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ORIGINAL RESEARCH

Hyperbaric Oxygen Reverses High-Glucose–Induced Stemness and Radioresistance in Non-Small Cell Lung Cancer Cells

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Background: Hyperglycemia may promote aggressive phenotypes and treatment resistance in non-small cell lung cancer (NSCLC). We investigated whether chronic high glucose (HG) exposure induces stem cell-like properties and radioresistance and whether hyperbaric oxygen (HBO) reverses these effects.

Methods: H1299 and A549 cells were maintained under normal glucose (NG; 5.5 mM D-glucose) or high glucose (HG; 25 mM D-glucose) conditions. Acute effects were evaluated by switching NG-grown cells to NG or HG and performing MTT assays. For all other experiments, cells were conditioned in HG for 3 months. Protein expression of glucose transporter-1 (Glut1), hypoxia-inducible factor-1α (HIF-1α), CD133, SOX2, and OCT4 was assessed by immunoblotting. Stem-like behavior and radiosensitivity were evaluated by tumorsphere formation and clonogenic survival assays following irradiation. HBO (100% O_2, 1.5 ATA, 90 min/session, once daily) was applied as indicated.

Results: Acute HG increased cell viability, whereas long-term HG conditioning did not significantly alter short-term viability but increased tumorsphere formation and clonogenic survival after irradiation. HG upregulated Glut1 and HIF-1α and increased SOX2 and OCT4 expression. Additional analyses showed that 4 weeks, but not 1 week, of continuous HBO exposure reduced HIF-1α, SOX2, and OCT4 expression under HG conditions, whereas Glut1 expression was not markedly altered. HBO suppressed HG-enhanced tumorsphere formation and reversed HG-associated radioresistance.

Conclusion: Chronic HG promoted stem-like and radioresistant phenotypes in NSCLC cells. Prolonged HBO exposure for 4 weeks attenuated these effects and was associated with reduced HIF-1α, SOX2, and OCT4 expression, supporting HBO as a potential preclinical adjunct strategy for mitigating HG-associated therapeutic resistance.

Keywords: non-small cell lung cancer, hyperglycemia, high glucose, hyperbaric oxygen, radioresistance, cancer stem-like cells

Introduction

Radiotherapy is the cornerstone of lung cancer treatment, particularly non-small cell lung cancer (NSCLC); however, its efficacy is often limited by intrinsic and acquired radioresistance.[1] Tumor cells that survive radiation can lead to treatment failure, local recurrence, and metastasis. Clinically, radioresistance is a major obstacle to curing lung cancer because escalating radiation doses are constrained by normal tissue toxicity and yield diminishing survival benefits.[1]

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Hypoxic tumor microenvironment and aberrant cancer cell metabolism are well-recognized contributors to radiotherapy resistance. Hypoxia in solid tumor cells stabilizes hypoxia-inducible factor-1α (HIF-1α), a transcription factor that promotes cell survival pathways, angiogenesis, and a glycolytic "Warburg" metabolism, all of which can hinder radiation response.[2–5] Tumor hypoxia is strongly associated with poor prognosis and reduces the effectiveness of radiotherapy and other treatments of multiple cancers.[2] Conversely, improved tumor oxygenation can enhance radiosensitivity by increasing radiation-induced DNA damage via oxygen fixation.[2] These observations underscore the need for addressing tumor hypoxia and metabolic conditions as part of a strategy for overcoming radioresistance in patients with lung cancer.

Metabolic conditions such as high glucose (HG) availability and hyperglycemia might further influence lung cancer behavior and therapeutic responses. Chronic hyperglycemia is associated with worse treatment outcomes in patients with lung cancer. For instance, elevated hemoglobin A1c levels correlate with significantly higher locoregional recurrence rates after radical radiotherapy in patients with lung cancer.[6] Hyperglycemia and excess glucose can fuel glycolytic metabolism and increase HIF-1α levels under hypoxic conditions,[7] thereby upregulating expression of HIF-1 target genes such as glucose transporter-1 (*Glut1*).[7] *Glut1* is often overexpressed in patients with solid tumors to support increased glucose uptake; its elevated expression is linked to radioresistance and aggressive tumor behavior.[8] High *Glut1* expression substantially correlates with radiation resistance involving various cancers and that targeting Glut1 or its upstream pathways (eg. PI3K/Akt–HIF-1 signaling) could increase radiosensitivity.[8] Consistently, experimental inhibition of HIF-1α or Glut1 has been reported to radiosensitize cancer cells and impair the metabolic advantage conferred by hypoxia.[9–11] These findings suggest that a high-glucose, hypoxia-driven metabolic phenotype (characterized by HIF-1α activation and Glut1-mediated glycolysis) would foster radioresistance in lung cancer cells.

Another key factor in lung cancer radioresistance is the presence of cancer stem-like cells (CSCs). CSCs are a subpopulation of tumor cells with self-renewal capacity and quiescence, making them less susceptible to radiation and chemotherapy. Markers of stemness such as cluster of differentiation 133 (CD133; also known as prominin-1) and the embryonic stem cell transcription factors SRY-related HMG-box gene 2 (SOX2) and octamer-binding transcription factor 4 (OCT4) are often used for identifying CSCs in lung tumor cells. $CD133^+$ cells in patients with NSCLC have been directly implicated in radioresistance: CD133-enriched cells showed higher clonogenic survival after irradiation and more efficient DNA double-strand break repair than did their $CD133^-$ counterparts.[12] These $CD133^+$ CSCs could expand post-radiation and promote tumor regrowth.[12] Similarly, SOX2, a pluripotency factor that maintains stem cell traits, drives radioresistance in NSCLC cases by inducing a dedifferentiated stem-like state.[13] Clinically, NSCLC with high SOX2 or CD133 expression tend to be treatment-refractory and aggressive.[14] Notably, intratumoral hypoxia can induce a stem-like phenotype; HIF-1 signaling under low-oxygen conditions directly induces the expression of embryonic stem cell markers such as OCT4, Nanog homebox, and SOX2 in cancer cells.[15] Mathieu et al reported that HIF activation led to upregulation of OCT4/SOX2 expression and enhanced sphere-forming ability of cancer cells,[15] linking the hypoxia–HIF axis to the maintenance of CSC persistence. Thus, hypoxia and HG not only support cancer cell metabolism, but also converge on promoting "stemness", together conferring greater resistance to radiotherapy and other therapies.

Given the central role of hypoxia/HIF-1α and CSCs in lung cancer radioresistance, strategies for modulating the tumor microenvironment and metabolic state are of great interest. One promising approach is hyperbaric oxygen (HBO) therapy, which involves breathing 100% oxygen at elevated atmospheric pressure to significantly increase tissue oxygenation. HBO has been safely used for decades to treat ischemic and hypoxic conditions; it does not promote tumor growth or recurrence.[16] In contrast, HBO could alleviate tumor hypoxia and has the potential for inhibiting tumor progression in certain contexts.[16] In preclinical studies on lung cancer, HBO has demonstrated multiple beneficial effects on the malignant phenotype under hypoxic or HG conditions. For instance, exposing A549 and H1299 NSCLC cells to HBO (hyperoxia) suppresses hypoxia-induced HIF-1α accumulation and its downstream signals.[17] Zhang et al observed that HBO abrogated the hypoxia-induced Warburg effect in these cells, reducing glucose uptake, lactate production, and ATP generation, ultimately slowing proliferation and even reversing epithelial–mesenchymal transition changes.[17] Additionally, HBO might diminish stem-like therapy-resistant subpopulations of cancer cells. Wang et al reported that HBO "rescued" lung cancer cells from $CoCl_2$-induced chemical hypoxia by restoring cellular differentiation and sensitizing them to apoptosis, effectively countering the acquisition of a low-differentiation, radioresistant phenotype.[18] HBO, as an adjunct to conventional treatment, has also been clinically tested combined with chemotherapy

and regional hyperthermia, with some promising signs of enhanced tumor control in patients with advanced NSCLC.[2,16,19] These findings support the concept that enriching tumors with oxygen could improve radiosensitivity (by overcoming hypoxia/HIF-1-mediated radioresistance) and reduce the cancer stem-like traits that drive relapse.

In this study, we investigated the effects of HG conditions and HBO on lung cancer cell behavior, with a particular focus on proliferation, stem-like characteristics, and radiosensitivity.

Materials and Methods

Cell Lines and Culture Conditions

Human NSCLC cell lines, H1299 (ATCC; CRL-5803; USA) and A549 (BCRC; 60074; Taiwan), were used in this study. H1299 cells were maintained in RPMI-1640 (Gibco; 31800–022; USA), and A549 cells were maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco; 31600–034; USA). The media were supplemented with fetal bovine serum (10% FBS) (Sigma-Aldrich; FC926; USA) and 1% Pen-Strep-Ampho.B Solution (biorion; AACR0003B; Taiwan), unless otherwise stated. Cells were cultured at 37 °C in a humidified incubator with 5% CO_2 and routinely passaged at approximately 70–80% confluence.

Glucose Conditions and Long-Term HG Conditioning

Two glucose conditions were used in this study: normal glucose (NG; 5.5 mM D-glucose) and HG (25 mM D-glucose). Culture media with defined glucose concentrations were prepared using a glucose-free base medium supplemented with D-glucose to achieve final concentrations of 5.5 mM or 25 mM. The acute glucose-switch experiment was performed exclusively for the acute 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay (Thermo; L11939.06; USA), in which cells were first maintained under NG conditions. At the start of the assay (day 0), the cells were switched to either NG or HG medium; cell viability was subsequently assessed over time. Regarding all other experiments, including long-term MTT, Western blotting, tumor-sphere formation, and clonogenic survival assays (with or without HBO and irradiation), cells in the HG group were continuously cultured in HG medium for 3 months prior to experimentation, whereas NG control cells were maintained in NG medium for the same duration. A 3-month conditioning period was selected to model chronic metabolic adaptation/hyperglycemic memory, rather than an acute glucose-switch response.[20,21] The culture media were refreshed every 2–3 days; furthermore, the cells were passaged as needed to maintain exponential growth. Long-term glucose-conditioned cells were used in all subsequent experiments unless otherwise specified.

HBO Exposure

Cells assigned to the HBO treatment were exposed to HBO at 1.5 ATA with 100% O_2 for 90 min per session, once daily. The control cells were handled in parallel under normobaric conditions for the same duration. For experiments evaluating the reversal of HG-associated radioresistance, HG-conditioned cells were exposed to HBO once daily (1.5 ATA, 100% O_2, 90 min/session) for 4 weeks before irradiation. To evaluate the time-dependent molecular effects of HBO under HG conditions, HG-conditioned H1299 and A549 cells were additionally exposed to HBO for 1 week or 4 weeks, followed by Western blot analysis of GLUT1, HIF-1α, SOX2, and OCT4.

MTT Cell Viability Assay

Cell viability was assessed by an MTT assay. Cells were seeded into 24-well plates at 3×10^4 cells/well in the appropriate culture medium and allowed to attach overnight when applicable. At indicated time points, MTT solution (final concentration, 0.5 mg/mL) was added to each well and incubated for 3–4 h at 37 °C. The medium was then removed, and formazan crystals were dissolved in dimethyl sulfoxide (100–200 μL per well). The absorbance was measured at 570 nm (reference: 630–690 nm) using a microplate reader (SpectraMax iD3).

Acute MTT (Glucose Switch)

To evaluate the acute effects of glucose, cells pre-cultured in NG were switched from day 0 to NG or HG, and MTT was measured on the indicated days.

Long-Term Conditioned MTT (± HBO)

In regard to long-term comparisons, NG- or HG-conditioned cells for 3 months were seeded and analyzed by MTT under the indicated conditions, including ± HBO where applicable. Viability was expressed as a percentage relative to that on day 0 (100%).

Western Blot Analysis

Protein expression was assessed using Western blotting. The cells were washed with cold phosphate-buffered saline and lysed in radio-immunoprecipitation assay buffer supplemented with protease and phosphatase inhibitors. Lysates were clarified by centrifugation; protein concentrations were determined by the bicinchoninic acid assay. Equal amounts of protein (50 μg) were resolved by SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were blocked with bovine serum albumin (5%) and incubated with primary antibodies against Glut1 (1:1000; ABclonal; A6982; USA), HIF-1α (1:1000; Proteintech; 20960-1-AP; USA), CD133 (1:500; Cell Signaling; #64326), SOX2 (1:500; 11,064-1-AP; Proteintech; USA), OCT4 (1:500; 11,263-1-AP; Proteintech; USA), and β-actin (1:20000; MAB1501R; Sigma-Aldrich; USA) overnight at 4°C. After incubation with horseradish peroxidase-conjugated goat anti-rabbit (1:2000; C04003; Croyez; Taipei, Taiwan) or goat anti-mouse (1:2000; C04001; Croyez; Taipei, Taiwan) antibody, the bands were visualized using ECL and imaged using a chemiluminescence-detection system (Western Lightning Plus, PerkinElmer; USA). Band intensities were quantified by the ImageJ software. Target protein levels were normalized to β-actin and expressed relative to the control conditions.

Tumor-Sphere Assay

To evaluate the stemness-associated self-renewal capacity, tumor-sphere assays were performed using long-term glucose-conditioned cells. Cells were plated in ultra-low attachment plates (24-well) at 1×10^2 cells/well in serum-free sphere medium consisting of DMEM supplemented with 5.5 mM D-glucose, 10 nM hEGF (E9644; Sigma; USA), and 10 nM bFGF (F3685; Sigma; USA). To minimize confounding by acute differences in sphere culture medium, the same sphere medium was used for both NG- and HG-conditioned cells; therefore, the relevant experimental variable in this assay was the prior long-term glucose conditioning of the cells rather than the glucose concentration of the sphere medium. The spheres were cultured for 7–10 days. Spheres with a diameter of ≥50 μm were counted.

In the HBO groups, cells were exposed to HBO once daily (1.5 ATA, 100% O_2, 90 min) during the sphere formation period. At the end point, the spheres were imaged using an inverted microscope. The sphere number was quantified as the number of spheres per field/well. Sphere size was measured as sphere diameter (μm) by ImageJ.

Irradiation and Clonogenic Survival Assay

Radiosensitivity was assessed by the clonogenic survival assay. Long-term glucose-conditioned cells (NG-conditioned- and HG-conditioned for 3 months) were seeded into tissue culture plates at densities optimized for each radiation dose. The cells were irradiated with 0, 1, 2, 4, or 8 Gy of radiation using a calibrated irradiator. Considering HBO experiments, cells were treated with HBO treatment (1.5 ATA, 100% O_2, 90 min, once daily) before the planned irradiation. After irradiation, the cells were incubated for 14 days to allow colony formation. The colonies were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Colonies containing 50 cells were counted.

Plating efficiency (PE) and surviving fraction (SF) were calculated as follows:

PE = (colonies formed/cells seeded) × 100%

SF = colonies counted/(cells seeded × PE of non-irradiated controls).

Statistical Analysis

Data are presented as mean ± standard error of the mean. Statistical analyses were performed using GraphPad Prism version 9. Comparisons between the two groups were conducted using Student's *t*-test; multiple comparisons were analyzed using one- or two-way analysis of variance followed by Tukey's post hoc test. A value of $P < 0.05$ was considered statistically significant.

Results

HG Exposure Enhances Cell Viability and Promotes Radioresistance in H1299 and A549 Cells

To determine whether elevated glucose availability could alter cell growth, H1299 and A549 cells were initially maintained in NG (5.5 mM D-glucose) and then subjected to NG or HG (25 mM D-glucose), followed by MTT-based viability measurements. Under this acute glucose challenge, HG significantly increased cell viability in a time-dependent manner compared with that after NG treatment in both cell lines (Figure 1A). In H1299 cells, HG treatment led to a significant increase in viability beginning on day 1; this difference became more pronounced on days 2–3 (Figure 1A). In A549 cells, HG did not noticeably alter viability on day 1; however, it significantly increased viability on days 2 and 3 (Figure 1A). To evaluate the impact of long-term glucose conditioning, H1299 and A549 cells were cultured in NG (5.5 mM) or HG (25 mM) for 3 months prior to repeating the MTT assay. After prolonged conditioning, NG- and HG-adapted cells exhibited broadly comparable growth trajectories. Although isolated pairwise differences were observed at individual time points, these differences were cell line-specific and were not sustained; importantly, no single time point showed a significant HG-associated increase in both cell lines simultaneously (Figure 1B). These data indicated that although acute HG exposure robustly enhanced cell viability, this proliferative advantage was not maintained after long-term adaptation to HG (Figure 1A and B). Finally, we assessed whether chronic glucose exposure affected radiosensitivity. After 3 months in the NG or HG, radiosensitivity was assessed by a clonogenic (colony formation) survival assay following irradiation (0–8 Gy). HG-conditioned cells exhibited a higher surviving fraction than did NG-conditioned cells at all irradiation doses, indicating reduced radiosensitivity (increased radioresistance) after chronic HG exposure (Figure 1C). Statistically significant increases in survival were observed at 4 and 8 Gy in H1299 cells and at 4 Gy in A549 cells (Figure 1C).

HG Culture Upregulates Glut1/HIF-1α Signaling and Increases Stemness-Associated Proteins in H1299 and A549 Cells

To investigate whether exposure to HG was associated with molecular changes linked to metabolic adaptation and stem-like properties, protein expression was determined in H1299 and A549 cells cultured in NG (5.5 mM D-glucose) or HG (25 mM D-glucose). Western blotting showed that HG increased the levels of Glut1 and HIF-1α in both cell lines compared with those with NG, with β-actin used as a loading control (Figure 2). Densitometric analysis confirmed that Glut1 protein was significantly elevated to approximately two-fold in H1299 and A549 cells under HG (*$P < 0.05$), while HIF-1α increased to approximately 2.1-fold in H1299 and 2.7-fold in A549 cells (**$P < 0.01$) (Figure 2). As HIF-1α signaling has been linked to acquisition of stem-like characteristics, we next assessed the expression of stemness-associated markers CD133, SOX2, and OCT4. Under HG conditions, CD133 protein levels showed an upward trend in both H1299 and A549 cells (approximately 1.7–1.9-fold); however, this increase did not reach statistical significance (Figure 2). In contrast, HG significantly increased SOX2 and OCT4 expression in both cell lines (Figure 2). SOX2 expression increased approximately to 2.0-fold in H1299 cells and approximately 2.2-fold in A549 cells (*$P < 0.05$), whereas OCT4 expression increased to approximately 2.3-fold in H1299 cells (**$P < 0.01$) and approximately 2.1-fold in A549 cells (*$P < 0.05$) (Figure 2). Collectively, these findings indicated that HG culture enhanced Glut1/HIF-1α signaling and elevated key stemness-associated proteins, suggesting that HG could promote metabolic and phenotypic changes consistent with a more stem-like state in lung cancer cells (Figure 2).

HG Enhances Tumor-Sphere-Forming Capacity in H1299 and A549 Cells

To functionally evaluate whether HG exposure could promote stem-like properties, we performed a tumor-sphere (sphere formation) assays in H1299 and A549 cells cultured under NG (5.5 mM D-glucose) or HG (25 mM D-glucose) conditions. Representative images showed that HG-cultured cells formed more spheres and generated larger spheres than those of NG-cultured cells in both cell lines (Figure 3). Consistent with the microscopic findings, quantitative analysis demonstrated a significant increase in sphere-forming output under HG conditions. In H1299 cells, HG significantly increased the number of spheres (approximately from 13 to 28; *** as indicated) and also increased the mean sphere size (diameter) (approximately from 115 μm to 170 μm; ** as indicated) compared with NG (Figure 3). Similarly, in A549 cells, HG increased the number of spheres (approximately from six to 20; ***) and sphere size

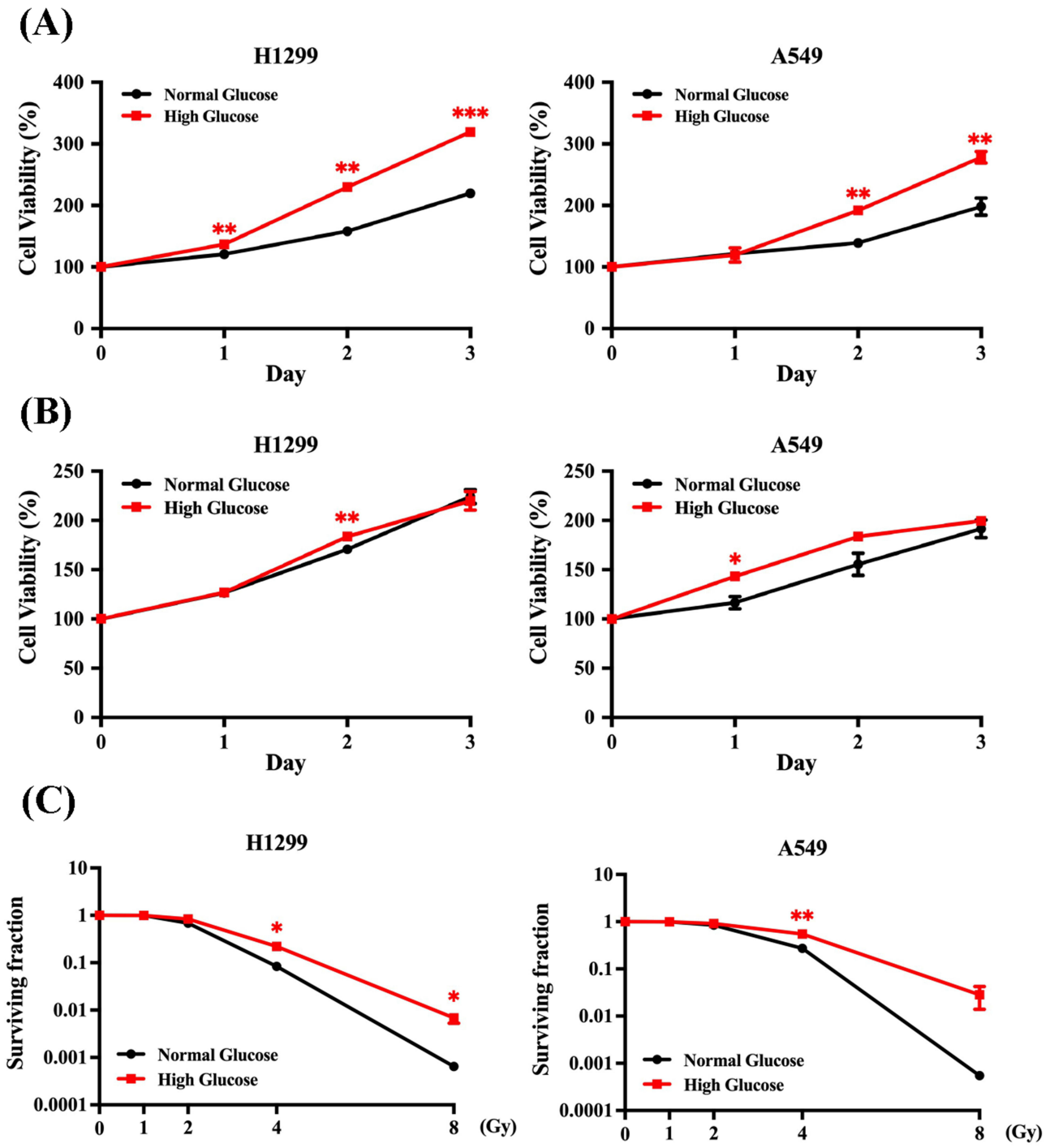

Figure 1 Acute and chronic high glucose exposure increases cell viability and induces radioresistance in H1299 and A549 cells. (**A**) Cells initially maintained in normal glucose (NG; 5.5 mM D-glucose) were cultured in NG (5.5 mM) or high glucose (HG; 25 mM D-glucose) and analyzed by MTT assay over the indicated days to determine relative cell viability. (**B**) Cells were cultured in NG (5.5 mM) or HG (25 mM) for 3 months, followed by MTT assay to compare cell viability over time. (**C**) After 3 months of culture in NG (5.5 mM) or HG (25 mM), radiosensitivity was assessed by clonogenic survival assay after irradiation (0–8 Gy), and surviving fractions were plotted. Asterisks indicate statistical significance compared with the NG group at the same time point/dose (*P < 0.05, **P < 0.01, ***P < 0.001).

(approximately from 100 μm to 145 μm; ***) relative to NG (Figure 3). Collectively, these results indicated that HG culture enhanced both the frequency and growth of spheres, supporting a functional increase in the sphere-forming (stem-like) capacity of H1299 and A549 cells (Figure 3).

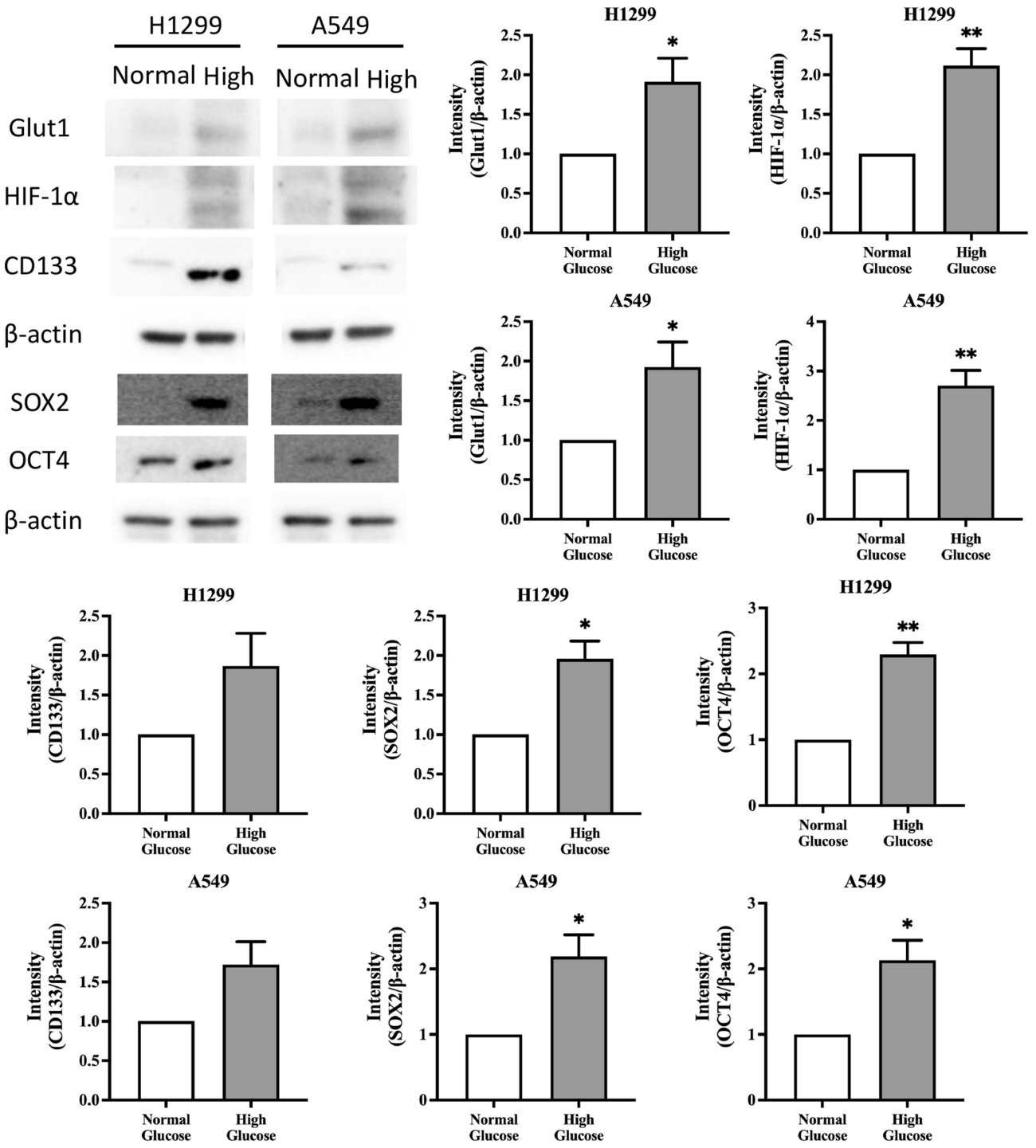

Figure 2 High glucose increases Glut1 and HIF-1α expression and stemness-associated markers in H1299 and A549 cells. H1299 and A549 cells were cultured in normal glucose (NG; 5.5 mM D-glucose) or high glucose (HG; 25 mM D-glucose) conditions and analyzed by Western blotting. Representative immunoblots show protein expression of Glut1, HIF-1α, and the stemness-associated markers CD133, SOX2, and OCT4, with β-actin as the loading control. Bar graphs depict densitometric quantification of each target protein normalized to β-actin and expressed compared with that in the NG group. *P < 0.05 and **P < 0.01 versus the NG group.

HBO Treatment Enhances Radiosensitivity of H1299 and A549 Cells Without Marked Effects on Short-Term Cell Viability

To determine whether increasing oxygen availability could modulate the radiation response of lung cancer cells, H1299 and A549 cells were treated with HBO, and radiosensitivity was assessed by a clonogenic survival assay. HBO treatment shifted

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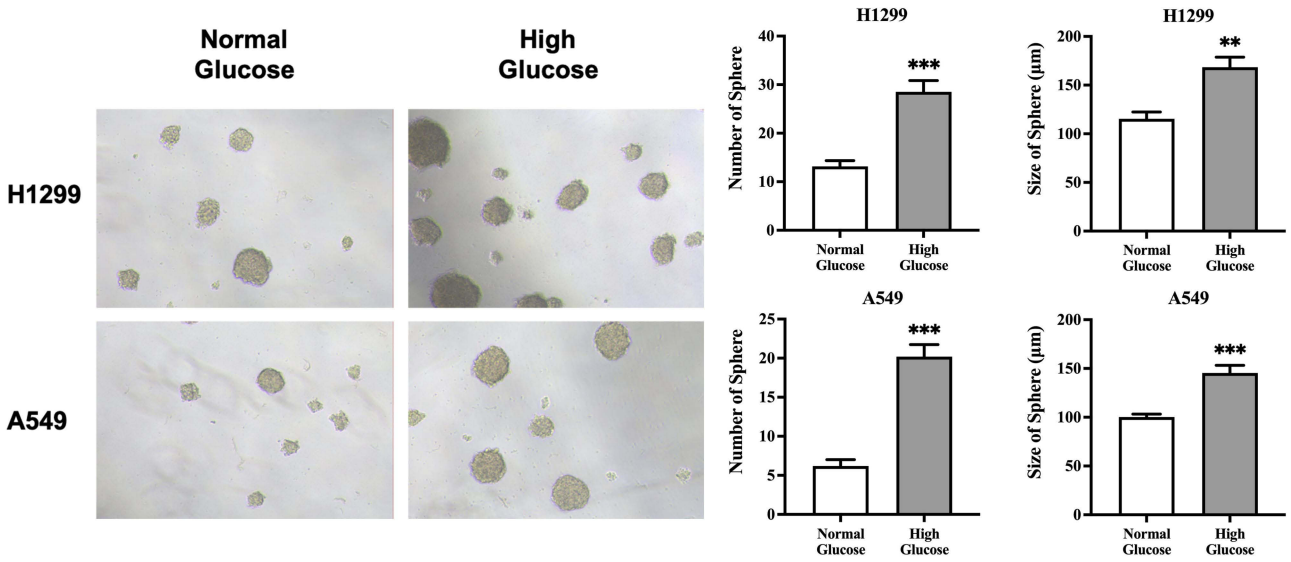

Figure 3 High glucose promotes tumor-sphere formation and increases sphere size in H1299 and A549 cells. H1299 and A549 cells cultured in normal glucose (NG; 5.5 mM D-glucose) or high glucose (HG; 25 mM D-glucose) were subjected to a sphere formation (tumor-sphere) assay. Representative images show the spheres formed under NG and HG conditions for each cell line. Bar graphs show quantification of sphere number and sphere size (μm) in H1299 and A549 cells. Error bars indicate the variability among replicate measurements. Asterisks denote statistical significance compared with the NG group. **P < 0.01 and ***P < 0.001 versus the NG group.

the survival curves downward in both cell lines, indicating enhanced radiosensitivity compared with that in the untreated control (Con) (Figure 4A). In H1299 cells, HBO significantly reduced the surviving fraction at 1, 2, and 8 Gy, relative to that in the Con group (Figure 4A). Similarly, in A549 cells, HBO significantly decreased clonogenic survival at 2, 4, and 8 Gy compared with that in the Con (Figure 4A). Notably, the divergence between HBO and Con was most prominent at a high dose (8 Gy), supporting the robust radiosensitizing effect of HBO in both models (Figure 4A). As a reduction in clonogenic survival could potentially reflect nonspecific cytotoxicity, we evaluated whether HBO altered short-term cell viability under different glucose conditions using an MTT assay. H1299 and A549 cells cultured under NG (5.5 mM D-glucose) or HG (25 mM D-glucose) conditions were compared in the presence or absence of HBO. Across the 3-day observation period, all groups displayed time-dependent increases in viability; moreover, HBO did not cause marked suppression of viability under either NG or HG conditions (Figure 4B). In H1299 cells, HG showed a modest but significant difference on day 1 (as indicated), whereas on days 2 and 3, the growth curves among the four groups were comparable (Figure 4B). In A549 cells, viability trajectories were also similar among the groups, with only a small but statistically significant difference at day 3 (denoted by "#" in the figure) (Figure 4B). Together, these findings suggest that HBO primarily enhanced radiosensitivity rather than exerting strong cytotoxic effects on baseline cell viability (Figure 4A and B).

HBO Reverses HG-Associated Radioresistance in H1299 and A549 Cells

To determine whether HBO could modulate the HG-associated radiation response, H1299 and A549 cells were evaluated by clonogenic survival assay after irradiation under four conditions: normal glucose (NG; 5.5 mM D-glucose), high glucose (HG; 25 mM D-glucose), NG + HBO, and HG + HBO (Figure 4C). Consistent with the radioresistant phenotype, HG increased clonogenic survival compared to NG. In H1299 cells, HG significantly increased the surviving fraction at 2, 4, and 8 Gy relative to NG (Figure 4C). In A549 cells, HG also resulted in significantly higher survival than NG at 2 and 8 Gy, indicating that HG promotes resistance, especially at higher radiation doses (Figure 4C). In contrast, HBO reduced clonogenic survival under both glucose conditions, demonstrating its radiosensitizing effects. In H1299 cells, HBO significantly decreased survival in the NG condition at 4 Gy (NG vs. NG + HBO) and markedly reduced survival in HG-conditioned cells at 2, 4, and 8 Gy (HG vs. HG + HBO) (Figure 4C). Similarly, in A549 cells, NG + HBO showed significantly lower survival at 2, 4, and 8 Gy than did NG, and HBO reduced survival in HG-cultured cells at 2 and 8 Gy (HG vs. HG + HBO) (Figure 4C). Importantly, the HG+HBO survival curve shifted downward toward (or below) the NG curve, indicating that HBO counteracted the HG-associated radioresistant phenotype and restored a more radiosensitive response in both lung cancer cell lines (Figure 4C).

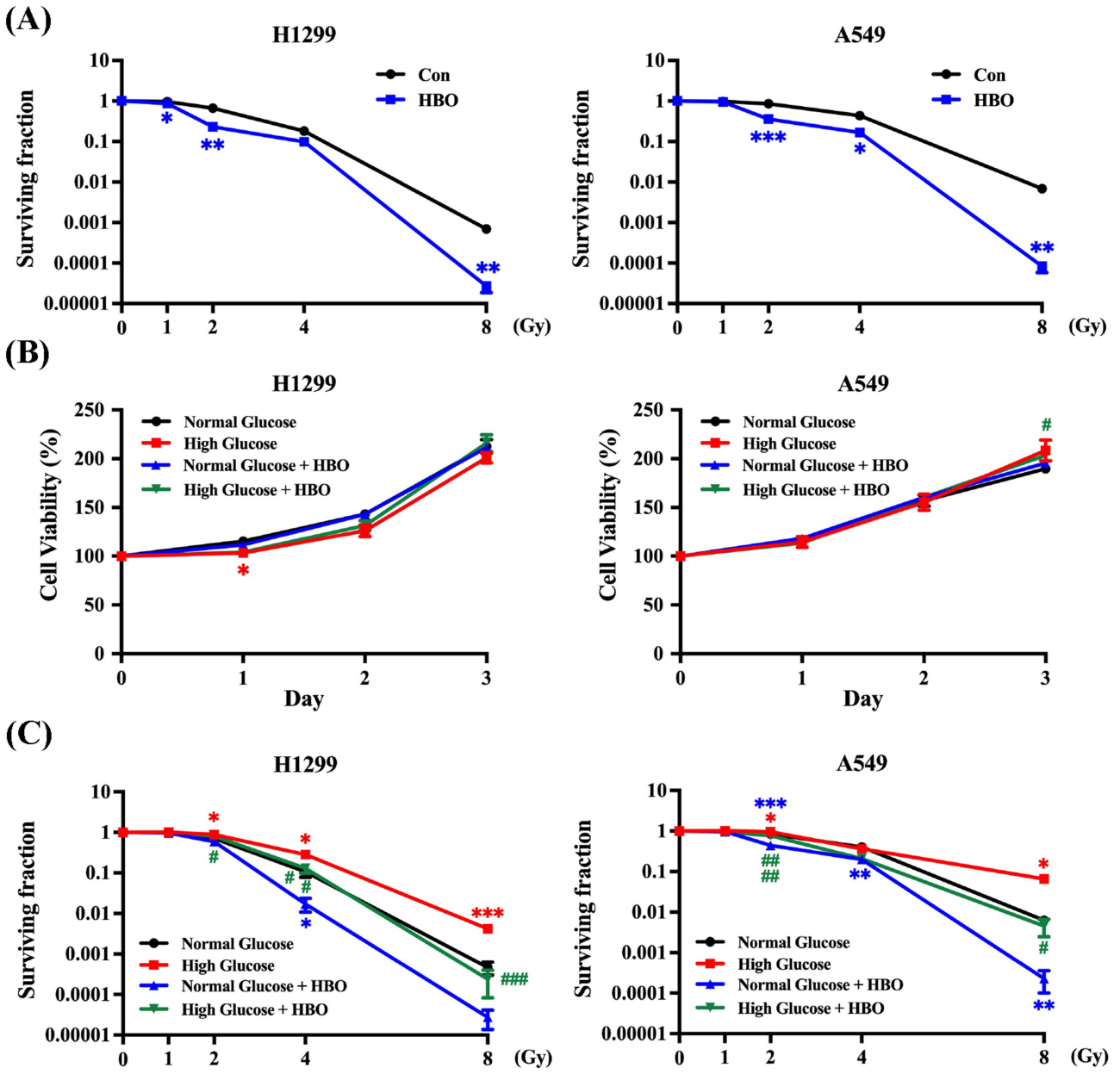

Figure 4 Hyperbaric oxygen (HBO) treatment radiosensitizes H1299 and A549 cells with minimal effects on short-term cell viability under normal or high glucose conditions. (**A**) H1299 and A549 cells were treated with HBO or left untreated (control) and exposed to ionizing radiation (0–8 Gy). Radiosensitivity was evaluated by clonogenic survival assay; the surviving fractions were plotted as a function of the radiation dose. (**B**) H1299 and A549 cells cultured in normal glucose (NG; 5.5 mM D-glucose) or high glucose (HG; 25 mM D-glucose) were assessed for short-term viability by the MTT assay in the presence or absence of HBO. Cell viability is presented as a percentage relative to day 0. (**C**) H1299 and A549 cells were cultured under NG or HG conditions and treated with or without HBO, followed by ionizing radiation (0–8 Gy). Radiosensitivity was assessed by clonogenic survival assay. Surviving fractions were plotted as a function of radiation dose for the NG, HG, NG + HBO, and HG + HBO groups. In (**A** and **B**), asterisks indicate statistically significant differences between the indicated groups at the same dose or time point (*P < 0.05, **P < 0.01, ***P < 0.001). In (**B**), the "#" symbol indicates a statistically significant difference between the NG + HBO and HG + HBO groups. In (**C**), red asterisks denote significant differences for HG vs. NG, blue asterisks denote significant differences for NG + HBO vs. NG, and green hash symbols denote significant differences for HG + HBO vs. HG (*P < 0.05, **P < 0.01, ***P < 0.001; #P < 0.05, ##P < 0.01, ###P < 0.001).

HBO Suppresses HG-Enhanced Tumor-Sphere Formation in H1299 and A549 Cells

To assess whether HBO would modulate the stemness-like phenotype induced by glucose availability, H1299 and A549 cells were subjected to a tumor-sphere (sphere formation) assay under four conditions: NG (5.5 mM D-glucose), HG (25 mM D-glucose), NG + HBO, and HG + HBO. The representative images showed that HG markedly increased both the number and size of spheres in H1299 and A549 cells compared with those under NG conditions, whereas HBO-treated

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cultures displayed visibly fewer and/or smaller spheres, particularly under HG conditions (Figure 5). Quantification confirmed that HG significantly increased the sphere output in both cell lines. In H1299 cells, HG increased the number of spheres (approximately 13 in NG vs. 28 in HG; *** as indicated) and sphere size (approximately 115 μm in NG vs. 170 μm in HG; ** as indicated) (Figure 5). Importantly, HBO attenuated the HG-driven increase in sphere formation: Under HG, HBO significantly reduced sphere number (HG vs. HG + HBO; *** as indicated) and markedly decreased sphere size (HG vs. HG + HBO; *** as indicated), bringing values closer to those observed under NG conditions (Figure 5). In addition, HBO reduced the sphere size under NG (NG vs. NG + HBO; * as indicated), suggesting that elevated oxygen availability can limit sphere growth, even under baseline glucose conditions (Figure 5). In A549 cells, HG likewise induced a strong increase in sphere number (approximately 6 in NG vs. 20 in HG; ***) and sphere size (approximately 100 μm in NG vs. 145 μm in HG; ***) (Figure 5). HBO significantly reversed the HG-associated increase, reducing the number of spheres (HG vs. HG + HBO; ***) and sphere size (HG vs. HG + HBO; ***) (Figure 5). Notably, in A549 cells, HBO modestly increased the sphere number under NG (NG vs. NG + HBO; *); however, under HBO conditions, the HG group formed significantly fewer spheres than those in the NG + HBO group (NG + HBO vs. HG + HBO; *), indicating that the pro-sphere effect of HG was abolished and even partially reversed in the presence of HBO (Figure 5). Overall, these findings demonstrate that HG promotes tumor-sphere formation, while HBO markedly suppresses HG-enhanced sphere number and sphere growth, supporting a role for HBO in counteracting glucose-associated acquisition/maintenance of sphere-forming capacity in H1299 and A549 cells (Figure 5).

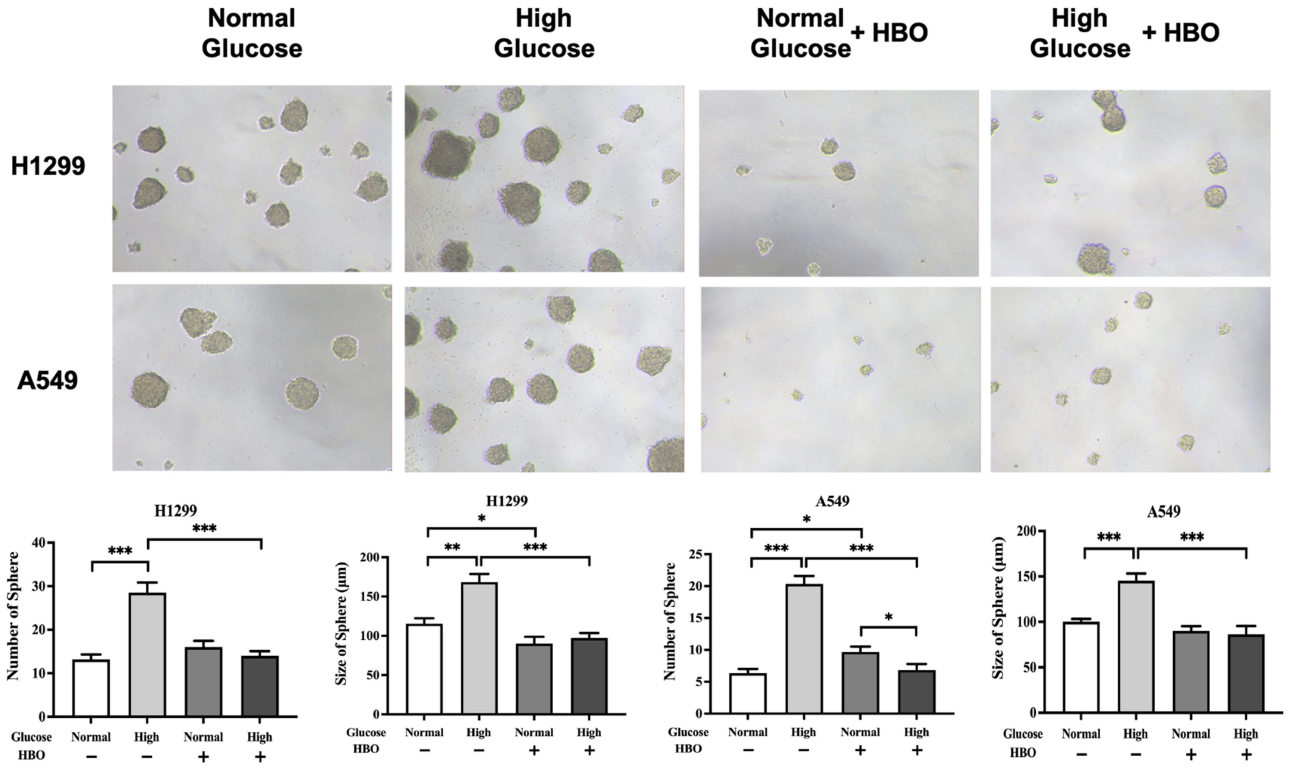

Figure 5 Hyperbaric oxygen attenuates high glucose–induced tumor-sphere formation in H1299 and A549 cells. H1299 and A549 cells were cultured under normal glucose (NG; 5.5 mM D-glucose) or high glucose (HG; 25 mM D-glucose) conditions in the presence or absence of hyperbaric oxygen (HBO) and then subjected to a tumorsphere formation assay. Representative images show tumorspheres formed under NG, HG, NG + HBO, and HG + HBO conditions in each cell line. Sphere-forming capacity was quantified by measuring sphere number and sphere size (μm), as shown in the bar graphs. In the condition labels, "–" and "+" indicate the absence and presence of the indicated condition, respectively; under the HG row, "–" denotes NG and "+" denotes HG, whereas under the HBO row, "–" denotes without HBO and "+" denotes with HBO. Error bars indicate variation among replicates. Statistical significance for bracketed comparisons is indicated as *P < 0.05, **P < 0.01, and ***P < 0.001.

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Prolonged Hyperbaric Oxygen Exposure Suppresses HIF-1α and Stemness-Associated Proteins in High-Glucose-Conditioned H1299 and A549 Cells

To determine whether repeated hyperbaric oxygen (HBO) exposure modulates high glucose (HG)-associated metabolic and stemness-related signaling, HG-conditioned H1299 and A549 cells were exposed to HBO for 1 week or 4 weeks, and protein expression was examined by Western blotting. As shown in the representative blots and densitometric analyses, Glut1 expression remained largely unchanged after either 1 or 4 weeks of HBO treatment in both cell lines (Figure 6), indicating that prolonged HBO did not markedly affect Glut1 under HG conditions. In contrast, HIF-1α expression was significantly reduced after 4 weeks of HBO exposure in both H1299 and A549 cells, whereas 1 week of HBO produced no significant change (Figure 6). In H1299 cells, HIF-1α decreased to approximately 0.6-fold of the HG control after 4 weeks ($P < 0.01$), while in A549 cells it decreased to approximately 0.4-fold ($P < 0.001$). Similarly, prolonged HBO exposure markedly suppressed the stemness-associated proteins SOX2 and OCT4. In H1299 cells, 4 weeks of HBO reduced SOX2 and OCT4 to approximately 0.5-fold and 0.2-fold, respectively, compared with the HG control ($P < 0.001$). In A549 cells, 4 weeks of HBO also significantly decreased SOX2 and OCT4 to approximately 0.1–0.3-fold of control levels ($P < 0.001$). By contrast, 1 week of HBO did not significantly alter SOX2 or OCT4 expression in either cell line (Figure 6). Collectively, these findings indicate that prolonged HBO exposure exerts a time-dependent suppressive effect on HG-associated HIF-1α and stemness-related proteins, whereas Glut1 expression is relatively unaffected under the same conditions.

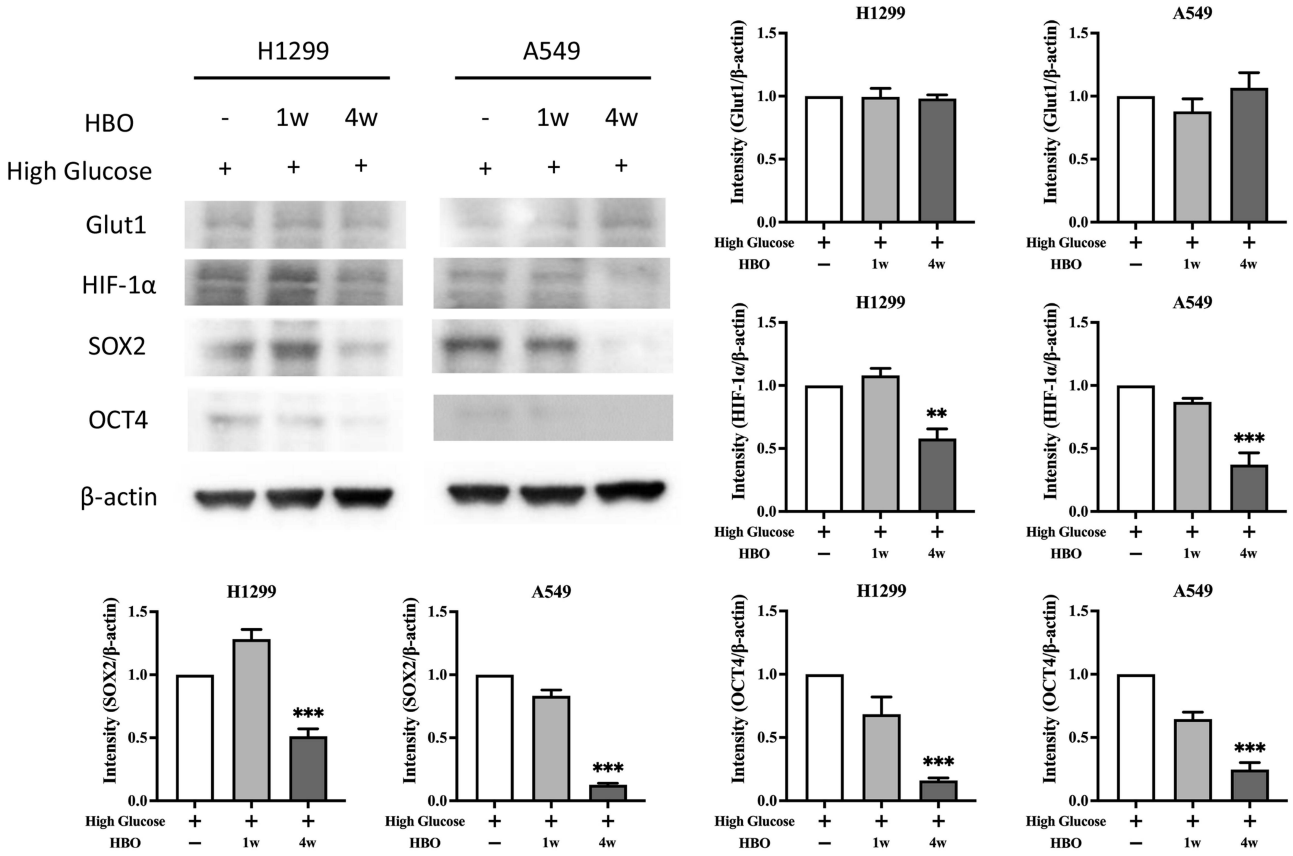

Figure 6 Prolonged hyperbaric oxygen exposure suppresses HIF-1α and stemness-associated proteins in high-glucose-conditioned H1299 and A549 cells. H1299 and A549 cells maintained under high-glucose (HG; 25 mM D-glucose) conditions were exposed to hyperbaric oxygen (HBO) for 1 week (1w) or 4 weeks (4w). Protein expression of Glut1, HIF-1α, SOX2, and OCT4 was analyzed by Western blotting, with β-actin used as the loading control. Representative immunoblots are shown on the left, and bar graphs on the right and bottom panels show densitometric quantification of each target protein normalized to β-actin and expressed relative to the HG control without HBO (set to 1). Prolonged HBO exposure for 4 weeks significantly reduced HIF-1α, SOX2, and OCT4 expression in both H1299 and A549 cells, whereas Glut1 expression was not markedly altered. Asterisks indicate statistically significant differences compared with the HG control group in the corresponding cell line (**$P < 0.01$, ***$P < 0.001$).

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Discussion

In this study, we demonstrated that acute exposure to HG (25 mM) increased MTT-based viability in NSCLC cells, whereas long-term HG conditioning did not sustain enhanced short-term viability but instead promoted stem-like properties and radioresistance. Specifically, acute HG challenge significantly increased cell viability in a time-dependent manner, with a more pronounced late response in A549 cells (Figure 1A), consistent with previous reports that hyperglycemia can promote malignant phenotypes in patients with lung cancer.[22] In contrast, after 3 months of glucose conditioning, NG- and HG-adapted cells exhibited overlapping MTT growth curves without significant differences, indicating that the acute viability advantage conferred by HG was attenuated following prolonged adaptation. Importantly, the absence of sustained differences in short-term MTT readouts did not preclude functional alterations: Chronic HG conditioning markedly enhanced tumor-sphere formation (Figure 3) and increased clonogenic survival after irradiation (Figure 1C), suggesting that prolonged HG exposure preferentially promoted stem cell-like traits and radioresistance rather than persistent acceleration of bulk growth.

At the molecular level, HG exposure significantly upregulated Glut1 and HIF-1α in both H1299 and A549 cells (Figure 2), supporting the induction of a glycolytic and stress-adaptive phenotype. Although these experiments were conducted under normoxic conditions, HG levels can activate hypoxia-inducible signaling via oxygen-independent mechanisms. In particular, activation of the receptor for advanced glycation end products–NADPH oxidase axis under HG conditions leads to increased reactive oxygen species and stabilization of HIF-1α.[22] Consistent with these findings, our data showed increased HIF-1α expression under HG, which in turn might drive Glut1 upregulation and reinforce glucose uptake and glycolytic metabolism. Notably, despite sustained HG exposure, HIF-1α levels did not continue to indefinitely increase, suggesting metabolic homeostasis establishment during long-term adaptation. Recent proteomic analysis of human NSCLC tissues has likewise shown that lung tumors undergo metabolic adaptation to oxidative stress, characterized by increased glutathione biosynthesis and reduced advanced glycation end-product formation, supporting the concept that lung cancer cells can reprogram glucose-related metabolism to sustain survival under stress conditions.[23] Nevertheless, chronic HG conditioning resulted in a phenotype characterized by persistently elevated metabolic signaling and functional resistance to radiation.

In addition to metabolic adaptation, HG significantly enhanced the stem cell-like characteristics of NSCLC cells. The expression of the stemness-associated markers SOX2 and OCT4 increased under HG conditions (Figure 2), accompanied by a robust increase in tumor-sphere number and size (Figure 3). These functional assays provided strong evidence that HG could enrich the sphere-forming stem cell-like subpopulation. This observation is consistent with that in previous studies demonstrating that hyperglycemia and diabetic conditions can promote stemness-associated phenotypes across multiple cancer types.[22,24–26] Importantly, enhancement of tumor-sphere formation following long-term HG conditioning occurred despite the absence of sustained differences in MTT viability, indicating that HG selectively augmented self-renewal capacity rather than general proliferative activity.

Mechanistically, metabolic stress and inflammatory signaling induced by chronic hyperglycemia have been implicated in maintaining stem cell-like states. HG environments are associated with increased oxidative and inflammatory cues that converge on transcriptional programs regulating pluripotency and cellular plasticity. In particular, HIF-1α interacts with stemness-related transcriptional networks and supports CSC maintenance.[15] Although the present study did not directly investigate epithelial–mesenchymal transition or inflammatory mediators, the observed upregulation of stemness markers and enhanced sphere-forming capacity strongly supported the finding of a functional shift toward a stem cell-like phenotype under HG conditions.

A central finding of this study was that chronic HG conditioning conferred pronounced radioresistance, as evidenced by increased clonogenic survival following irradiation. This effect was observed at multiple radiation doses in both NSCLC cell lines, indicating that HG broadly enhanced survival following genotoxic stress. Clinically, tumors with elevated glucose metabolism, often assessed by ^{18}F-fluorodeoxyglucose (FDG) uptake on FDG-positron emission tomography, have been associated with poor local control and survival after radiotherapy.[27] Our data provided mechanistic support for these clinical observations by demonstrating that HG directly increased the clonogenic survival capacity of irradiated lung cancer cells.

Previous studies have identified specific pathways linking hyperglycemia to radioresistance. Notably, Kwon et al reported that HG inhibits radiation-induced anoikis in NSCLC cells by disrupting death-associated protein kinase-mediated apoptotic signaling, thereby promoting survival following irradiation.[27] Although the apoptotic pathways were not directly studied in the present study, the increased clonogenic survival observed under HG conditions was consistent with the impaired elimination of irradiated cells. In addition, the enhanced glycolytic metabolism under HG conditions might provide metabolic resources that support DNA damage tolerance and post-irradiation survival. Taken together, these findings support the hypothesis that hyperglycemia might represent a modifiable contributor to radioresistance in lung cancer cells.

Importantly, we demonstrated that HBO effectively counteracted both HG-induced radioresistance and stem-like phenotypes. HBO treatment significantly reduced clonogenic survival following irradiation under both NG and HG conditions while exerting minimal effects on short-term MTT viability. These results indicated that HBO enhanced radiosensitivity primarily by impairing long-term reproductive survival rather than by inducing acute cytotoxicity. The radiosensitizing effect of HBO is consistent with the well-established oxygen effect in radiobiology, by which increased oxygen availability enhances the fixation of radiation-induced DNA damage.[28]

In addition to radiosensitization, HBO markedly suppressed HG-enhanced tumor-sphere formation (Figure 5), indicating a reduction in stem cell-like properties. Hyperoxic conditions can disrupt hypoxia-driven maintenance of stemness by attenuating HIF-1α signaling and altering the tumor microenvironment.[17,18,28] Our findings extended these observations by demonstrating that HBO effectively reversed HG-associated stem cell-like phenotypes, highlighting its dual impact on radiosensitivity and cellular plasticity.

Importantly, we further examined whether HBO directly affects HG-associated hypoxia/stemness-related proteins. Additional Western blot analyses showed that 4 weeks, but not 1 week, of continuous HBO exposure significantly reduced HIF-1α, SOX2, and OCT4 expression in HG-conditioned H1299 and A549 cells, whereas Glut1 expression remained largely unchanged (Figure 6). These findings provide direct molecular evidence that prolonged HBO attenuates selected HG-associated hypoxia/stemness-related proteins. Together with the reduced tumorsphere formation and improved clonogenic radiosensitivity observed under HG + HBO conditions (Figures 4C and 5), these data support the interpretation that the effective reversal of HG-associated radioresistance in our model requires prolonged HBO exposure. At the same time, because Glut1 was not significantly altered, our results suggest that HBO may not broadly reverse all HG-induced molecular changes, and the precise downstream mechanisms require further investigation.

Collectively, our results suggested that chronic HG exposure could reshape NSCLC cell behavior by promoting stemness and radioresistance, even in the absence of sustained proliferative advantages. In this in vitro preclinical model, prolonged HBO exposure attenuated these adverse effects at both the functional and molecular levels. These findings support further investigation of oxygen-based or metabolic interventions in hyperglycemic or metabolically aggressive lung cancer models.

This study has several limitations. First, all experiments were conducted in vitro, and in vivo validation is required to determine the therapeutic relevance of HBO in hyperglycemic tumor settings. Second, only two NSCLC cell lines were evaluated, which limits generalizability across molecular subtypes. Third, although prolonged HBO reduced HIF-1α, SOX2, and OCT4 expression under HG conditions, the present study did not include mechanistic intervention experiments to establish causality. In addition, post-transcriptional regulatory mechanisms, including APA/eQTL-associated variation in genes such as NIT2, may also contribute to malignant phenotypes in lung adenocarcinoma, underscoring the broader molecular heterogeneity of lung cancer beyond glucose availability alone.[29] Future studies integrating metabolic, transcriptomic, and in vivo approaches are warranted.

In conclusion, chronic HG promoted stem-like and radioresistant phenotypes in NSCLC cells. Although the short-term viability advantage observed under acute HG challenge was not maintained after long-term adaptation, chronic HG conditioning markedly enhanced tumorsphere formation and clonogenic survival after irradiation. Importantly, prolonged HBO exposure for 4 weeks effectively radiosensitized both H1299 and A549 cells, suppressed HG-enhanced tumorsphere formation, and reduced HIF-1α, SOX2, and OCT4 expression under HG conditions. Collectively, these findings indicate that the effective reversal of HG-associated radioresistance in this in vitro model requires continuous HBO

exposure over 4 weeks and support further preclinical investigation of HBO as a strategy to mitigate HG-associated therapeutic resistance in NSCLC.

Abbreviations

CD133, cluster of differentiation 133; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; Glut1, glucose transporter 1; HBO, hyperbaric oxygen; HG, high glucose; HIF-1α, hypoxia-inducible factor-1 alpha; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NG, normal glucose; NSCLC, non-small cell lung cancer; OCT4, octamer-binding transcription factor 4; PE, plating efficiency; SF, surviving fraction; SOX2, SRY-related HMG-box gene 2.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from Hung-Pei Tsai upon reasonable request.

Ethics Approval and Informed Consent

This study used established human NSCLC cell lines and did not involve human participants, primary human samples, identifiable human data, or animals. Therefore, institutional review board approval and informed consent were not required.

Author Contributions

All authors made a significant contribution to the work reported, whether that was in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors have no conflicts of interest in this work.

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