

Hyperbaric oxygen promotes malignant glioma cell growth and inhibits cell apoptosis

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Abstract. Glioblastoma multiforme (GBM) is the most frequently diagnosed intracranial malignant tumor in adults. Clinical studies have indicated that hyperbaric oxygen may improve the prognosis and reduce complications in glioma patients; however, the specific mechanism by which this occurs remains unknown. The present study investigated the direct effects of hyperbaric oxygen stimulation on glioma by constructing an intracranial transplanted glioma model in congenic C57BL/6J mice. Bioluminescent imaging (BLI) was used to assess the growth of intracranial transplanted GL261-Luc glioma cells *in vivo*, while flow cytometric and immunohistochemical assays were used to detect and compare the expression of the biomarkers, Ki-67, CD34 and TUNEL, reflecting the cell cycle, apoptosis and angiogenesis. BLI demonstrated that hyperbaric oxygen promoted the growth of intracranially transplanted GL261-Luc glioma cells *in vivo*. Flow cytometric analysis indicated that hyperbaric oxygen promoted GL261-Luc glioma cell proliferation and also prevented cell cycle arrest. In addition, hyperbaric oxygen inhibited the apoptosis of the transplanted glioma cells. Immunohistochemical analysis also indicated that hyperbaric oxygen increased positive staining for Ki-67 and CD34, while reducing staining for TUNEL (a marker of apoptosis). The microvessel density was significantly increased in the hyperbaric oxygen treatment group compared with the control group. In conclusion, hyperbaric oxygen treatment promoted the growth of transplanted malignant glioma cells *in vivo* and also inhibited the apoptosis of these cells.

Introduction

Gliomas originate from glial cells and are the most common type of intracranial tumor, accounting for 40-60% of brain tumors (1,2). Glioblastoma multiforme (GBM) is the most frequently diagnosed intracranial malignant tumor in adults (3). The prognosis for GBM patients is invariably poor, even when the patients receive complete surgical resection combined with chemoradiotherapy (1,4). According to a previous study, the postoperative median survival time for GBM patients was ≤ 15 months, and only 26.5% of patients survived for 2 years after diagnosis (1).

Hypoxia-induced apoptosis and angiogenesis are key indicators for the diagnosis of GBM. A large hypoxic area in the GBM tissue is associated with poorer prognosis in GBM patients (5,6). Previous studies have indicated that glioblastoma stem cells were present in the hypoxic area and developed resistance to chemotherapy and radiotherapy (7). By contrast, well-differentiated glioblastoma cells were located in the marginal and well-perfused areas of tumor tissues (8,9). Previous studies proposed that hyperbaric oxygen stimulation prior to chemotherapy and radiotherapy may increase the efficacy of the treatment and improve prognosis, as hyperbaric oxygen may increase the oxygen content of tumor tissue (10,11). Hyperbaric oxygen stimulation of GBM patients has previously resulted in longer median survival rates and reduced side effects (12-15). Temozolomide (TMZ) is a novel drug for the treatment of glioma. The cytotoxicity effects of TMZ on chemotherapy-resistant and chemotherapy-sensitive cells increased when the cells were also stimulated with hyperbaric oxygen (16). *In vivo*, TMZ treatment combined with hyperbaric oxygen may reduce angiogenesis, increase apoptosis and inhibit drug resistance in tumor cells (17).

The direct effect of hyperbaric oxygen stimulation on malignant glioma cells remains unknown. Stuhr *et al* (18) proposed that hyperbaric oxygen may inhibit the growth of subcutaneous transplanted glioma cells in C57BL/6J mice; however, no cytological experiments were performed and the subcutaneous transplanted gliomas were quite different from intracranial gliomas, which originate from glia cells *in situ* (19).

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The influence of hyperbaric oxygen on non-tumor cells is complicated: Short exposure to hyperbaric oxygen has been demonstrated to promote tumor cell proliferation, while long exposure results in apoptosis and inhibits proliferation (20). In addition, another previous study demonstrated that hyperbaric oxygen promoted angiogenesis by inducing oxidative stress under physiological conditions (21). In the present study, an intracranial transplanted glioma model in congenic mice was constructed to investigate the direct effects of hyperbaric oxygen stimulation on transplanted glioma cells *in vivo*.

Materials and methods

Cell culture. The human glioma GL261/GL261-Luc cell line was obtained from the Division of Cancer Treatment and Diagnosis Tumor Repository, National Cancer Institute (NCI; Frederick, MD, USA). This cell line was routinely cultured in RPMI 1640 medium (GE Healthcare Life Sciences, Logan, UT, USA) supplemented with heat-inactivated fetal bovine serum (Gibco Life Technologies, Carlsbad, CA, USA) in a humidified atmosphere with 5% CO₂ at 37°C.

Tumor xenograft assay. Inbred 10-week-old female congenic C57BL/6J mice (NCI) were maintained under pathogen-free conditions. GL261-Luc glioma cells were injected into the caudate nucleus of the right brain hemisphere, as a 5 μ l suspension of 5 \times 10⁵ cells (1 \times 10⁸ cells/ml) in an equal volume of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). A total of 12 mice were divided into experimental (n=6) and control groups (n=6) and treated accordingly; the two groups were raised in the same conditions, however, the mice in the experimental group were exposed to the hyperbaric intervention process. *In vivo* bioluminescence imaging (BLI) was applied to measure the tumor volumes. This study was approved by the ethics committee of Beijing Sanbo Brain Hospital (Beijing, China).

Hyperbaric oxygen intervention process. Mice in the hyperbaric oxygen treatment group were placed into an NG90-IIIB medical hyperbaric oxygen chamber (Ningbo Hyperbaric Oxygen Chamber Plant, Ningbo, China) and pressurized to 2.5 atmospheres at a rate of 0.015 MPa/min for 10 min. This pressure was maintained for 60 min and decompression was performed at the same rate for 10 min. The hyperbaric oxygen intervention process was performed daily for 10 days.

Immunohistochemical staining. Following 10 days of exposure to the hyperbaric oxygen intervention process, the C57BL/6J mice injected with GL261-Luc glioma cells were sacrificed. Next, the tumors were removed, fixed in 10% formalin for 24 h, embedded in paraffin and sectioned at 5 μ m thickness. Following rehydration, antigen retrieval was performed by heating in EDTA (pH 8.0) for 15 minutes, and endogenous peroxidase activity was blocked using 3% hydrogen peroxide at 37°C for 10 min. Nonspecific binding was blocked using 3% skimmed milk for 2 h at 37°C. Rabbit anti-human polyclonal Ki-67 (1:500; cat. no. ab15580; Abcam, Cambridge, MA, USA) and rat anti-human monoclonal CD34 (1:100; cat. no. ab8158; Abcam) antibodies and TUNEL reagent

(cat. no. ab66108; Abcam) were applied to each slide and incubated at 37°C for 2 h. The slides were then incubated using an Envision™ kit (Dako, Glostrup, Denmark) for 30 min following 3 washes, while 3,3'-diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA) was used as a chromogen. The sections were counterstained with hematoxylin (Sigma-Aldrich), dehydrated with graded ethanol and xylene and mounted in neutral balsam (CWBio, Inc., Beijing, China).

Flow cytometry. GL261 glioma cells (~1 \times 10⁶) were harvested by 0.25% EDTA and then fixated in pre-cooled 75% ethanol for 2 h at 4°C. Subsequently, the cells were washed twice with phosphate-buffered saline (PBS), resuspended in 500 μ l PBS with 1 μ g/ml RNase A (Life Technologies, Grand Island, NY, USA) and 40 μ g/ml propidium iodide (PI; Biotool, Houston, TX, USA), and then incubated at 37°C for 30 min. An Annexin V/PI kit (Biotool) was used to detect the externalization of phosphatidylserine in apoptotic cells and all experiments were conducted according to the manufacturer's instructions. The cells were immediately analyzed by flow cytometry (BD Biosciences) with Cell Quest Lysis II software (BD Biosciences), and 5,000 events were observed.

Statistical analysis. The data from at least three independent experiments were analyzed using a two-tailed Student's t-test. The data are presented as the mean \pm standard error (SE), in which the SE is presented as the error bars. $P < 0.05$ was considered to indicate a statistically significant difference. SPSS software package (version 14.0; SPSS, Inc., Chicago, IL, USA) was used for data analysis.

Results

Hyperbaric oxygen promoted growth of transplanted glioma. After 10 days of GL261-Luc inoculation, BLI was employed to gain real-time images of the tumors *in vivo* (Fig. 1A). The photon area and photon sum were also determined using the subsidiary software. No statistically significant difference was identified between the light-emitting area of the hyperbaric oxygen group and the control group (476.67 \pm 190.46 vs. 498.83 \pm 273.73; $P = 0.872$). The difference between the sum of emitted photons (x1,000) in the two groups was also not statistically significant (5,677.83 \pm 2,346.48 vs. 6,069.17 \pm 3,430.37; $P = 0.822$; Fig. 1B and C).

To investigate the effects of hyperbaric oxygen on intracranial glioma cells, 6 congenic C57BL/6J mice were subjected to repeated hyperbaric oxygen stimulation at day 10 after inoculation. After a further 10 days from the repeated hyperbaric oxygen treatment, further real-time images were obtained in the same congenic C57BL/6J mice (Fig. 1A). The light-emitting area of the intracranial glioma in the hyperbaric oxygen group was significantly increased compared with the control group (2,767.50 \pm 811.44 vs. 1,744.50 \pm 572.04; $P = 0.030$). The sum of emitted photons (x1,000) was also significantly increased in the hyperbaric oxygen group compared with the control group (45,642.50 \pm 17,613.99 vs. 22,217.83 \pm 7,273.73; $P = 0.013$; Fig. 1B and C).

Hyperbaric oxygen promoted glioma cell proliferation and escape from cell cycle arrest. Flow cytometric analysis

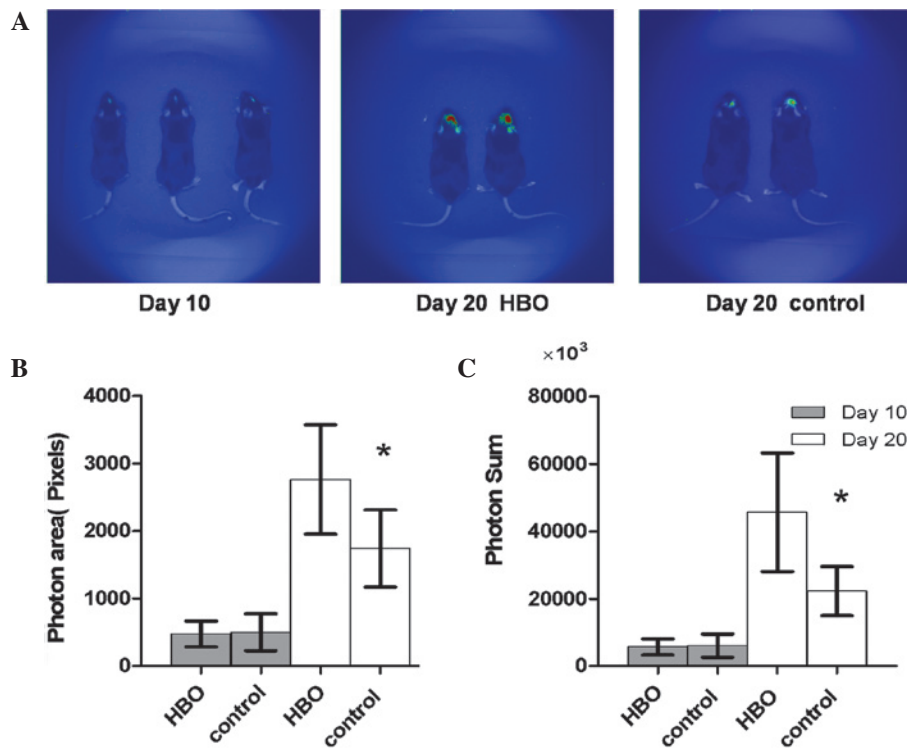


Figure 1. Hyperbaric oxygen (HBO) promoted the growth of intracranially transplanted GL261-Luc glioma cells *in vivo*. (A) *In vivo* bioluminescent imaging of tumors inoculated in HBO and control groups. (B) Photon light-emitting area and (C) photon sum of the HBO and control groups. Data are expressed as the mean ± standard deviation. *P<0.05 vs. HBO group.

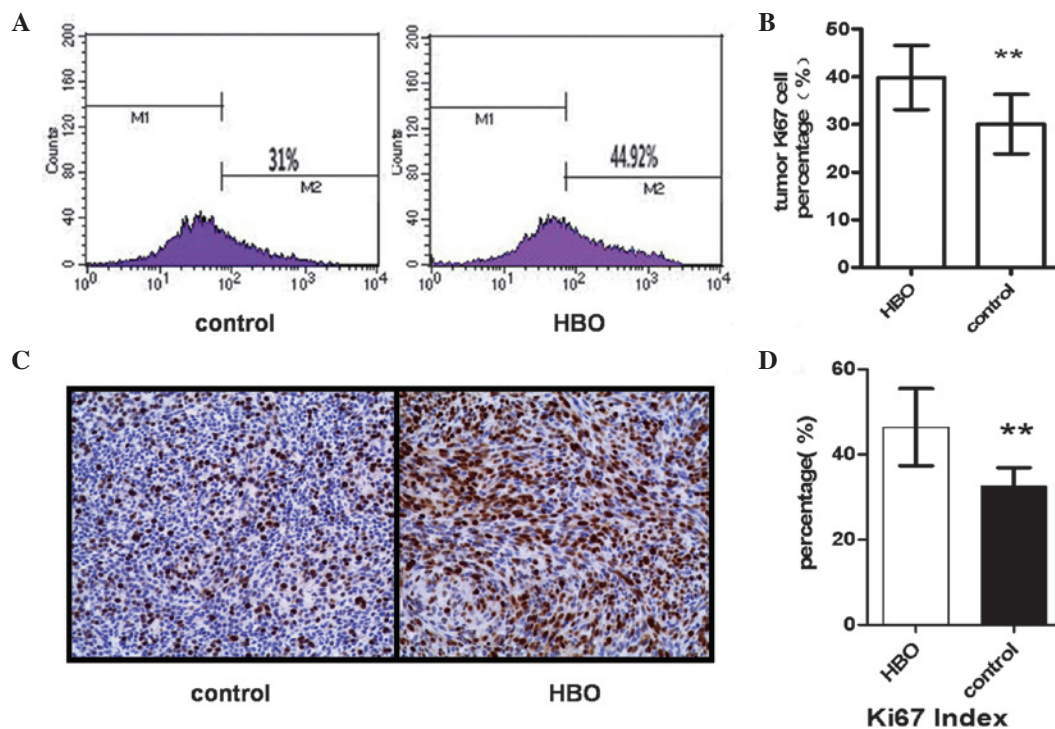


Figure 2. Hyperbaric oxygen (HBO) promoted the proliferation of glioma cells. (A) Flow cytometric analysis of tumors in the HBO and control groups. (B) Percentage of Ki67-positive glioma cells identified using flow cytometry in tumors inoculated in the HBO and control groups. (C) Images of Ki67 expression (stain, hematoxylin; magnification, x400) and (D) plot of the percentage of Ki67-positive glioma cells, identified using immunohistochemical analysis in tumors inoculated in the HBO and control groups. Data are expressed as the mean ± standard deviation. **P<0.01 vs. HBO group.

indicated that the proportion of Ki67-positive glioma cells in the hyperbaric oxygen group was significantly increased compared with the control group (39.82 ± 6.69 vs. 30.06 ± 6.22 ;

$P=0.009$; Fig. 2A and B). Immunohistochemical analysis also indicated that the percentage of Ki67-positive cells was increased in the hyperbaric oxygen group compared with

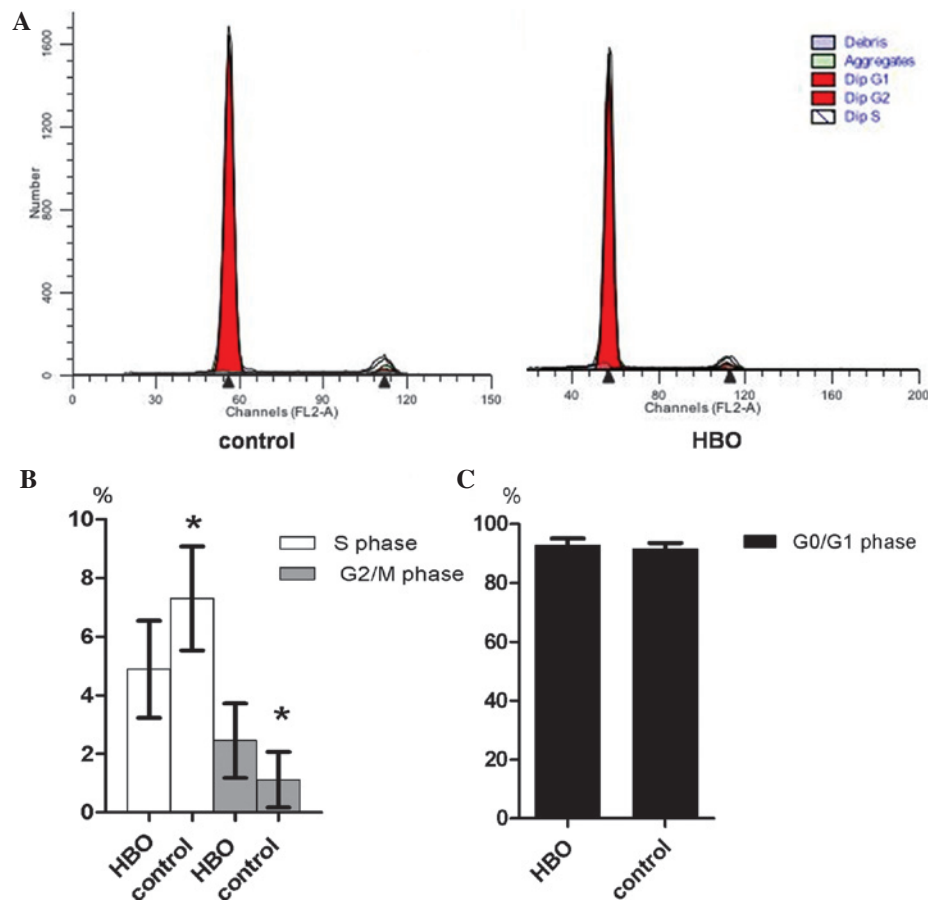


Figure 3. Hyperbaric oxygen (HBO) promoted glioma cells escaping cell cycle arrest. (A) Cell cycle analysis of tumors inoculated in the HBO and control groups. The percentages of glioma cells in the (B) S and G₂/M phases, and (C) in the G₀/G₁ phase of the cell cycle in tumors inoculated in HBO and control groups are demonstrated. Data are expressed as the mean \pm standard deviation. * $P < 0.05$ vs. HBO group.

the control group (46.33 ± 9.03 vs. 32.50 ± 4.42 ; $P = 0.007$; Fig. 2C and D).

Flow cytometric analysis of the cell cycle status demonstrated that the majority of glioma cells in the hyperbaric oxygen and control groups were in G₀/G₁ phase, while a small number of cells were in G₂/M phase (Fig. 3A and B). The proportion of glioma cells in S phase of the hyperbaric oxygen group was significantly reduced compared with the control group (4.88 ± 1.66 vs. 7.30 ± 1.77 ; $P = 0.014$; Fig. 3C). In addition, the proportion of glioma cells in G₂/M phase in the hyperbaric oxygen group was significantly increased compared with the control group (2.45 ± 1.27 vs. 1.12 ± 0.95 ; $P = 0.033$; Fig. 3C). However, no statistically significant difference was observed in the proportion of glioma cells in G₀/G₁ phase between the two groups (92.67 ± 2.35 vs. 91.58 ± 1.98 ; $P = 0.334$; Fig. 3D).

Hyperbaric oxygen inhibited the apoptosis of glioma cells and promoted angiogenesis in transplanted glioma. Flow cytometric analysis indicated that the proportion of apoptotic glioma cells (Annexin V/PI double positive) in the hyperbaric oxygen group was significantly increased compared with the control group (13.75 ± 8.84 vs. 8.27 ± 3.19 ; $P = 0.023$; Fig. 4A and B). By contrast, the proportion of normal apoptotic brain cells in the hyperbaric oxygen group was significantly reduced compared with the control group (14.70 ± 6.73 vs. 24.63 ± 9.66 ; $P = 0.032$; Fig. 4A and B). Immunohistochemical analysis also indicated that the number of TUNEL-positive glioma cells in

the hyperbaric oxygen group was increased compared with the control group (Fig. 4C).

CD34 expression is associated with the angiogenesis capacity of vascular endothelial cells. Therefore, an increase in CD34 immunostaining was observed in the hyperbaric oxygen group compared with the control group (Fig. 5A), indicating that the angiogenesis capacity of the vascular endothelial cells of the transplanted glioma was increased by hyperbaric oxygen treatment.

The present study also compared the microvessel density (MVD) of glioma following hyperbaric oxygen treatment. The MVD in the hyperbaric oxygen group was significantly increased compared with the control group (9.18 ± 2.53 vs. 19.80 ± 3.33 , $P = 0.0003$; Fig. 5B), which further demonstrated that the angiogenesis capacity of vascular endothelial cells in the transplanted glioma was increased in the hyperbaric oxygen group.

Discussion

A number of previous studies have indicated that combining hyperbaric oxygen treatment with chemoradiotherapy may improve the prognosis for glioma patients and reduce complications (13-15,22); however, the specific mechanism by which this occurs remains unknown. A previous study proposed that hyperbaric oxygen may induce apoptosis in tumor cells and decrease the vascular density in subcutaneous

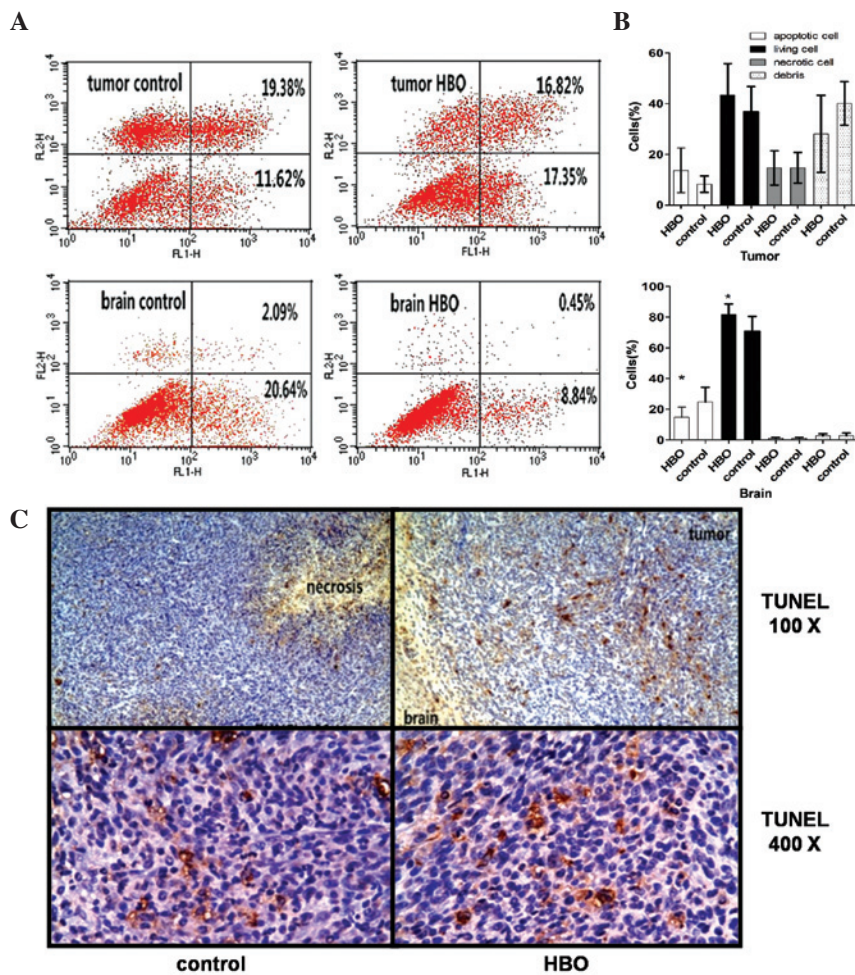


Figure 4. Hyperbaric oxygen (HBO) suppressed apoptosis in glioma cells. (A) Cell apoptosis of tumors inoculated in HBO and control groups, analyzed using flow cytometry. (B) Percentage of glioma cells in different survival status in the HBO and control groups. (C) Immunohistochemical analysis results of TUNEL staining in tumors inoculated in the HBO and control groups. Data are expressed as the mean \pm standard deviation. * $P < 0.05$ vs. control group.

transplanted glioma tumors (18). Due to the blood-brain barrier, numerous therapies that have proven effective for treating subcutaneous transplanted gliomas, may not be effective in treating intracranial primary gliomas (19). Therefore, the present study produced an intracranial transplanted glioma model in congenic mice to investigate the direct effects of hyperbaric oxygen stimulation on glioma cells.

In vivo bioluminescent imaging (BLI) was applied in the present study for the C57BL/6J mice tumorigenicity assay, in order to continuously and dynamically monitor the biological function of tumor cells in real-time, without affecting the physiological function of C57BL/6J mice (23,24). A previous study indicated that hyperbaric oxygen increased the oxygen partial pressure of the intracranial tumor and this effect remained for 40 min following hyperbaric oxygen therapy (11). To avoid the interference of oxygen partial pressure, BLI was employed 12 h following hyperbaric oxygen therapy. In the present study, BLI indicated that the light-emitting area and the sum of emitted photons were increased in mice treated with hyperbaric oxygen, indicating that hyperbaric oxygen promoted growth of the intracranial glioma.

Ki67 is expressed in all phases of the cell cycle, with the exception of the G_0 phase, and therefore its expression is

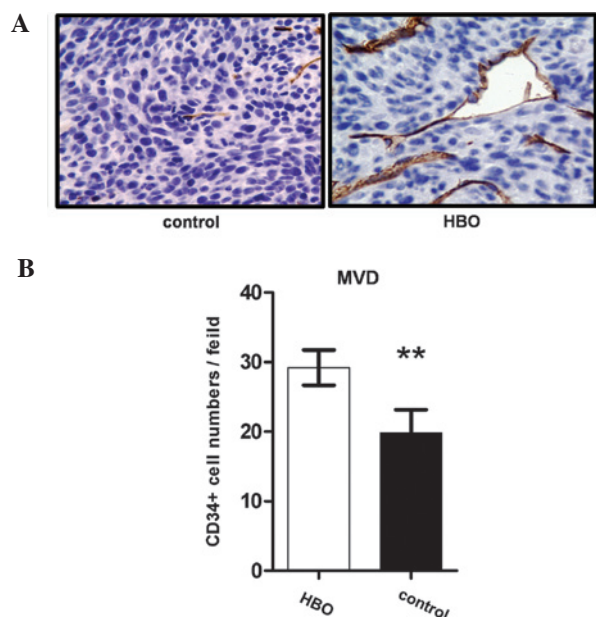


Figure 5. Hyperbaric oxygen (HBO) promoted angiogenesis of intracranially transplanted GL261-Luc glioma *in vivo*. (A) Immunohistochemical image of CD34 staining in tumors inoculated in the HBO and control groups (stain, hematoxylin; magnification, x400). (B) Number of CD34⁺ cells per field in tumors of HBO and control groups. Data are expressed as the mean \pm standard deviation. ** $P < 0.01$ vs. HBO group.

associated with cell proliferation. The results of immunohistochemical and flow cytometric analyses indicated that the expression of Ki67 in tumors repeatedly exposed to hyperbaric oxygen was increased compared with the control group. In contrast to the subcutaneous model, hyperbaric oxygen also increased the level of apoptosis in intracranial glioma cells in C57BL/6J mice. The proportion of TUNEL-positive cells was $\geq 60\%$ in the control group, whereas the proportion of Ki67-positive cells was $\leq 10\%$. In addition, in a previous study (15), for subcutaneous glioma cells, the proportion of apoptotic and proliferative cells was $\geq 60\%$ and $\leq 10\%$, respectively, whereas in the present study, the proportion of apoptotic cells in the intracranial glioma was $\leq 20\%$, while the proportion of proliferative cells was $\geq 30\%$. This difference may be due to the differences in the surroundings of the glioma cells. Therefore, the subcutaneous microenvironment may delay the growth of glioma cells. The increased proportion of apoptosis was associated with an increased capacity of self-renewal, which may have resulted from the glioma cells adapting to ambient pressure. Furthermore, the decreased proportion of proliferation may be associated with an adverse influence on the growth of glioma cells.

The present study also assessed the angiogenesis of tumor cells. Cells originating from glioma may participate in angiogenesis; glioma cells have been observed around blood vessels (25,26), leading to the apoptosis of vascular cells and the destruction of vascular integrity, resulting in angiogenesis. The increased density and morphological change of capillaries is associated with an increased degree of malignancy and a poorer prognosis (27,28). In the present study, the results demonstrated that increased MVD was associated with the proliferation of glioma cells, thus promoting angiogenesis; however, the opposite was observed in a subcutaneous model (18) and in breast cancer (29). The aforementioned observations indicate that the same intervention in different microenvironments may influence the growth of glioma cells differently (19). In addition, another previous study indicated that MVD was an effective prognostic factor, although no direct association was observed between MVD and therapeutic effect (30).

In conclusion, repeated exposure to hyperbaric oxygen promoted the proliferation and angiogenesis of intracranial glioma cells, inhibited apoptosis and prevented cell cycle arrest. Therefore, hyperbaric oxygen therapy may be a potentially effective therapeutic option and may improve the prognosis for patients with glioma.

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