Hyperbaric oxygen as an adjuvant to temozolomide nanoparticle inhibits glioma growth by inducing G2/M phase arrest

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Aim: To study the effects of combinational treatment of hyperbaric oxygen (HBO) and nanotemozolomide in glioma. **Materials & methods:** Temozolomide (TMZ)-loaded porous silicon nanoparticles (TMZ/PSi NPs) were prepared. *In vitro* and *in vivo* evaluations were performed. **Results:** The cell uptake of TMZ/PSi NPs could be tracked by autofluorescence of porous silicon. The concentration of oxygen in tumor was improved and the antitumor rate was increased to 84.2% in the TMZ/PSi NPs combined with HBO group. The viability of hypoxia-induced glioma C6 cells was decreased and cell cycle was arrested at G2/M phase in response to TMZ/PSi NPs treatment with HBO compared with continuous treatment with hypoxia. **Conclusion:** The combinational treatment of TMZ/PSi NPs and HBO could be a promising therapeutic strategy for glioma.

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Keywords: cell cycle • combinational treatment • glioma • hyperbaric oxygen • hypoxia • hypoxia-induced • nanodrug • porous silicon nanoparticle • temozolomide • xenograft

Glioma is the most common and lethal primary brain cancer in humans in Europe and the USA [1]. The current standard treatment for this disease is maximal safe resection followed by radiation- or chemotherapy [2,3]. Temozolomide (TMZ), an oral imidazotetrazinone methylation agent that inactivates the DNA repair enzyme O⁶-alkylguanine-DNA alkyltransferase, is a primary choice for chemotherapy of glioma in clinic [4]. TMZ improves the glioma patient survival rate and prolongs the progression-free survival after surgery therapy [5]. However, considerable toxicity of TMZ limits its therapeutic dosage [6]. The 5-year survival rate is still less than 8% under current standard therapy due to the high malignancy of glioma [2,7,8]. Thus, strategies to improve conventional chemotherapeutic drug against glioma are sorely needed.

Nanodrugs have the intrinsic advantages of accumulating into tumor by the enhanced permeability and retention effect (EPR) [9,10]. Kim *et al.* had administered a cationic liposome (DOTAP: DOPE) encapsulating TMZ directly into the brain tumor in animal model [11]. The drug was more effective than standard TMZ in both TMZ-resistant and TMZ-sensitive glioma. Poly(β -L-malic acid), poly(lactide-*co*-glycolide), amphiphilic chitosan-grafted-functionalized polylactic acid and mesoporous silica were used to encapsulate TMZ to induce the glioma cell apoptosis *in vitro* [6,12–14]. Porous silicon nanoparticles (PSi NPs) are characterized with adjustable pore size with a large drug-loading capacity and strong intrinsic fluorescence for real-time imaging [15,16]. Moreover, PSi NPs have good biocompatibility and biodegradability [17,18]. Therefore, PSi NPs are an ideal drug-delivery system of TMZ for glioma therapy *in vivo*.

Hypoxia is suggested to contribute to the resistance of glioma to TMZ [19]. It is widely accepted that hypoxia promotes tumor growth, angiogenesis and invasion [20]. Increasing oxygen concentration in tumor tissue may enhance chemotherapy effects. Hyperbaric oxygen (HBO) therapy holds promise as an approach to overcome the problem of oxygen deficiency in the poorly oxygenated regions of the neoplastic tissue. HBO therapy is the use of oxygen under elevated atmospheric pressure, which is at a pressure higher than 1 atm [21]. The pO₂ level can be

high for prolonged periods (15–60 min) post-treatment of HBO, which was beneficial for improving hypoxia [20]. HBO improves the radiation or chemotherapy response in gliomas [22–24]. However, in anticancer therapy, HBO alone has a limited curative effect and is usually not applied by itself [25–28]. HBO treatment combined with chemotherapy has increased sensitivity in the treatment of solid tumors in mice [29–31]. Enhanced therapeutic effects were observed in combinational treatment of HBO and TMZ in C6 glioma rat model and on glioma U251 cell lines, respectively [19,32]. Considering the enhanced cellular uptake of nanodrug delivery [33], it is worth to investigate whether the combinational treatment of HBO and TMZ delivered by nanoparticle is more effective for glioma *in vivo*.

In this study, we investigated the effects of combinational treatment of TMZ encapsulated in PSi NPs and HBO, and underlying mechanism on glioma in mouse model. Interestingly, the combinational treatment of TMZ/PSi NPs and HBO, which inhibited tumor growth through arresting the tumor cell cycle progression at the G2/M phase, was proved to be a powerful strategy for anti-glioma.

Materials & methods

Materials

The boron-doped p-type silicon wafers (0.8–1.2 m Ω /cm resistivity, (100) orientation) were purchased from Virginia Semiconductor, Inc. (VA, USA). Chemotherapy drug TMZ was obtained from Aladdin Reagent Co. Ltd. (Shanghai, China). 40,6-Diamidino-2-phenylindole was obtained from Beyotime Institute of Biotechnology (Shanghai, China).

Rat glioma C6 cell line was purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and preserved in our lab. Male BALB/c/nude mice (6–8 weeks old) were obtained from the Beijing HFK Bioscience Co. Ltd. (Beijing, China). The animal studies were approved by the Animal Experimentation Ethics Committee of College of Life Science and Technology, Huazhong University of Science and Technology. All procedures were carried out in strict with the animal care guidelines of the Science and Technology Department of Hubei Province.

Preparation of PSi & TMZ/PSi NPs

Preparation of PSi NPs was according to the report previously [34,35]. Briefly, PSi NPs were prepared by electrochemical etching of single-crystal silicon wafers in aqueous hydrofluoric acid /ethanol (4:1, v/v) solution at a constant current density of 165 mA/cm² for 300 s using a Teflon etch cell (Wuhan, China). The porous silicon film was fractured into NPs by ultrasonication in ultrapure water, centrifuged at 8000 rpm for 20 min for removing deposits, heated at 60°C for 3 h to activate and then PSi NPs were collected by ultrafiltration.

Loading of TMZ into PSi NPs was conducted through adding TMZ dissolved in methanol into the NPs dropwise at a weight ratio of 1:5 under stirring at room temperature overnight. TMZ-loaded PSi NPs (TMZ/PSi NPs) were collected by ultrafiltration centrifugation at 4500 rpm for 10 min and washed three-times with methanol. The amount of TMZ loaded into PSi NPs was measured using ultraviolet spectrophotometer.

Characterization of PSi & TMZ/PSi NPs

The morphology and the size of PSi and TMZ/PSi NPs were characterized by transmission electron microscopy (JEM-2010; JEOL, Tokyo, Japan). The hydrodynamic diameter and mean zeta potential of the NPs were determined by dynamic light scattering (ZetaSizer Nano-ZS 90, Malvern Instruments Ltd., Worcestershire, UK). To evaluate the fluorescence property of the materials, a Fluorolog-3 spectrofluorometer (Horiba Jobin Yvon, Longjumeau, France) was used at $\lambda_{\text{ex}} = 488$ nm.

The photostability of the NPs was evaluated in ultrapure water on a Fluorolog-3 spectrofluorometer when PSi NPs were illuminated with a 100 W mercury lamp at 488 nm for different time courses.

Drug release test

TMZ-loaded NPs were collected by ultrafiltration, put into dialysis bag and then shaken at 150 rpm. Release kinetics of TMZ from TMZ/PSi NPs in PBS at 37°C was measured by filtering out TMZ from the solution at each time point and the amount of TMZ was measured by UV-Vis absorption spectra at 328 nm.

Cell uptake analysis

C6 cells were cultured in DMEM containing 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in the presence of 5% CO₂. 2×10^5 cells/well were plated onto plates for 24 h, and then the cell culture medium was replaced by the fresh culture medium containing 400 µmol/l TMZ/PSi NPs for 2 h. Cell samples were washed three-times with PBS buffer, and stained with 40,6-diamidino-2-phenylindole according to the kit manual. The subsequent cell observation was performed with laser scanning confocal microscopy (LSM710 NLO, Zeiss, Germany) through a 488 excitation filter.

In vivo antitumor effects of combinational treatment of TMZ/PSi & HBO

3×10^6 C6 cells were inoculated into a nude mouse to establish the subcutaneous tumor model. The mice bearing tumor were randomly divided into six groups: model group, PSi group, HBO group, TMZ/PSi group, TMZ combined with HBO group, and TMZ/PSi combined with HBO group. On day 8 after inoculation of tumor cells, the tumor burden mice were administered intratumor injection with TMZ or TMZ/PSi NPs at the dose of 5 mg/kg TMZ followed with or without HBO treatment under 2.5 atm for 2 h on days 1, 3, and 5 in the related groups, respectively. The sizes of the tumors were measured every 2 days. On day 22, all the mice were sacrificed, and the tumors were fixed within 4% paraformaldehyde, sectioned and stained for tumor proliferation analysis.

Proliferation analysis of tumor tissue

Immunohistochemical staining for the nuclear proliferation-associated antigen Ki-67 was conducted using the mouse monoclonal antibody MIB1 according to the report [36]. The percentage of stained cell nuclei (marked antigen Ki-67) among all nuclei visible in the field was estimated.

Analysis of oxygen fields in tumor tissue

For assessing changes of tumor oxygen concentration after HBO treatment, hypoxic marker pimonidazole was applied. Pimonidazole protein adducts are only detected in hypoxic cells after systemic administration, which can be determined by immunohistochemistry [37]. In this study, after a single dose of HBO treatment for 2 h in mice bearing tumor, pimonidazole (dissolved in saline) was intravenous injected via tail vein at a dosage of 60 mg/kg according to the manual of Hypoxyprobe-1™ Kit (Hypoxypore, Inc., MA, USA). In the control group, the experiment was conducted without HBO treatment. 90-min after injection, tumor tissues were harvested and fixed. Antipimonidazole mouse IgG1 monoclonal antibody was used for the detection of pimonidazole protein adducts.

Cell viability assay of TMZ after treatment with different oxygen concentrations

To test C6 cell viability under different concentrations of oxygen at different concentrations of TMZ (50, 100, 200, 400, 800, 1600 µM), C6 cells were divided into normobaric, hypoxia and HBO groups, respectively. C6 cells (6×10^3 cells/well) were plated into 96-well plates. Cells were cultured in normal incubator with 5% CO₂ in the normobaric oxygen group, whereas cells were incubated under 1% O₂ and 5% CO₂ in the hypoxia group for 48 h before adding TMZ. In the HBO group, cells were incubated under hypoxia condition for 48 h, and then incubated under 100% O₂ for 2 h/day after adding TMZ. After treatment with TMZ for 72 h, 20 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/ml) was added to each well. Then, formazan crystals were solubilized with 150 µl of DMSO 4 h later. The absorbance at 490 nm was used to determine the relative cell viability.

Evaluation of TMZ/PSi on cell viability after treatment with different oxygen concentrations

According to the cell viability assay about TMZ, one toxicity concentration of TMZ was used to evaluate the TMZ/PSi NPs effect on C6 cells. C6 cells were incubated with PSi, TMZ or TMZ/PSi NPs at the different oxygen concentrations. After rinsing with PBS, the cells were fixed with trichloroacetic acid and then dyed with 70 µl of sulforhodamine B (1%) for 30 min. After that, cells were washed with 1% acetic acid to remove the unbound sulforhodamine B. The plates were dried at room temperature, and 100 µl of TRIS base (10 mM) was used to solubilize the bound stain. Absorbance at 540 nm was used to determine the relative cell viability.

Figure 1. Morphologies and sizes of porous silicon and temozolamide/porous silicon, respectively. (A) Morphologies and sizes of PSi and TMZ/PSi under transmission electron microscope. **(B)** Diameters of dynamic light scattering of PSi and TMZ/PSi were 103.7 and 117.3 nm, respectively. PSi: Porous silicon; TMZ: Temozolamide.

Cell cycle analysis

To analyze cell cycle progression, C6 cells were plated into 6-well plates at a density of 2×10^5 cells/well. TMZ or TMZ/PSi NPs was added into cells after treatments with different oxygen concentrations. After that, cells were harvested and washed three-times with PBS, and then fixed in 70% ethanol at 4°C overnight. Cells were then dyed with 100 μ l propidium iodide (PI) solution (50 μ g/ml PI and 100 μ g/ml RNase A in PBS) at 4°C for 30 min. Then, flow cytometry was used for PI detection. Results were analyzed using the CXP analysis software (Beckman Coulter, KS, USA).

Statistical analysis

Experiments were repeated at least three-times. Data were analyzed by Student's t-test, and $p < 0.05$ was regarded as statistically significant.

Results & discussion

Characterization of TMZ/PSi NPs

From transmission electron microscope images, the average size of PSi and TMZ/PSi NPs was about 100 nm, respectively (Figure 1A). The irregular shapes of two kinds of NPs were observed. The results of dynamic light scattering showed that the hydrodynamic diameters of PSi and TMZ/PSi were about 103.7 and 117.3 nm,

Figure 2. Stability and release of temozolomide/porous silicon. (A) Diameter of temozolomide/PSi nanoparticle remains stable in a week at room temperature. **(B)** The fluorescence intensity increased along with oxygen time exposed to air. **(C)** The release of temozolomide was completed within 24 h. PSi: Porous silicon.

respectively (Figure 1B), implying a potential passive targeting ability through EPR effect. The zeta potential of PSi and TMZ/PSi was -18.8 and -17.6 mV, respectively, implying less size variation and protein adsorption *in vivo* [38].

The NPs remained similar size at room temperature for 1 week (Figure 2A). The intensity of fluorescence of TMZ/PSi NPs was increased along with the oxygenation time when it was exposed to air (Figure 2B), thereby allowing potential intracellular location according to their fluorescence. The drug loading of TMZ was about 7%. TMZ release (pH 6.5) was completed within 24 h (Figure 2C), suggesting that TMZ is effectively encapsulated within the porous silicon and has a sustained-release property.

Cell uptake of TMZ/PSi NPs

TMZ acts as a prodrug of the cytotoxic compound 5-(3-methyltriazene-1-yl) imidazole-4-carboxamide and alkylates guanine in genomic DNA at position O⁶ with subsequent impaired DNA repair [39]. Given that TMZ plays an essential role in cell nucleus, the internalization of TMZ/PSi NPs by tumor cells was evaluated in C6 cells. *In vitro*, cell uptake of PSi or TMZ/PSi NPs could be tracked by autofluorescence of porous silicon (Figure 3). PSi or TMZ/PSi NPs exhibited a remarkable cellular uptake after 2-h incubation. The data showed that TMZ/PSi NPs were localized in perinuclear region after internalization, providing additional advantages in possibly bypassing pump resistance.

Antitumor effects of TMZ/PSi NPs in combination with HBO

In our study, the combined therapy of nanomedicine of TMZ/PSi and HBO presented ideal antitumor effects. The relative tumor volume changes were plotted and shown in Figure 4A. The mice injected with PBS buffer exhibited a rapid growth of tumor volume. The tumor growth rate after treatment with PSi or HBO alone presented no significant difference compared with that in the model group, suggesting PSi or HBO has no obvious effect on antitumor. In comparison, the significant inhibition of tumor growth was observed in TMZ/PSi, TMZ combined

Figure 3. C6 cell uptake of temozolomide/porous silicon. TMZ/PSi was internalized and located near nuclear. Nuclear was stained by DAPI as blue color and red color represented PSi. Scale bar, 10 μ m. DAPI: 40,6-Diamidino-2-phenylindole; PSi: Porous silicon; TMZ: Temozolomide.

Figure 4. Antitumor effects of combination treatment of temozolomide/porous silicon and hyperbaric oxygen. Drugs were administered three-times in 2-day intervals. Tumor sizes were serially measured with calipers every 2 days. **(A)** Changes of tumor volume after treatments. **(B)** Tumors from mice after different treatments. **(C)** Tumor weight. $n = 5$. $**p < 0.01$; $***p < 0.001$; versus saline. $\#p < 0.05$; TMZ/PSi + HBO versus TMZ/PSi. HBO: Hyperbaric oxygen; ns: No significant difference. PSi: Porous silicon; TMZ: temozolomide.

Figure 5. Cell proliferation in tumor after different treatments. Immunohistochemical staining for the nuclear proliferation-associated antigen Ki-67 (left). Brown color represents positive staining. (A) Saline, (B) PSi, (C) TMZ/PSi, (D) HBO, (E) TMZ + HBO and (F) TMZ/PSi + HBO. Relative optical density in each tumor slices of immunohistochemical staining as above (right).

*** $p < 0.001$, versus saline.

HBO: Hyperbaric oxygen; PSi: Porous silicon; TMZ: Temozolomide.

Figure 6. Oxygen fields were detected in tumor tissue by hypoxic probe. Hypoxic marker pimonidazole (green) was found in tumor in the control, and nuclei (blue) were stained by DAPI (A). Quantification of intensity of fluorescence (B).

* $p < 0.05$. Scale bar, 100 μm .

DAPI: 40,6-Diamidino-2-phenylindole; HBO: Hyperbaric oxygen.

with HBO, and TMZ/PSi combined with HBO groups. An increased effect for inhibiting tumor growth was observed in the mice treated by TMZ or TMZ/PSi NPs combined with HBO, indicating that HBO can be used as an adjuvant to TMZ or TMZ/PSi NPs in glioma therapy. In contrast, significant inhibition of tumor growth by TMZ/PSi NPs combined with HBO was observed compared with other groups on day 22 postinoculation of tumor cells (Figure 4B). The results of tumor weight change were in line with the results of tumor size (Figure 4C).

Figure 7. Cell viability after treatment with temozolomide or temozolomide/porous silicon nanoparticles under different oxygen concentrations. Under normobaric, hypoxia or hypoxia/HBO, cell viability was decreased along with increasing of TMZ concentration, whereas no significant change of cell viability was detected in hypoxia group (50–800 μmol/l TMZ) (A). Cell viability of hypoxia-induced C6 cells was decreased significantly when treated with TMZ/PSi at 400 μmol/l TMZ followed with HBO (100% O₂) treatment (B).

p < 0.01; *p < 0.001.

HBO: Hyperbaric oxygen; PSi: Porous silicon; TMZ: Temozolomide.

The tumor inhibition rate was about 84.2% in the TMZ/PSi combined with HBO group. These results revealed that the integration of TMZ/PSi NPs and HBO can significantly enhance the antitumor efficacy.

Proliferation inhibition of tumor tissue after treatment with TMZ/PSi NPs in combination with HBO

In order to evaluate the level of tumor cell proliferation *in vivo*, we countered cells positively reacting to Ki-67 by the immunoperoxidase method [40]. The expression level of Ki-67 protein in a tumor is related to a proliferative rate [19]. In this study, after treatment of TMZ/PSi in combination with HBO, tumor tissue sections were stained with the Ki-67 antibody on day 22 postinoculation of tumor cells. HBO treatment alone has few effects on proliferation of tumor, which is consistent with the report that hyperoxic treatment showed a slight but insignificant decrease in tumor cell proliferation [20]. As shown in Figure 5A, the number of Ki-67-positive cells (brown color) was lower in the combinational treatment of TMZ/PSi and HBO than that in the TMZ/PSi group. Therefore, HBO could be used as an adjuvant for TMZ/PSi therapy. Cell proliferations in the tumor tissue were significantly suppressed in the TMZ combined with HBO group, too (Figure 5B). Therefore, combinational treatment of TMZ/PSi and HBO is a potential strategy for glioma therapy.

Modification of hypoxia region in subcutaneous tumor tissue after treatment with HBO

Hypoxia is a typical feature in solid tumor tissue. To detect the change of hypoxia regions in tumor after treatment of HBO, the C6 glioma subcutaneous tumor model was established. Hypoxia fields in tumors with or without HBO treatment were detected on day 8 postinoculation of tumor cells. Hypoxia fields detected by the probe were obviously decreased after treatment with HBO (Figure 6A). Above 50% hypoxia regions were modified (Figure 6B). The increase of oxygen tension in tumors improved the chemotherapy response in solid tumors [23]. Therefore, improved oxygen regions in tumor after HBO treatment may render glioma cells sensitive to TMZ or TMZ/PSi.

Effects of TMZ & TMZ/PSi NPs on cell viability at different oxygen concentrations

To further demonstrate tumor cell viability affected by oxygen concentration after treatment with TMZ or TMZ/PSi NPs, C6 cells were cultured under different oxygen concentrations *in vitro*. As shown in Figure 7A, the significant

Figure 8. Cell cycle measured by flow cytometer. Compared with hypoxia treatment, proportion of G2/M phase cell was increased after treatment with hypoxia followed by HBO in TMZ and TMZ/PSi groups. HBO: Hyperbaric oxygen; PSi: Porous silicon; TMZ: Temozolomide.

decrease of C6 cell viability was induced by TMZ at 1600 $\mu\text{mol/l}$ under hypoxia condition, whereas it was as low as 200 $\mu\text{mol/l}$ after HBO treatment. It demonstrated that hypoxia-induced C6 cells were sensitive to TMZ after HBO treatment.

Furthermore, the effect of TMZ/PSi NPs (400 $\mu\text{mol/l}$, TMZ) on C6 glioma cells at different oxygen concentrations was studied. The decreased cell viability of TMZ/PSi NPs was observed after treatment with HBO in hypoxia-induced C6 cells compared with that without HBO treatment (Figure 7B). The internalization of TMZ/PSi NPs significantly decreased cell viability even under safety dose of TMZ when combined with HBO. Taken together, HBO could enhance the sensitivity of hypoxia-induced C6 cells to TMZ or TMZ/PSi NPs treatment.

HBO potentiates the cell cycle arrest

TMZ alkylates guanine in genomic DNA at position O⁶ inducing impaired DNA repair and G2/M arrest [39]. To further illustrate the underlying mechanism that HBO enhancing cell sensitivity to TMZ or TMZ/PSi NPs, the cell cycle was detected by fluorescence-activated cell sorting analysis. As shown in Figure 8, C6 cells arrested at G2/M phase of cell cycle was increased to 27% after treatment with TMZ under normobaric oxygen, whereas it was 18% under hypoxia condition. Similarly, increased proportion of G2/M phase cells (23%) was induced by combinational treatment of HBO and TMZ/PSi NPs (TMZ). G2/M phase arrest can inhibit tumor cell proliferation. Therefore, HBO enhanced the C6 cell sensitivity to TMZ or TMZ/PSi NPs through inducing cell cycle arrested at G2/M phase.

Conclusion

TMZ/PSi NPs combined with HBO treatment suppressed tumor growth significantly. Enhanced sensitivity of hypoxia-induced tumor cell to TMZ/PSi NPs was detected after treatment with HBO, which may be attributed to arrest tumor cells at G2/M phase. Taken together, the combinational treatment of TMZ/PSi NPs and HBO may be a promising therapeutic strategy for glioma.

Summary points

- Hypoxia is a critical hallmark of solid tumors and involved in drug resistance.
- Combinational treatment of hyperbaric oxygen (HBO) therapy and temozolomide (TMZ) loaded in porous silicon nanoparticles (PSi NPs) was used for glioma.
- The cell uptake of TMZ/PSi NPs could be tracked by autofluorescence of porous silicon.
- The concentration of oxygen in tumor was improved after treatment with HBO.
- The antitumor rate was about 84.2% in the TMZ/PSi NPs combined with HBO group.
- The cell proliferation in tumor was inhibited after combinational treatment of TMZ/PSi NPs and HBO.
- After treatment with HBO, the decreased viability in hypoxia-induced glioma C6 cells was observed in TMZ/PSi NPs treatment group.
- Inhibition of tumor cell proliferation is ascribed to the mechanisms that HBO induces cell cycle arrested at the G2/M phase in response to TMZ/PSi NPs.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval. All procedures were carried out in strict with the animal care guidelines of the Science and Technology Department of Hubei Province.

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