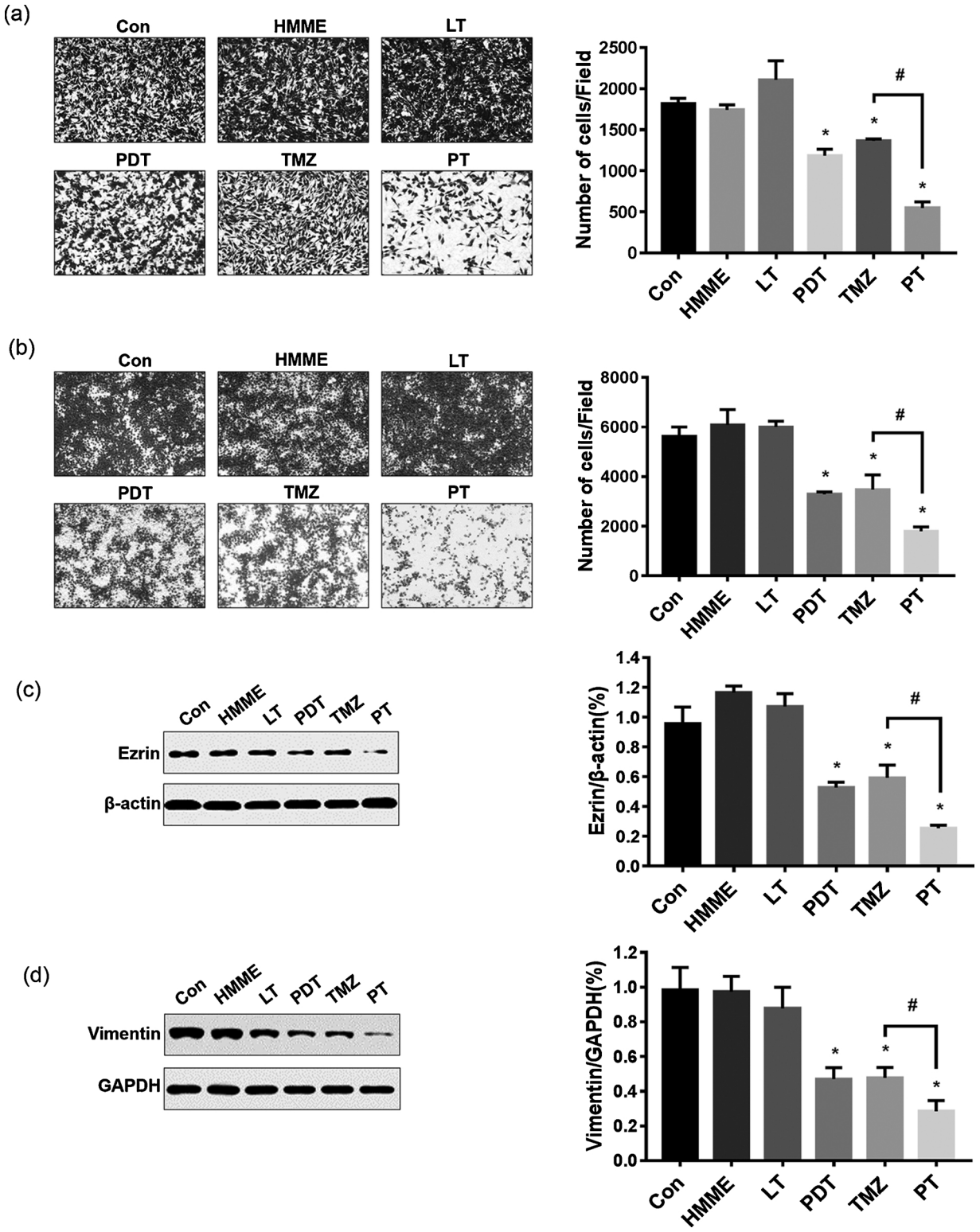

Fig. 4. Migration and invasion of C6 cells. (a) The capacity of C6 cells to migrate through Transwell chambers (8 μm pore size) under six different treatment conditions for 24 h as described above; the data were analyzed by image-pro Plus 6.0. Data are the means ± S.E.M. (n = 3). *P < 0.05 versus Con; #P < 0.05 versus TMZ. (b) The same method was used to detect the invasiveness of C6 cells except for the addition of 1:500 Matrigel matrix to the wells. The statistical analysis of the histogram is the same as above. (c) Ezrin expression under six different conditions as described above for 24 h was analyzed by Western blot. Data were normalized by β-Actin in each sample. Data are the means ± S.E.M. (n = 3). *P < 0.05 versus Con; #P < 0.05 versus TMZ. (d) Western blot analysis of vimentin under the conditions as described above for 24 h. β-Actin was used as a loading control; data were analyzed using GraphPad Prism 7 software. Data are the means ± S.E.M. (n = 3). *P < 0.05 versus Con; #P < 0.05 versus TMZ.

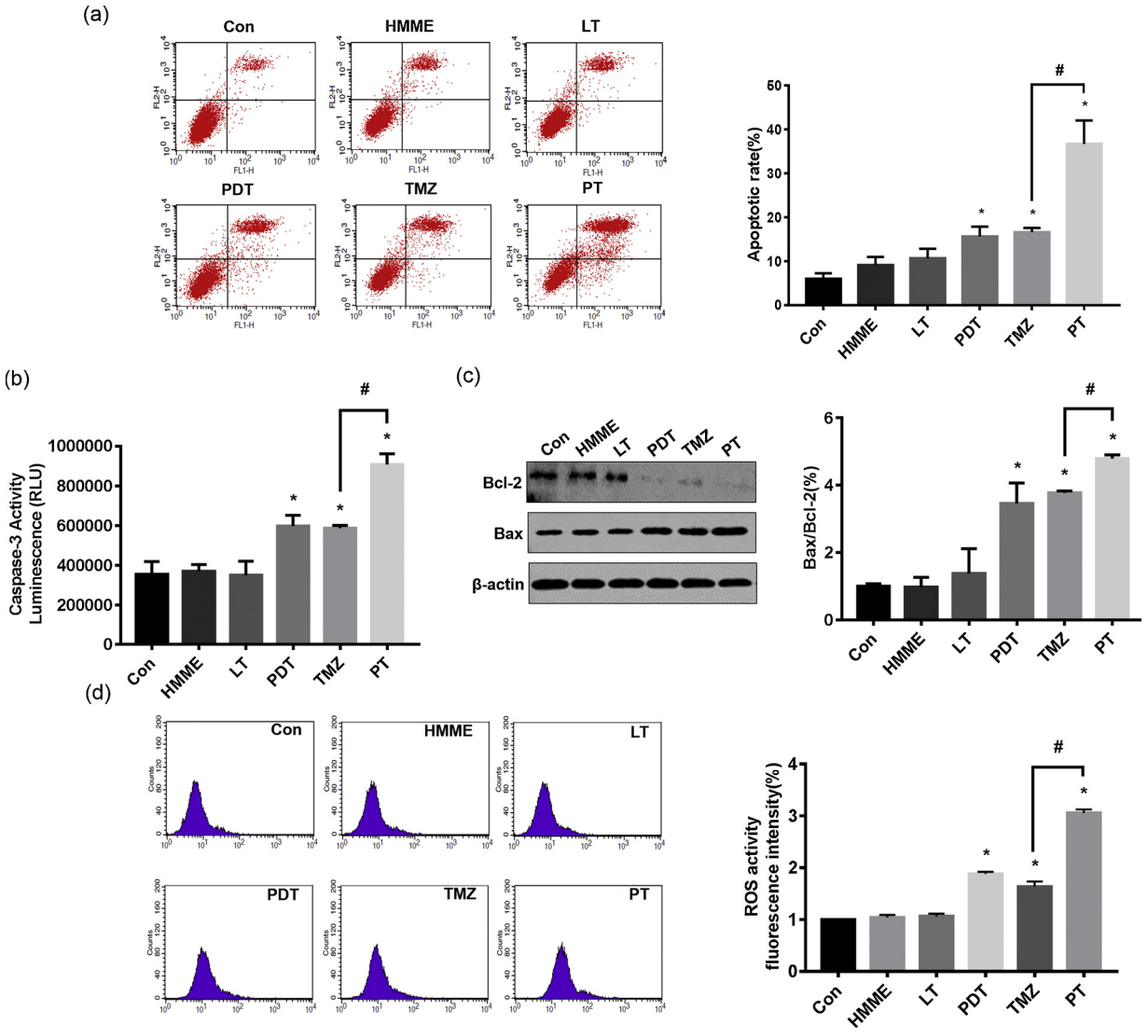

Fig. 5. Effects of different conditions on apoptosis in C6 cells. (a) A flow cytometry assay was used to evaluate C6 cells that were exposed to six different experimental conditions as described above for 24 h; the histogram was analyzed using GraphPad Prism 7 software. Data are the means ± S.E.M. (n = 3). *P < 0.05 versus Con; #P < 0.05 versus TMZ. (b) The activity of caspase-3 was detected using a caspase-3 activation kit. Data are the means ± S.E.M. (n = 3). *P < 0.05 versus Con; #P < 0.05 versus TMZ. (c) The expression levels of the apoptotic-related proteins Bcl-2 and Bax were analyzed by Western blot; relative expression levels of apoptotic-related proteins under different treatment conditions for 24 h were then normalized to β-Actin. Data are the means ± S.E.M. (n = 3). *P < 0.05 versus Con; #P < 0.05 versus TMZ. (d) The expression of ROS by was evaluated by flow cytometry under different conditions; the histogram was analyzed using GraphPad Prism 7 software. Data are the means ± S.E.M. (n = 3). *P < 0.05 versus Con; #P < 0.05 versus TMZ.

induced an increase in NHE1 expression, and increasing the TMZ concentration gradually reduced NHE1 expression. Therefore, TMZ has a dose-dependent effect on NHE1 levels in C6 cells. Our experiments innovatively combined PDT with TMZ therapy, which can more effectively reduce the activation of NHE1, reducing the migration and invasion of glioma cells and promoting apoptosis.

Gliomas have strong migration and invasion capacities. Ezrin plays an important role in tumor cell migration and invasion [12]. Increased ezrin expression can promote tumor cell metastasis, whereas decreased expression has the opposite effect [22]. Tynninen et al. also showed that the overall survival rate decreased in glioma patients with high ezrin expression [23]. Ezrin and NHE1 bind to the lamellipodia of the cell membrane and synergistically enhance the migration and invasion of glioma cells after TMZ treatment [9]. In our study, PDT could simultaneously inhibit the expression of NHE1 and ezrin, which jointly inhibited the migration and invasion of C6 cells. The inhibitory effect in the PT group was more obvious. Vimentin, as a component of the middle filament, plays an important role in maintaining cytoskeletal integrity and is highly expressed in tumors [24]. Negatively regulating the level of vimentin can inhibit EMT, which can promote glioma cell migration and invasion [25]. In this study, we proved that the vimentin protein was significantly downregulated after combined treatment with PT. Therefore, it can be inferred that PT affects the migration and invasion of glioma cells by inhibiting the EMT pathway; however, specific experiments to address this question were not performed in this paper, and further verification is needed (for example, to analyze the changes in relevant proteins in the EMT pathway, such as N-cadherin and E-cadherin).

Previous literature has suggested that intracellular alkalinization and extracellular acidification through the activation and upregulation of pH_i-regulating systems can be activated by NHE1 [26], and the authors suggested that the lactic acid produced by the cells was the main cause of cell death when NHE1 was mutated [27]. Therefore, NHE1 plays an important role in the pH_i-regulating system, and pH_i regulation can be a novel and effective method of antitumor therapy. According to our flow cytometry results, apoptosis is significantly changed after PDT

treatment in C6 cells. We further determined whether pH_i changes after therapy and found that the degree of acidification increased over time and that the pH_i changes were consistent with the observed changes in NHE1 protein expression levels. In addition, downregulated NHE1 expression attenuates extracellular acidification, which has been linked to multidrug resistance due to the neutralization of weak base chemotherapeutic drugs (which made the drugs less efficient) [28]. The drug resistance of TMZ was decreased through the inhibition of pH_i and the induction of apoptosis; similar results were also reported in a leukemia study [29]. The mechanisms that lead to apoptosis are complex and diverse, and mitochondrial-associated apoptosis is one of the most important pathways. Mitochondrial damage can release Cyt-c, which is followed by a series of related reactions, ultimately inducing apoptosis [30]. Bcl-2 family members play important roles in this process. In this study, compared to those of cells treated with PDT and TMZ alone, Bcl-2, which can inhibit apoptosis, was significantly downregulated, and Bax, which can promote apoptosis, was upregulated after PT treatment in C6 cells. Bcl-2 on the outer mitochondrial membrane can form heterodimers with Bax and change the membrane permeability, thereby participating in the release of Cyt-c [31,32]. Our experiments also demonstrated the release of large amounts of ROS after PT treatment by damaging the mitochondria, which can downregulate NHE1 protein levels [1]. Therefore, the increase in ROS can cause the pH_i to decrease. A previous study reported that low pH_i could accelerate the rates of formation of Cyt-c and Apaf-1 polymers, further facilitating caspases activation [33]. Moreover, our research also demonstrated that the activity of caspase-3 was significantly increased in the PT group. Therefore, we concluded that PT can influence the mitochondrial signaling pathway to induce apoptosis by decreasing the NHE1 expression in glioma cells.

However, our study has certain limitations. Only high concentrations of TMZ were studied, which may require evaluation before being applied in the clinic. A previous study showed that low TMZ concentrations can upregulate NHE1 expression and, as a result, promote the migration and invasion of glioma tumor cells [9]. Therefore, we do not know whether the low concentration of TMZ combined with PDT can produce the same experimental results as this study; therefore, further research is needed.

In summary, this study demonstrated that TMZ regulates the expression of NHE1 in C6 cells in a dose-dependent manner and that PDT can reduce the resistance induced by TMZ chemotherapy. In addition, PT can synergistically inhibit the migration and invasion of C6 cells by inhibiting NHE1 protein expression. Cotreatment can also induce mitochondrial-associated apoptosis by downregulating NHE1 in vitro. Combining PDT with TMZ therapy may be a potential treatment for glioma in the future.

Conflicts of interest.

The authors declare that there are no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.pdpdt.2019.05.007.

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