Isoflurane enhances the malignant potential of glioblastoma stem cells by promoting their viability, mobility in vitro and migratory capacity in vivo

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Abstract

Background: Isoflurane is one of the most common general anaesthetics used during surgical procedures, including tumour resection. However, the effects of isoflurane on the viability and migration capacity of cancer cells, specifically in the context of brain cancer cells, remain unclear. Therefore, the aim of this study was to evaluate the influence that isoflurane has on the function of glioblastoma stem cells (GCSs) in regards to cell proliferation, survival and migration.

Method: U251-GSCs were exposed to isoflurane at clinically relevant concentrations and incubation times. The effects on proliferation, survival and migration capacities of the cells were evaluated in vitro. The potential risk was assessed in mice by intracranial injection of U251-GSCs pretreated with isoflurane. Furthermore, the average tumour volume and migration distance of U251-GSCs from the tumour centre were calculated.

Results: Exposure of U251-GSCs to 1.2% isoflurane for 6 h resulted in increased proliferation (P<0.05) and decreased apoptosis rate (P<0.05) when compared with the control group. In addition, isoflurane exposure caused increased migration capacity in vitro (P<0.05) and the distance migrated was increased in vivo (P<0.05).

Conclusion: Clinically relevant concentrations and incubation times of isoflurane could promote the viability and mobility of U251-GSCs, suggesting this general anaesthetic may have detrimental effects in glioblastoma by facilitating its growth and migration.

Key words: glioblastoma; isoflurane; proliferation and invasion; stem cells

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Glioblastoma is one of the most common primary malignant brain tumours in adults, with an annual incidence of 5.26 per 100 000 population or 17 000 new diagnoses per year.1 2 Although treatment options have been expanding and improving, the overall prognosis remains poor, with an average overall survival time of 15–17 months.3 Early surgical resection may be first-line treatment, even in suspected cases of low-grade glioma.4 There are numerous events within the perioperative and postoperative periods that can influence tumour recurrence, metastasis, and survival. The effect of anaesthetics has been implicated in tumour growth, with a number of retrospective studies suggesting that general anaesthetics are associated with an increased risk of recurrence or metastasis in a variety of tumours, including melanomas5 and cancers of the breast,6 prostate,7 colon and rectum.8 9

Isoflurane, one of the most commonly used general anaesthetics in clinical practice, has been shown to have cytotoxic properties in different types of cultured cells.10-13 Moreover, an increasing number of studies suggest that isoflurane may be cytotoxic in vitro14-17 and in vivo.18-20 These studies suggest that different effects may be observed based on the particular cell type used. It has been reported that isoflurane increases cell proliferation and enhances malignancy in head and neck squamous cell carcinoma cell lines, ovarian cancer cell lines and renal cell carcinoma cell lines.21-23 However, few studies have evaluated the effects of isoflurane on glioblastoma, specifically the viability and migration capacity of glioblastoma stem cells (GSCs). Thus, the aim of this study was to investigate the effects of clinically relevant concentrations of isoflurane on cell proliferation, apoptosis and migration of human GSCs in vitro and in vivo.

Methods

GSCs isolation and cell culture

The human glioblastoma cell line U-251 MG (Glioblastoma U251) was obtained from the China Center for Typical Culture Collection (CCTCC, Wuhan, China). The CD133-positive glioblastoma stem cells (GSCs) from U251 were isolated using flow cytometry. The stemness of the cells was verified using Nestin and DAPI staining (Supplementary data, Fig. 1). U251-GSCs were cultured in GSC complete media (DMEM/F12 (GIBCO) with 20 ng ml⁻¹ epidermal growth factor (Millipore), 20 ng ml⁻¹ fibroblastic growth factor (Millipore), 2% B27 and 1% penicillin/streptomycin (GIBCO)). U251-GSCs were then seeded on laminin-coated plates (Sigma; 1 mg cm⁻²) or cultured as GSC spheres with GSC complete media. These cells were maintained in a humidified incubator at 37°C and an atmosphere of 5% CO₂.

Anaesthetic exposure

To evaluate the anaesthetic effects on U251-GSCs, the cells were exposed to different concentrations of isoflurane and for different durations. Isoflurane was delivered from an agent-specific vaporizer carried by humidified air 1 min⁻¹/5% CO₂. The flow rate to the sealed plastic chamber was initially 5 litre min⁻¹ for the first 2 min for anaesthesia maintenance. An infrared Ohmeda 5330 agent monitor (Coast to Coast Medical, Fall River, MA, USA) was used to continuously monitor the delivered isoflurane concentration.

Cell proliferation assays

Cell proliferation was evaluated by testing mitochondrial dehydrogenase activity, which reduces 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma). U251-GSCs were exposed to isoflurane for 3, 6, 9, 12 or 24 h then cultured in complete media for 3 days (Supplementary data, Fig. 2) prior to assay.

Nuclear Ki-67 expression was used to identify U251-GSCs in the proliferative phase. Cells were exposed to isoflurane for 3 or 6 h at various concentrations (0.6, 1.2 and 2.4%). After 3 days, the cells were fixed in 4% paraformaldehyde and treated with 0.1% Triton X-100 prior to incubation with human anti-Ki67 (RM-9106; Thermo). The secondary antibody (Alexa 594; GIBCO) and DAPI were used for visualization and counterstaining the cell nuclei. The percentage of Ki-67-positive cells was recorded with a fluorescence microscope at 200× magnification from nine random fields and quantified by a single blinded observer.

Cell apoptosis analysis

Annexin V and propidium iodide (PI) were used (Joincare Biosciences, China) to detect apoptosis of U251-GSCs. The assay was performed 2 days after treatment. Cells were stained with annexin V for 15 min and counterstained with PI for 5 min. Annexin V and PI incorporation were quantified via flow cytometry (Becton Dickinson). Three replicate experiments were performed for each sample and data were analysed with FlowJo 7.6.1.

In vitro cell migration assay

Boyden transwell chambers (Corning) were used to analyse the migration capacity of U251-GSCs. Cells were pretreated in different concentrations of isoflurane (0.6, 1.2 and 2.4%) for different durations (3 or 6 h) before being added to the upper chamber. Cells were allowed to migrate into the lower chamber into GSC complete medium at 37°C and then non-migrated cells were removed with a cotton swab. Migrated cells were fixed in 100% methanol for 2 min, stained with haematoxylin-eosin solution and counted at 200× magnification. Nine randomly selected fields per membrane were analysed from independent experiments.

In vivo studies

All animals were treated in accordance with the ethical guidelines set by Huazhong University of Science and Technology (HUS) and relevant sections of the ARRIVE Guidelines were followed. To investigate the effect of isoflurane on U251-GSCs in vivo, 4–6-week-old male BALB/c athymic nude mice were divided into three groups (n=15 per group) and stereotactically injected with U251-GSCs [control (Ctrl) group] or GSCs pretreated for 6 h with either 0.6% isoflurane or 1.2% isoflurane into the right
striatum. General anaesthesia was induced by intraperitoneal (i.p.) injections of chloral hydrate (Sigma, St Louis, MO, USA) in phosphate-buffered saline (PBS) at a dose of 400 mg kg\(^{-1}\). Anaesthesia was assessed by loss of righting reflex, which was usually between 3 and 6 min. Then the head of each mouse was immobilized in a Kopf stereotaxic instrument (Kopf Instruments, Tujunga, CA, USA) and unilateral striatal injection of GSCs was performed at the following coordinates: \(\text{AP}=-0.5\) mm, \(\text{L}=2.7\) mm, \(\text{V}=3.2\) mm.

After 4 weeks, the mice were killed under deep anaesthesia (i.p. injection of 1000 mg kg\(^{-1}\) chloral hydrate), followed by transcardial perfusion with 20 ml 0.1 M PBS and then 20 ml of the fixative solution (4% paraformaldehyde in 0.1 M PBS). Brains were extracted, cryosectioned at a thickness of 20 \(\mu\)m, collected in six series, and stained. The sections were rinsed and pre-incubated in PBS containing 0.1% Triton X-100 with 10% goat serum prior to incubation with primary antibodies against human nuclei (Millipore MAB4383). After washing with PBS, the secondary antibody (Alexa 488; GIBCO) was used and DAPI (Sigma) was used for counterstaining. To quantify tumour area, tumour mass was outlined based on the distribution of DAPI and human nuclei-positive staining, using ImageJ software. Tumour mass volumes were calculated using the sum of the tumour areas corrected for section thickness and sample frequency: tumour volume (mm\(^3\)) = sum of areas (mm\(^2\)) x 20 \(\mu\)m x 6.25

To quantify the migratory potential capacity of U251-GSCs in vivo, migrating cells were measured from the centre of the tumour mass. A minimum of 150–200 migrating cells were counted for each animal.

**Results**

**Proliferation**

U251-GSCs exposed to isoflurane for 3 h had significantly increased numbers of Ki-67-positive cells when compared with cells not exposed to isoflurane (Fig. 1A and B). Those cells exposed to 2.4% isoflurane for 3 h had the greatest number of Ki-67-positive cells, suggesting an isoflurane dose response. Cells exposed to 1.2 or 2.4% isoflurane for 6 h had a higher number of Ki-67-positive cells compared with the control cells (Fig. 1C and D).

**Apoptosis**

U251-GSCs exposed to isoflurane had less apoptosis compared with cells not exposed to isoflurane (Fig. 2B and D). Those cells pretreated with 2.4% isoflurane for 3 h or 1.2% isoflurane for 6 h had the fewest apoptotic cells compared with other groups.

**Migration**

There was increased migration of GCCs exposed to isoflurane compared with cells without isoflurane (Fig. 3A and B). Those...
Fig 2 Apoptosis of U251-GSCs in vitro. (A) Representative image of flow cytometry analysis of annexin V and propidium iodide as a measure of apoptosis and (B) quantitative analysis of apoptosis in U251-GSCs exposed to isoflurane for 3 h. (C) Representative image of flow cytometry analysis of annexin V and propidium iodide as a measure of apoptosis and (D) quantitative analysis of apoptosis in U251-GSCs exposed to isoflurane for 6 h. Mean (SD) shown; n=3 per group. *P<0.05, **P<0.01, ***P<0.001.

Fig 3 Migration capacity of U251-GSCs in vitro. (A) Representative image of transwell analysis of migration and (B) quantitative analysis of migration of U251-GSCs exposed to isoflurane for 3 h. (C) Representative image of transwell analysis of migration and (D) quantitative analysis of migration of U251-GSCs exposed to isoflurane for 6 h. Mean (SD) shown; n=9 per group. *P<0.05, **P<0.01, ***P<0.001.
Fig 4. Tumour migratory potential capacity and tumour volume in vivo. (A) Representative pictures of DAPI (blue) and human nuclei (green) staining in mice brain after injection of GSCs pretreated with isoflurane. (B) Schematic diagram showing quantification of tumour volume and (C) quantitative analysis of tumour volume analysis in mouse brain. (D) Schematic diagram showing quantification of migration distance and (E) quantitative analysis of tumour volume analysis in mouse brain. Scale bars=100 μm. Mean (±SD) shown; n=15 per group. *P<0.05, **P<0.01, ***P<0.001.
cells exposed to 2.4% isoflurane for 3 h or 1.2% isoflurane for 6 h had a higher number of migrating cells compared with control cells (Fig. 3a and b).

**Tumour volume**

All mice developed detectable tumour parenchyma at the site of injection, but there was no significant difference between animals injected with GSCs pre-exposed to isoflurane compared with controls (Fig. A–C). In animals injected with cells not exposed to isoflurane, the average distance migrated was similar to that seen in mice given cells pre-exposed to 0.6% isoflurane for 6 h (Fig 4a). However, in those mice injected with GSCs pre-exposed to 1.2% isoflurane for 6 h, the distance migrated was increased when compared controls (Fig. 4b).

**Discussion**

Glioblastoma is the most common primary malignant intracranial neoplasm in adults and surgical resection is the standard treatment for most patients.26–28 Balanced anaesthesia combined with isoflurane is used in the majority of neurosurgical procedures, but it is not known whether isoflurane affects the proliferation, survival and migration of glioblastoma cells. In this study we investigated the effects of isoflurane on the viability and migration capacity of U251-GSCs in vitro and in vivo. We found that U251-GSCs pre-exposed to isoflurane possessed higher growth potential due to increased proliferative behaviour as well as increased survival rate compared with cells not exposed to isoflurane. Furthermore, cells pre-exposed to isoflurane had higher migration capacity in vitro and migrated further in vivo.

Previous studies have reported that anaesthetics can affect the characteristics of cancer cells. Studies have shown that isoflurane has neuroprotective effects in cerebral ischemia models13 and increases the viability and mobility of various carcinoma cell lines.21–23 Likewise, Zhao and colleagues24 found that isoflurane promoted proliferation of neural progenitor cells at low concentrations, while Zhang and colleagues25 reported that isoflurane induced caspase 3 in neurons but not in neural progenitor cells. In our study, we found that pretreating GSCs with isoflurane could alter their ability to proliferate and migrate such that exposure of cells to clinically relevant levels of isoflurane increased proliferation and mobility in vitro and migratory capacity in vivo.

It has been reported that isoflurane regulates cellular signalling pathways, which may affect various characteristics of human cancer cell lines.26 Xu and colleagues27 reported that isoflurane preconditioning attenuated activation of the inducible nitric oxide synthase–nitric oxide–glutamate pathway, which can stimulate a protective effect and enhance the survival rate of microglial cells. We found that isoflurane enhanced the survival by significantly decreasing the rate of apoptosis. Isoflurane has also been reported to increase the expression of hypoxia-inducible factor (HIFs).21 HIFs are transcription factors that regulate cell proliferation and survival and have been shown in active pathways in glioblastomas leading to enhanced invasion.24 Sevoflurane has also been reported to promote expansion of glioma stem cells through activation of HIFs.25 In concordance with these findings, we found in vitro migration of U251-GSCs pre-exposed in vivo to isoflurane was significantly greater and the migrated distance was increased.

We did not find any effect of isoflurane pre-exposure of GSCs on tumour volume after injection into mice. This may be related to insufficient time for the tumour to develop.

There have been several retrospective studies reporting the relationship between anaesthetic technique and cancer recurrence in patients.36–38 Experimental, preclinical and clinical studies suggest that general anaesthesia might increase the risk of tumour recurrence when compared with regional anaesthesia.39–40 Compared with general anaesthesia, epidural anaesthesia may improve the prognosis and reduce the recurrence rate for ovarian tumour patients,41 and patients with colon cancer given epidural anaesthesia had longer survival times without recurrence compared with general anaesthesia.42 Thus general anaesthesia during surgical procedures might induce immunosuppression that could affect the ability of cancer cells to proliferate and invade.35 Although the mechanism by which isoflurane drives viability, survival and mobility remains unclear, our study suggests that this common anaesthetic may promote the stem cell properties in glioblastoma, potentially driving a more malignant phenotype of this disease.

However, it is acknowledged that our study lacks an in-depth analysis of the effects of isoflurane in vivo. Other anaesthetics, including propofol and sevoflurane, may also mediate similar effects, and the effect of those agents on GSCs needs to be analysed in a similar fashion. Future studies should evaluate the effects on different cell lines, specifically in primary human glioblastoma cell lines.

In conclusion, this study indicates that exposure to isoflurane under relevant conditions can promote the proliferation, survival and migration distance of human glioblastoma stem cells and provides preliminary evidence suggesting that isoflurane may have potentially detrimental effects in glioblastoma by facilitating its growth and migration capacities.

**Authors’ contributions**

Conception and design, collection and assembly of data, data analysis and interpretation, and manuscript writing: M.Z. and M.L.

Collection and assembly of data, data analysis, interpretation, and manuscript writing: Y.Z., S.D., K.U., and R.X.

Collection and assembly of data and illustrations: X.Q. and S.A.

Data analysis, interpretation, and manuscript writing: M.Z.

Data analysis and interpretation, and manuscript writing: X.Q.

Financial support: D.Y. and T.L.

Design, interpretation of data, manuscript writing, final approval of manuscript, and financial support: D.Y. and T.L.

**Declaration of interest**

None declared.

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**Supplementary material**

Supplementary material is available at British Journal of Anaesthesia online.

**References**

1. Dolecek TA, Propp JM, Stroup NE, Kruchko C. CBTRUS statistical report: primary brain and central nervous system


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