

Department of Neurosurgery<sup>1</sup>; Institute of Pathology and Southwest Cancer Center<sup>2</sup>, Southwest Hospital; Key Laboratory of Tumor Immunopathology of Ministry of Education of China<sup>3</sup>, Third Military Medical University, Chongqing, China

## Dihydroartemisinin exhibits anti-glioma stem cell activity through inhibiting p-AKT and activating caspase-3

LIU CAO<sup>1</sup>, WANGSHENG DUANMU<sup>1</sup>, YI YIN<sup>1</sup>, ZHIHANG ZHOU<sup>2,3</sup>, HONGFEI GE<sup>1</sup>, TUNAN CHEN<sup>1</sup>, LIANG TAN<sup>1</sup>, ANYONG YU<sup>1</sup>, RONG HU<sup>1</sup>, FEI LI<sup>1</sup>, HUA FENG<sup>1</sup>

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Hua Feng, M.D., Ph.D., Department of Neurosurgery, Southwest Hospital, Third Military Medical University, Chongqing (400038), China  
fenghua8888@vip.163.com

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Glioma stem cells (GSCs) have been proven to play key roles in tumorigenesis, metastasis and recurrence. Although dihydroartemisinin (DHA), a derivative of the antimalaria drug artemisinin, has been shown to have anti-cancer activity, it is still unclear whether DHA affects GSCs. This study investigated the effects of DHA on the growth and apoptosis of GSCs, as well as the possible molecular mechanism involved in these processes. GSCs were enriched using a non-adhesive culture system with serum-free neural stem cell medium. Their stemness characteristics were identified by assessment of tumor sphere formation, mRNA expression analysis, and immunofluorescence staining of stem cell markers (CD133, SOX2, and nestin). We found that DHA not only inhibited proliferation, which was determined with the cell counting kit-8 (CCK8) assay, but also induced apoptosis of GSCs, as evaluated with the annexin-V/PI flowcytometric assay. Interestingly, DHA treatment also induced a concentration-dependent cell cycle arrest in the G1 phase according to the cell cycle assay. To reveal the underlying mechanisms, we detected the expression levels of p-Akt and Cleaved Caspase-3. The data showed that Cleaved Caspase-3 increased significantly in a dose-dependent manner ( $p < 0.05$ ) after the GSCs sphere cells were treated with 20, 40, and 80  $\mu\text{M}$  of DHA for 24 h, which correlated with significantly decreased expression levels of p-Akt ( $p < 0.05$ ). These data indicate that DHA selectively inhibits proliferation and induces apoptosis of GSCs through the down-regulation of Akt phosphorylation, which is followed by Caspase-3 activation, and these findings offer a new approach for treating gliomas.

### 1. Introduction

Glioma is the most prevalent primary malignant brain tumour in adults. Although the incidence of brain tumors is relatively low compared with other cancers, the median survival with optimal therapy is only 15 months from diagnosis, and most tumors recur within 9 months of initial treatment (Taphoorn et al. 2005). The primary histological feature of malignant gliomas is invasion of the tumor cells into the surrounding normal brain tissue. To date, surgery is still the most effective treatment with curative potential, but even after standard surgery, radiotherapy, and chemotherapy, the prognosis of patients with malignant gliomas is still poor (Li et al. 2013). Therefore, numerous approaches have been attempted in the search for further options, including efficient chemotherapeutic agents. Because apoptotic resistance is a major challenge that hampers the efficacy of anticancer treatment, there have been increased efforts in the development of innovative compounds that more effectively kill therapy-resistant tumor cells, such as glioma cells.

Recent reports have characterized a subpopulation of cells, termed ‘cancer stem cells’ (CSCs), within tumors that drive tumor formation and growth. Glioma stem cells (GSCs) have been proven to play key roles in growth, invasion, angiogenesis

and immune evasion of glioma (Yi et al. 2011). They have also been identified as the sources of chemo- and radio- resistance. Even when the majority of tumor cells has been eliminated, the GSCs survive and recreate new tumor cells (Osuka et al. 2013). Therefore, effective therapies against gliomas, especially malignant glioblastoma (GBM), may be more beneficial if they specifically target GSCs to achieve better clinical outcomes (Zhao et al. 2013).

Because of their resistance to chemotherapeutic agents, inhibiting the growth of GSCs has been very difficult. Recently, some natural products have been reported to kill CSCs. Dihydroartemisinin (DHA), derived from the Chinese medicinal herb *Artemisia annua*, is a safe, effective, FDA-approved and WHO-recommended mainstay for treating malaria (Eastman and Fidock 2009; Hanpithakpong et al. 2008). Recent studies have suggested that DHA also exerts preferentially cytotoxic effects on several human malignancies, including lung cancer, ovarian cancer, hepatocellular carcinoma, and pancreatic cancer (Zhang et al. 2012). Furthermore, GSCs are responsible for glioma recurrence, metastasis, and the high mortality of glioma patients, and GSCs are considered as an important target for developing future cancer therapies or improving current therapeutic strategies (Modrek et al. 2014). Additionally the

serine/threonine kinase Akt plays an important role in tumorigenesis. The biological significance of Akt kinase activity in GSCs has been recently established. Overexpression of p-Akt can inhibit apoptosis (Liu et al. 2014a). Furthermore, activation of Akt can predict a poor prognosis in glioma. The phosphorylation of Akt may alter the activity of proteins, such as caspase-3, Bcl-2 family members, nuclear factor-kappa B (NF- $\kappa$ B) and other transcription factors that induce or inhibit apoptosis. Based on these findings, we hypothesized that DHA might induce apoptosis in GSCs by inhibiting p-Akt, which is important for GSCs survival. In the present study, we investigated the effect of DHA on GSCs, with a focus on apoptosis, and examined the involvement of p-Akt and caspase-3 in this process to elucidate the possible molecular mechanisms by which DHA acts on GSCs.

## 2. Investigations and results

### 2.1. Enrichment of GSCs by sphere formation

The GL261 cells cultured in DMEM-F12 with 10 % FBS adhered to the plate (Fig. 1 A). GL261 GSCs formed typical spheres in the serum-free neural stem cell medium (Fig. 1B).

### 2.2. The CD133, SOX2 and Nanog mRNA expression levels were increased in the GSC spheres

Quantitative real-time RT-PCR analysis showed that there was almost no expression of CD133, SOX2 and Nanog in the parental GL261 cells, while their expressions were evident in the tumor sphere cells (Fig. 1C).

### 2.3. GSC spheres expressed high levels of the putative stem cell markers

We also showed that high levels of the putative stem cell markers, CD133, SOX2 and nestin were detected in the tumor spheres (Fig. 1D). The normal GL261 cells rarely expressed the putative stem cell markers (data not shown).

### 2.4. DHA inhibits the growth of GSCs

A significant morphology change in the DHA-treated GSCs was apparent and dose-dependent (Fig. 2A). The results from the proliferation inhibition CCK-8 assay of DHA against the GL261 cells and GSCs are shown in Fig. 2B. The percentages of growth inhibition from various concentrations of DHA treatment on the GL261 cells and GSCs were determined as the percentage of viable treated cells versus viable cells from untreated controls. DHA exhibited a dose-dependent inhibitory effect on the GL261 cells and GSCs. Interestingly, DHA showed a greater inhibition to the GSC growth more effectively than the GL261 cell growth.

### 2.5. DHA inhibits GSC sphere formation

Fifty GSCs seeded in the serum-free neural stem cell medium without DHA generated  $37 \pm 3$  GSCs spheres per well. After treatment with 5, 10, and 20  $\mu$ M of DHA for 7d, the tumor sphere formation was significantly inhibited in a dose-dependent manner (Fig. 2C,  $p < 0.05$ ).

### 2.6. Dihydroartemisinin resulted in G1 arrest and induced apoptosis of GSCs

Incubation with DHA resulted in a significant suppression of cell proliferation, G1 arrest, and induction of apoptosis in a dose-dependent manner. The cell cycle was arrested in the G1 phase

with  $65.7 \pm 2.09$  % of the 80  $\mu$ M DHA treated cells in G0/G1 versus  $51.4 \pm 2.59$  % of the control cells ( $p < 0.05$ ; Fig. 3B). In addition, 24 h of incubation with 80  $\mu$ M DHA induced apoptosis ( $69.26 \pm 3.29$  % versus  $7.9 \pm 1.36$  % in the control group,  $p < 0.05$ ; Fig. 3C and Fig. 3D).

### 2.7. DHA inhibits phosphorylation of Akt and activates caspase-3

To explore the potential mechanisms underlying the growth inhibition effect of DHA on GSCs, the apoptotic proteins cleaved caspase-3, Akt and p-Akt were evaluated with Western blot analysis. The expression levels of p-Akt decreased significantly ( $p < 0.05$ , Fig. 4A) and cleaved caspase-3 increased significantly in a dose-dependent manner ( $p < 0.05$ , Fig. 4B) after the GSCs sphere cells were treated with 20, 40 and 80  $\mu$ M of DHA for 24 h.

## 3. Discussion

The prognosis of glioma patients remains poor due to therapeutic resistance and tumor relapse. Increasing scientific evidence suggests that gliomas are driven by a rare subset of tumor cells known as glioma stem cells (GSCs) (Auffinger et al. 2014). These cells have been identified as capable of dividing, differentiating, and initiating a new tumor. Additionally, this subpopulation is more resistant to conventional therapies than its differentiated counterpart. The inability of conventional treatments to exterminate all infiltrative tumor foci is one of the main causes of therapeutic failure and malignant recurrence in GBM (Ping et al. 2011). Although the radio-resistance properties of glioma cells are fairly well established, the underlying molecular mechanisms of chemo-resistance have been addressed in only a few studies. To date, the identification and treatment of cancer stem cells (CSCs) through targeted drugs or genes have already been important topics in basic and clinic cancer research (Cheng et al. 2012).

Dihydroartemisinin, the active constituent of the herb *Artemisia annua* (sweet wormwood), is widely used as an effective anti-malarial drug. It has additionally been reported that DHA inhibits cell proliferation and its anti-cancer properties have been observed in many cell lines isolated from breast cancer, prostate cancer, hepatocellular carcinoma, leukemia, bladder cancer and other types of cancers. We hypothesized that DHA has potential to inhibit glioma CSC and/or other CSC growth. However, there are no studies on the growth inhibition effect of DHA on glioma CSCs and/or other CSCs or on the underlying mechanism of such activity.

In this report, we studied the effects of DHA on GL261 GSCs. We found that DHA could significantly influence the morphology of the GSC spheres in a dose-dependent manner (Fig. 2C), suggesting that DHA might exert an inhibition effect on the growth of as well as induce the apoptosis of GSCs. It is further demonstrated that DHA exhibits a dose-dependent inhibitory effect on the growth of GL261 cells and GSCs, while DHA showed greater inhibition of the GSCs than the GL261 cells through the CCK-8 assay (Fig. 2B). Simultaneously, the GSC sphere formation was significantly inhibited in a dose-dependent manner (Fig. 2C). Furthermore, flow cytometric analysis showed that concentration-dependent cell cycle arrest occurred at the G1 phase in GSCs after incubation with DHA, resulting in cell cycle dysfunction (Fig. 3A and 3B). Apoptosis is a common mode of action by which chemotherapeutic agents treat cancer. The percentages of apoptotic cells present after 24 h treatment with 0, 20, 40, and 80  $\mu$ M of DHA are shown

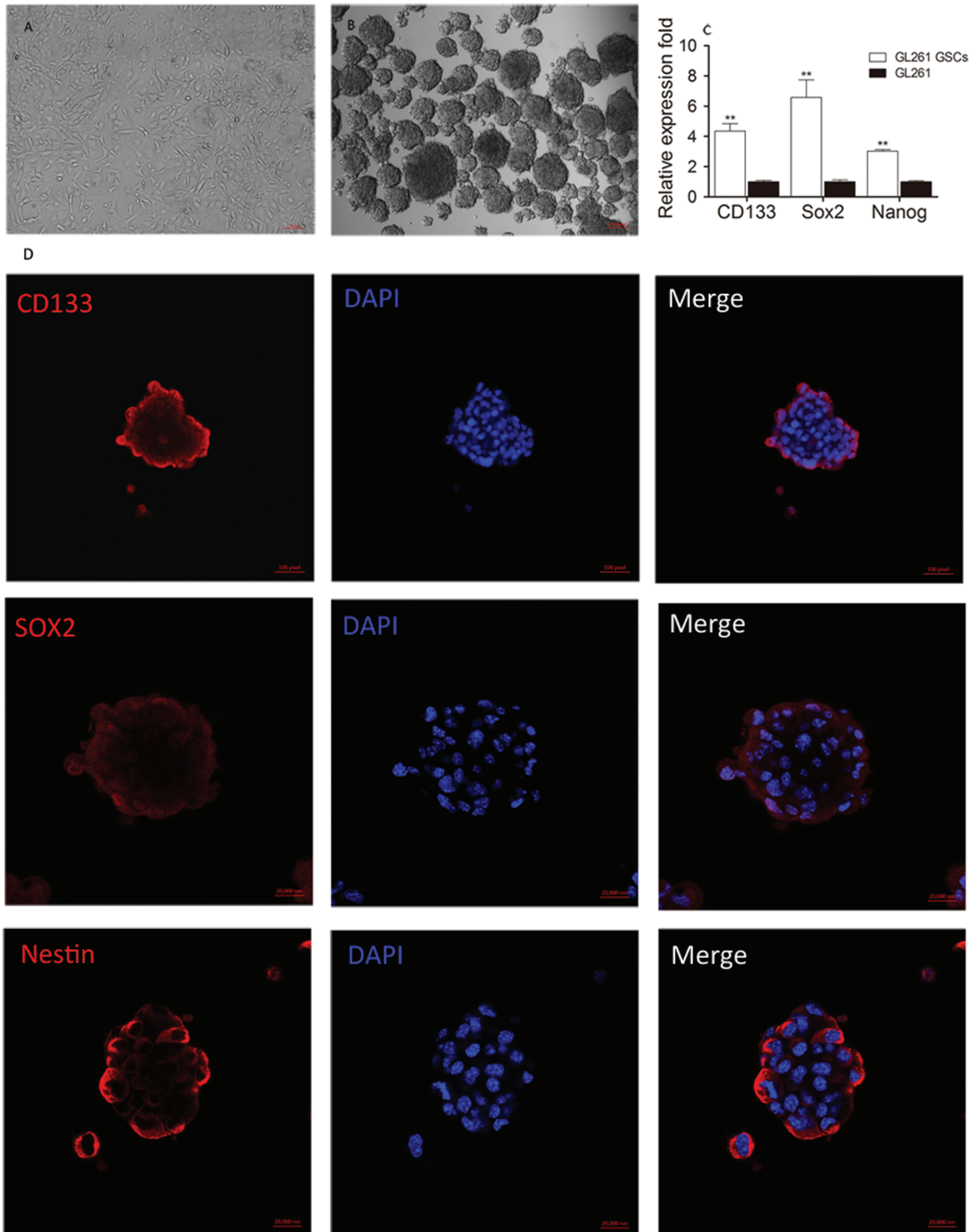


Fig. 1: The morphology and mRNA expression characteristic of the GL261 parental cells and GSC spheres. (A) Parental GL261 cells that were cultured with DMEM-F12 supplemented with 10 % FBS grew as an adherent monolayer. (B) GL261 cells cultured under non-adhesive culture system formed typical tumor spheres (magnification is  $40\times$ ). (C) The expression of the putative stem cell markers, CD133, Nanog and SOX2 in GL261 GSC spheres and GL261 cancer cell lines were determined by quantitative real-time RT-PCR. There was very little expressions of CD133, SOX2 and Nanog in the parental GL261 cells, while the expressions of these markers was evident in the tumor sphere cells. The data are shown as the mean  $\pm$  SEM for three independent experiments. (D) GL261 GSC spheres expressed high levels of the putativestem cell markers, CD133 and SOX2 and Nestin.

in Fig. 3C. It is indicated that DHA could selectively induce apoptosis of GSCs, comparing with its non-stem counterparts. Caspases, a family of intracellular cysteine proteases, are the effector components of triggering apoptosis, and they matured from inactive zymogens (pro-Caspases) (Thornberry and Lazebnik 1998). Caspases are divided into two functional subfamilies: initiator Caspases (Caspase-8, -9, and -10) that are involved in regulatory events and effector Caspases

(Caspase-3, -6, and -7) that are responsible for cell disassembly events. Activation of Caspase-3 is a key event in initiating programmed cell death (Cai et al. 2013). The execution of apoptosis is also perceptible by increased levels of Cleaved Caspase-3. Activation of the apoptotic pathway induces the proteolytic cleavage of Caspase-3, which was reflected as a high level of Cleaved Caspase 3 in the cells treated with DHA (Fig. 3A).

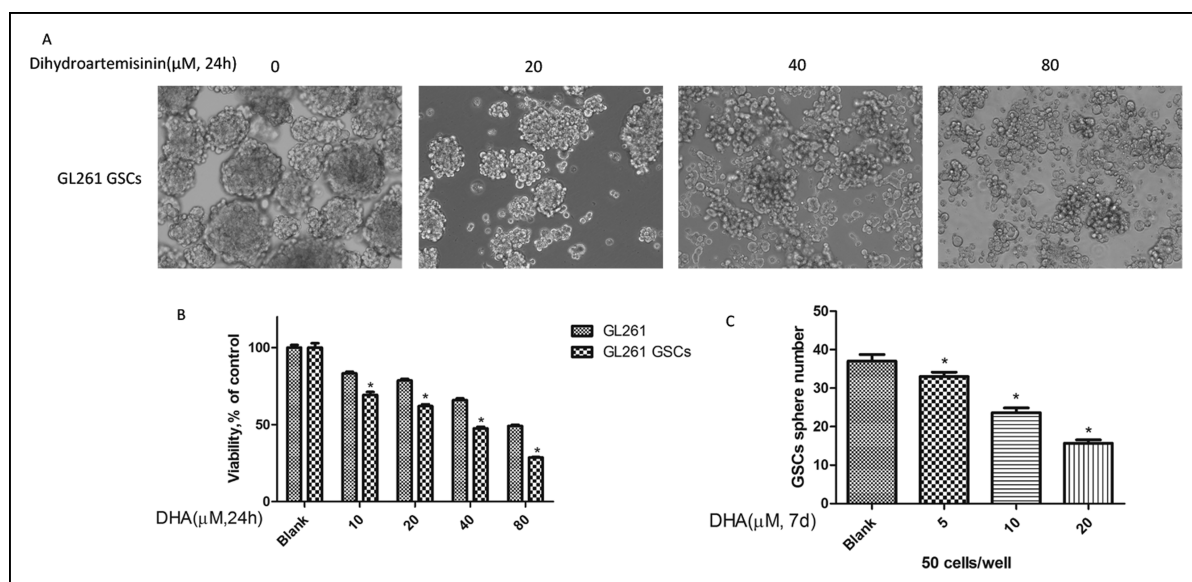


Fig. 2: Growth inhibition of dihydroartemisinin on the GL261 parental and GSC sphere cells. (A) DHA induces a significant morphology change in GSCs in a dose-dependent manner. (B) Both the GL261 parent and GSC sphere cells were seeded in 96-well plates and treated with dihydroartemisinin at different concentrations for 24 h, and the percentage of cell viability was determined by the CCK-8 assay. The OD values of each treated group were compared with those of the blank at the same time point; interestingly, DHA showed greater inhibition of the GSCs than the GL261 cells. The single asterisk (\*) indicates a significant difference compared to the GL261 cell group ( $p < 0.05$ ). (C) After treatment with 5, 10, and 20  $\mu\text{M}$  of DHA for 7 d, the tumor sphere formation was significantly inhibited in a dose-dependent manner. The single asterisk (\*) indicates a significant difference from the blank group ( $p < 0.05$ ). The results are given as the mean values  $\pm$  SEM of independent experiments performed in triplicate.

Akt, a serine–threonine kinase that is activated by a number of growth factor receptor signaling pathways, plays an important role in the regulation of cell apoptosis. Activation of Akt is represented by phosphorylation of Ser 473. Activated Akt can activate several downstream target proteins, including glycogen synthase kinase (GSK), Caspase-9, Bad and NF- $\kappa\text{B}$ , to promote proliferation, angiogenesis and anti-apoptosis of cells during chemotherapy or radiotherapy (Yang et al. 2006; Tang et al. 2013). Overexpression or dysfunction of Akt phosphorylation is common in human cancers, such as ovarian cancer, breast cancer, prostate cancer, lung cancer and malignant lymphoma (Lin et al. 2012). A recent study demonstrated that p-Akt expression could be a prognostic factor of glioma (Tang et al. 2013a). Furthermore, we observed that down-regulation of Akt phosphorylation after GSCs were treated for 24 h with 40 and 80  $\mu\text{M}$  of DHA correlated with increased apoptosis. This suggests that Akt phosphorylation may be related to the DHA-induced GSC apoptosis.

Additionally, inhibition of Akt phosphorylation affects cell proliferation and anti-apoptosis of glioma (Ding et al. 2013). Down-regulation of p-Akt may sensitize drug-resistant glioma cells (Millet et al. 2013). These results suggest that the sustained activation of Akt is an important factor in glioma drug resistance, and is thus a potential therapeutic target (Liu et al. 2014b and Xie et al. 2014). Recently, a few clinical trials have tested anti-tumor drugs targeting Akt activation (Bailey et al. 2006; Lam et al. 2005). However, there are still some practical problems, such as severe adverse events and poor selectivity. Our data show that DHA could attenuate Akt phosphorylation with high efficacy. Additionally, DHA is still widely used in the clinics with low toxicity, and it may be a safe and potential candidate for treating glioma by targeting Akt activation.

DHA seems to have potent inhibitory effects on the proliferation of GSCs. The mechanism for this might include p-Akt attenuation mediated apoptosis induction.

Taken together, our results demonstrate that DHA could selectively inhibit GSC growth by inhibiting proliferation and inducing apoptosis through attenuating Akt phosphorylation and

Caspase-3 activation. DHA thus presents a novel therapeutic agent for targeting GSCs in the treatment of glioma.

## 4. Experimental

### 4.1. Cell culture and treatment of cells with DHA

The murine glioma cell line GL261 (defined as GL261 adherent cells, GL261-AC) was obtained from ATCC (USA) and cultured in DMEM/F12 (Hyclone, USA) supplemented with 10 % FBS (Sigma, USA). The cells were then placed in a humidified atmosphere with 5 %  $\text{CO}_2$  at 37 °C. CSCs from the glioma cell line GL261 were obtained and cultured as we previously described. In summary, during the exponential growth phase, the GL261 glioma cells were harvested via a treatment of 0.25 % trypsin (Hyclone, USA) for 3 min at 37 °C. The prepared glioma cell suspension was centrifuged at 1000 rpm for 5 min, and resuspended in a serum-free neural stem cell medium (SFM) containing Dulbecco's Modified Eagle Medium/F12 (DMEM/F12) (Hyclone, USA), B27 (Invitrogen, USA), recombinant murine epidermal growth factor (20 ng/mL; Peprotech Inc., USA), and basic fibroblast growth factor (20 ng/mL; Peprotech Inc., USA). The number of cells was then counted using a regular hemacytometer, and the cells were seeded in 24-well plates with  $2 \times 10^4$  cells/well. Subsequently, one-third of the culture medium was replaced with an equal volume of fresh SFM after the cells were cultured for 24 h. After 3d in culture, all culture medium was discarded and the wells were filled with 600 mL of fresh SFM. Under these conditions, only GICs and early progenitor cells survived and proliferated, whereas the differentiated cells died. Approximately 8 % of these single cells could develop into spheres containing 10-20 cells after 5 days. In 2 weeks, the size of the spheres expanded by 10- to 30-fold (Wang et al. 2012).

For DHA (Huali, China) treatment, GSCs were treated at concentration of 20, 40 and 80  $\mu\text{M}$  for 24 h.

### 4.2. Immunofluorescence

The pre-coated glass-bottom cell culture dishes were prepared with 100  $\mu\text{g}/\text{ml}$  of Poly-D-lysine (sigma, P4707). All dishes, washed twice with sterile cell culture grade water, were seeded with GSC spheres 24 h after they had been coated with the aforementioned substrates. Half the volume of the culture medium was exchanged every 3 days. For fluorescence immunocytochemistry, GSC spheres that adhered to precoated dishes were fixed with 4 % paraformaldehyde in 0.01 M phosphate-buffered saline (pH 7.4) for 2 h at room temperature and blocked with 10 % normal goat serum (Boshide Biotech, China). Then

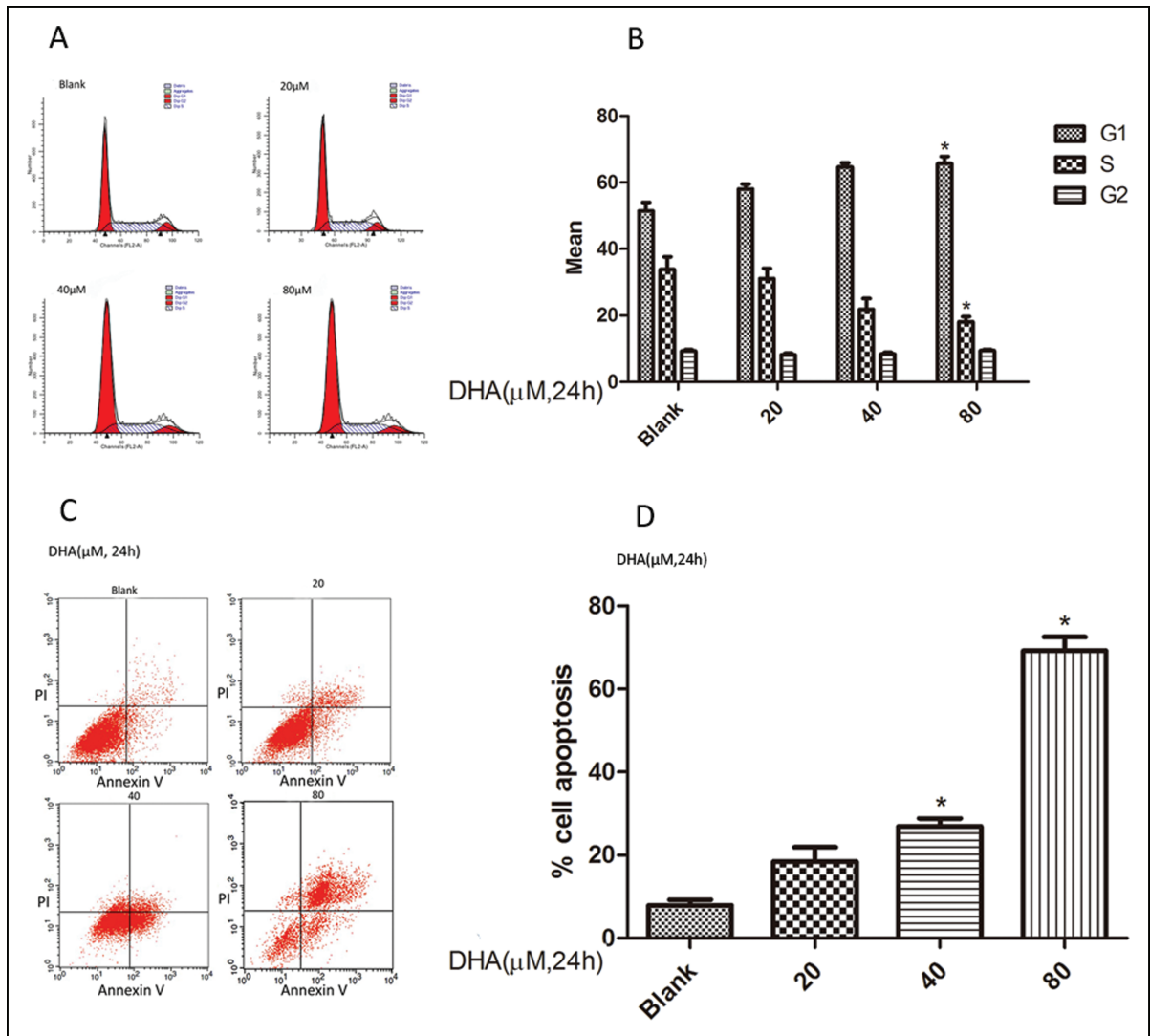


Fig. 3: Incubation with DHA resulted in G1 arrest and induction of apoptosis in a dose-dependent manner. (A) With 24 h of treatment with DHA, the number of GSCs in the G1 phase gradually increased. There were  $65.7 \pm 2.09\%$  of the  $80\ \mu\text{M}$  DHA treated cells in G0/G1 versus  $51.4 \pm 2.59\%$  of the control cells. (B) The summary of the cell cycle distribution from panel A. (C) DHA induces GL261 GSCs apoptosis in a dose-dependent manner. GL261 GSCs infected with DHA were labeled with FITC-Annexin V/PI, and apoptosis was assessed by flowcytometry. (D) The summary of the cell cycle distribution in panel C.

the GSC spheres were incubated with the following antibodies: rabbit anti-mouse nestin polyclonal antibody (1:200, Abcam, USA), rabbit anti-mouse CD133 polyclonal antibody (1:200, Abcam, USA) and rabbit anti-mouse SOX2 polyclonal antibody (1:200, Abcam, USA) overnight at  $4\ ^\circ\text{C}$ . The primary antibodies bound on the CSCs were reacted with FITC-conjugated goat anti-rabbit IgG (1:50, Sigma, USA) for 2 h at room temperature. The cells were then counterstained with the DNA-binding dye 4',6-diamidino-2-phenylindole (DAPI, 2 mg/ml, sigma) for 10 min at room temperature.

**4.3. RNA extraction and quantitative real-time RT-PCR**

Total RNA was extracted using the Trizol reagent (Life Technologies, USA) according to the manufacturer's instructions. The concentrations of RNA were determined using a NanoDrop ND-1000 (NanoDrop, USA). cDNA was synthesised with the PrimeScript RT reagent kit (TaKaRa, Dalian, China) using 500 ng total RNA as template. qPCR analyses were conducted to quantitate mRNA relative expression using SYBR Premix Ex Taq (TaKaRa, Dalian, China) with GAPDH as internal control. The primers used for PCR

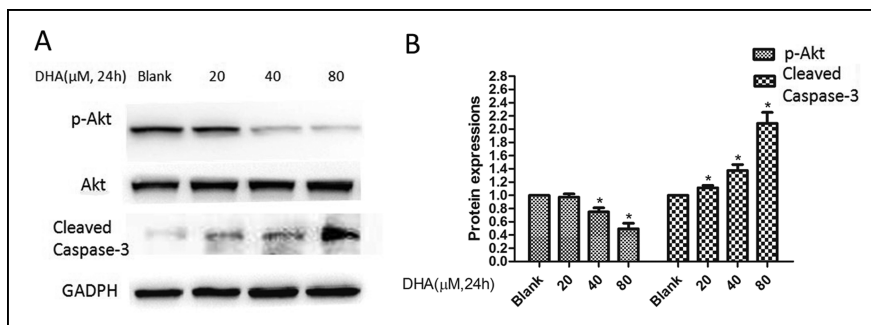


Fig. 4: Immunoblot analysis of Akt, p-Akt and Cleaved Caspase-3 in DHA-treated GL261 GSCs. Exponentially growing GL261 GSCs cultured in 6-well plates were exposed to different doses of DHA (0, 20, 40, and  $80\ \mu\text{M}$ ) for 24 h. GL261 GSCs were collected at 24 h and lysed. The lysates were separated by electrophoresis and immunoblotted with antibodies against Akt, p-Akt, and Cleaved Caspase-3. Immunoblotting of GADPH served as a loading control. All experiments were performed three times.

analysis were as follows (5'-3'): CD133 (forward: ACTGAGAAATCC-CCTACTGAAGT; reverse: GGCCTGTTTTCGGCTTTCCTT), Sox2 (forward: GCGGAGTGGAAACTTTTGTCC; reverse: GGAAGCGTG-TACTTATCCTTCT), Nanog (forward: CACAGTTTGCCTAGTTCT-GAGG; reverse: GCAAGAATAGTTCTCGGGATGAA). The relative expression levels were calculated with the  $2^{-\Delta\Delta Ct}$  method from the threshold cycle (Ct) (Wang et al. 2014).

#### 4.4. Cell proliferation assay

The Cell Counting Kit-8 (Beyotime, China) was employed to determine the viability of the GL261 cells and GSCs. In accordance with the manufacturer's instructions for the Cell Counting Kit-8, harvested cells were seeded in 96-well plates at  $5 \times 10^3$  per well ( $n = 6$  for each time point) in a final volume of 100  $\mu$ L. GSCs were cultured in 0, DMSO, 10, 20, 40, and 80  $\mu$ M for 24 h. CCK-8 solution (10  $\mu$ L) was added into each well, and the absorbance at 450 nm was measured after 2 h incubation at 37 °C to calculate the number of viable cells (Liu et al. 2014c).

#### 4.5. Cell cycle analysis by flow cytometry

The cell cycle analyses were performed using propidium iodide (Beyotime, China). For cell cycle analysis, cells were seeded in 6-well plates at  $3 \times 10^5$  per well. Twenty-four hours after adding the DHA, in accordance with the manufacturer's instructions, the GSCs were harvested and fixed in 70 % ethanol at 4 °C for 24 h and stained with 50  $\mu$ g/mL propidium iodide. The cell cycle distribution was analyzed by flow cytometry (Beckman Coulter, USA).

#### 4.6. Apoptosis FACS analysis

For the cell apoptosis analysis, CSCs were treated for 24 h in serum-free media with various concentrations of DHA. Apoptosis analysis was performed using the BD Pharmingen FITC Annexin V Apoptosis Detection Kit (BD, USA). For all FACS experiments, more than 10,000 cells per condition were analyzed by flow cytometry (Chen et al. 2009).

#### 4.7. Western blot analysis

Total protein from the cells was extracted in lysis buffer (Beyotime) and quantified using the Bradford method. Total protein was separated by SDS-PAGE (12 %). After transfer to a polyvinylidene fluoride (PVDF) membrane (Millipore, GER), the membranes were incubated overnight at 4 °C with antibodies against Cleaved Caspase-3 (1:1000, Abcam, USA), Akt (1:1000, CST, USA), P-AKT (1:1000, CST, USA) and GAPDH (1:500, Santa Cruz Biotechnology, USA). After incubation with peroxidase-coupled anti-rabbit IgG (Santa Cruz Biotechnology, USA) at 37 °C for 2 h, bound proteins were visualized using ECL (Pierce) and detected using BioImaging Systems (UVP Inc., Upland, CA). The relative protein levels were calculated with the GAPDH protein as a loading control.

#### 4.8. Statistical analysis

The results were expressed as the mean  $\pm$  SEM. Statistical analysis was performed using ANOVA and Student-Newman-Keuls comparison for parametric data sets. Differences were considered statistically significant at a  $p$  value of  $< 0.05$ .

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