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Original Research

Dietary 2-deoxy-D-glucose impairs tumour growth and metastasis by inhibiting angiogenesis

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Abstract Accumulating evidence suggests the antiangiogenic potential of the glycolytic inhibitor 2-deoxy-D-glucose (2-DG) among the anticancerous properties of this drug. In the present studies, we investigated the antiangiogenic effects of dietary 2-DG on tumour (Lewis lung carcinoma [LLC]) as well as ionising radiation–induced angiogenesis in mouse models. Dietary 2-DG reduced the serum vascular endothelial growth factor levels (~40%) in LLC-bearing mice along with a significant inhibition of tumour growth and metastases. *In vivo* Matrigel plug assays showed significant decrease in vascularisation, Fluorescein isothiocyanate (FITC)-dextran fluorescence and factor VIII–positive cells in the plugs from 2-DG–fed mice, supporting the notion that dietary 2-DG significantly suppresses the tumour-associated and radiation-induced angiogenesis. 2-DG inhibited the glucose usage and lactate production as well as ATP levels of human umbilical vein endothelial cells (HUVECs) in a concentration-dependent manner, accompanied by growth inhibition and loss of viability *in vitro*. Furthermore, 2-DG inhibited the capillary-like tube formation in Matrigel as well as migration and transwell invasion by HUVECs, which are functional indicators of the process of angiogenesis.

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These results suggest that dietary 2-DG inhibits processes related to angiogenesis, which can impair the growth and metastasis of tumours.

1. Introduction

Tumour growth and its metastatic spread mainly depend on neoangiogenesis, making it a potential target for tumour therapy. Normally, cells of endothelial origin rarely divide and remain quiescent with slower cellular metabolism. However, on activation in diseased condition, endothelial cells (ECs) change to a highly proliferative state dictated as the angiogenic switch, driven by a metabolic modulation besides angiogenic growth factors (e.g. vascular endothelial growth factor [VEGF]), making ECs responsive to metabolic targeting [1,2]. Antiangiogenic therapy has gained interest recently; however, it has not been quite successful for various reasons such as development of resistance to chemotherapy and radiation [3–5]. Current anti-angiogenic therapies include mainly the use of monoclonal antibodies and pharmacological inhibitors against VEGF [6,7], platelet-derived growth factors [8,9] and receptor tyrosine kinase [10,11].

Neovascularisation in response to proangiogenic stimuli such as hypoxia and growth factors develops extensively in the tumour bed. However, it is not able to deliver sufficient oxygen and nutrients to the tumours because of the structural abnormality of these newly formed blood vessels as they are chaotic, leaky and irregular [12]. This insufficiency leads to more hypoxic regions, further enhancing the tumour growth and vasculature, and is a critical factor in impaired drug delivery during solid-tumour therapy [13,14]. Radiotherapy, which is an essential treatment modality of cancer management, exerts its antitumour effects by killing tumour cells and bringing changes in the tumour microenvironment (TME), including the ECs in the microvasculature [15]. At higher doses of ionising radiation (IR), ceramide-mediated apoptosis in the ECs within the tumour bed has been observed, thus contributing to reduced tumour growth [16]. On the other hand, there are recent clinical and experimental observations demonstrating proangiogenic and prometastatic effects of IR [17,18]. During radiotherapy, the tissue surrounding the tumour area receives low doses of IR compared with those delivered inside the tumour mass as multiple radiation beams (fractionated) are given to prevent damage of the adjoining organs at risk [19]. Low-dose IR has been shown to promote angiogenesis, resulting in accelerated tumour regrowth and metastasis [5,18]. Stimulated angiogenesis involves proliferation, migration and capillary formation by ECs, requiring increased energy demand [20–22]. Moreover, the angiogenic switch significantly upregulates EC expression of glucose transporter 1 (GLUT-1) and glucose uptake, where almost all the energy (ATP) is derived from the catabolism of glucose generated by glycolysis [20,21,23]. High blood glucose levels and accelerated angiogenesis have often been correlated with aggressive growth of gliomas in mice [24–26]. On the other hand, dietary restriction has been shown to induce an antiangiogenic effect, suggesting that reduced energy intake significantly contributes to the local tumour control through a shift from a proangiogenic to an antiangiogenic state [27]. Therefore, approaches that can simultaneously target the glucose metabolism of tumour cells and of activated ECs inhibiting angiogenesis would be beneficial as a preventive tumour strategy.

Restricted caloric intake significantly compromises the tumour growth and angiogenesis of both non-invasive and highly invasive experimental mouse and human brain tumours [24,28,29]. We have previously shown that the dietary administration of the glycolytic inhibitor 2-deoxy-D-glucose (2-DG) as an energy restriction mimetic (ERM) agent impairs the process of tumourigenesis after implantation of Ehrlich ascites carcinoma (EAC) cells by modulating several key factors such as blood glucose, insulin, PI3K/Akt signalling, immune status and MMP-9 activity, besides targeting the glucose metabolism of the tumour [30]. Furthermore, a significantly lower vascular density in the tumours suggested antiangiogenic activity in the 2-DG–fed animals, which could be partly responsible for the impaired tumour growth [30]. This prompted us to evaluate the effects of 2-DG on the process of angiogenesis *in vivo* and *in vitro*. Therefore, the present studies were undertaken to investigate the effects of dietary administration of 2-DG on tumour-induced angiogenesis and radiation-induced angiogenesis (RIA). For tumour-induced angiogenesis, we implanted Lewis lung carcinoma (LLC) cells in C57BL/6 mice that facilitate tumour growth and its metastasis in the lung, whereas RIA was studied in nude mice. LLC tumours are highly angiogenic and invasive among transplantable tumour models with distant metastasis property in immunocompetent mice [31]. Furthermore, we also investigated the effects of 2-DG on the formation of capillary tubes and migration of human umbilical vein endothelial cells (HUVECs) that represent the *in vitro* processes of angiogenesis and metastatic potential. The results clearly show the antiangiogenic effects of dietary 2-DG

both in tumour-induced and RIA, which appears to be due to the inhibition of growth and compromised survival of ECs that may be linked to impaired glycolysis.

2. Materials and methods

2.1. Cell lines, animals and reagents

HUVECs and the endothelial growth medium (EGM) were purchased from HiMedia Pvt. Ltd. (Mumbai, India). HUVECs were cultured in 1% gelatin–coated tissue culture flasks in complete EGM containing 100 units/ml penicillin, 100 g/ml streptomycin and 3 ng/ml endothelial cell growth factor (ECGF) and maintained in an incubator at 5% CO_2. The LLC cell line was grown in RPMI-1640 (Sigma chemicals) with 10% foetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO_2. Male C57BL/6 mice (8–10 weeks old) for the tumour-associated angiogenesis (TAA) and metastasis study and 6 to 8 week-old athymic Balb C nu/nu female mice for the RIA study were obtained from the institute's in-house experimental animal facility. D2-DG was kindly provided by Dr. Reddy's Laboratories. Cobalt chloride ($CoCl_2$), Dispase, Dimethyl sulfoxide (DMSO), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and FITC-dextran were procured from Sigma chemicals. The growth medium RPMI-1640 was purchased from Sigma. The cell detachment solution Accutase for ECs was purchased from HiMedia. Matrigel (HC) was obtained from BD Biosciences (Bedford, MA) and used *in vitro* at 4 mg/mL and *in vivo* at 10 mg/mL concentration.

2.2. Ethics statement

Animal studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals in cancer research of the United Kingdom Coordinating Committee on Cancer Research [32]. The protocols were approved by the Committee on the Ethics of Animal Experiments of the Institute of Nuclear Medicine and Allied Sciences, Defence Research and Development Organization (Institutional Ethical Committee approval; INM/IAEC/2011/08/001).

2.3. Irradiation

All irradiation experiments were performed using IRfrom a ^{60}Co gamma source (Bhabhatron-II teletherapy unit, Panacea) at a dose rate of 1.9 Gy/min and source-skin-distance (SSD) of 80 cm.

2.4. Cell growth and toxicity assays

HUVECs were seeded (2.5×10^5 cells) in culture dishes in complete EGM. The next day, the cells were treated with 2-DG at different concentrations (0, 0.625, 6.25, 12.5 and 25 mM) for 48 h, and live/dead cells were counted after trypan blue staining. For MTT assay, in brief, 5×10^3 HUVECs/well were plated in 96-well plates in 100 μL of culture medium for Over night (O/N), and 24 h later, the medium was replaced with a fresh medium containing 2-DG (aforementioned doses). Twenty microlitresL (0.5 mg/ml) of MTT reagent was added to the cells at different time points, followed by another 2 h of incubation at 37 °C. The medium was removed, and 150 μL of DMSO was added to dissolve the formazan crystals formed. The absorbance was read at 570 nm on the BioTek plate reader (USA).

2.5. Estimation of glucose, lactate and intracellular ADP/ATP ratio

The levels of glucose and lactate were estimated (at 24 h) in the spent medium of HUVECs using kits according to the manufacturer (DIALAB, Austria). The intracellular ADP/ATP ratio was measured using a luciferin/luciferase-based assay (BioVision). In brief, the cells (HUVECs) were incubated with 2-DG at the given concentrations of 0, 0.625, 6.25, 12.5 or 25 mmol/L. After 24 h of incubation, aliquots containing an equal number of cells (1×10^4) were processed following the manufacturer's guidelines (Abcam).

2.6. Scratch assay

Migration of the ECs was assessed by the *in vitro* scratch assay. In brief, a total of 4×10^5 HUVECs were seeded in complete EGM in 24-well plates and allowed to form a confluent monolayer before making scratches using a sterile 200-μL pipette tip. In the low-dose radiation-induced migration assay, scratches were made after 0.5 Gy of radiation treatment, and the medium was replaced with 2-DG–containing media with or without the presence of $CoCl_2$ (150 uM). Bright-field micrographs were taken at 0 h and 24 h after scratching. The percentage of migration of treated cells was quantitated by measuring the width of the cell-free zone immediately after making the scratch at 0 h and 24 h later using image analysis software (AxioVision). Changes in migration potential of the treated cells were expressed as percentage of the (untreated) controls.

2.7. Matrigel tube formation assay

Each well of a prechilled 24-well cell culture plate was coated with 100 μL of unpolymerised Matrigel (4 mg/mL) and incubated at 37 °C and 5% CO_2 for 1 h. The HUVECs were harvested, and 8×10^4 cells were resuspended in 300 μL of complete EGM containing 2-DG at different concentrations, alone or in presence of $CoCl_2$, seeded in Matrigel-coated wells and incubated at 37 °C and 5% CO_2. Tube formation was assessed at 18 h, by bright-field microscopy (40X). Tube formation

(total tube length, number of tubes, covered area, branching points) in the microphotographs was analysed quantitatively using Wimasis Image Analysis automatic software.

2.8. Invasion assay

The transwell invasion assay was performed in 24-well plates using Hanging Millicell inserts (Millipore) with a Polyethylene Terephthalate (PET) membrane of a pore size of 8.0 μm. In brief, an insert was used to divide each well of the plate into lower and upper chambers. The lower chamber was filled with 750 μL of EGM supplemented with ECGF (50 μg/ml) as a chemoattractant. The HUVECs were seeded at a density of 5.0×10^4 cells/well in the upper chamber in 200 μL of EGM without ECGF and FBS and incubated with various concentrations of 2-DG. After incubation for 18 h, the cells present on the upper surface of the inserts were removed with cotton swabs. The inserts were fixed in 3.7% formaldehyde followed by permeabilisation with methanol and stained with Giemsa. The cells attached on the lower surface of the membranes were counted at 100× magnification in five randomised field views. The percentage of invasion was calculated from the number of invading cells in 2-DG treatment and control conditions.

2.9. Tumour study

The LLC cells were implanted, and 2-DG administration was started after implantation. In brief, male C57BL/6 mice were subcutaneously implanted with 0.5×10^6 LLC cells in the hind leg. After 24 h, the mice were given 2-DG–containing drinking water (0.0%, 0.2% or 0.4% w/v), which was continued till the termination of the experiment [30]. Tumours were measured regularly using electronic calipers. Twenty-one days after implantation, the mice were sacrificed, and the tumours were excised, weighed and fixed in 10% buffered formalin for further studies.

2.10. Tumour-associated and RIA (in vivo Matrigel plug) assay

For TAA, male C57BL/6 mice were randomised into one of the three groups (N = 6 per group). In brief, 500 μL of unpolymerised Matrigel (~10 mg/mL) with 0.5×10^6 LLC cells per mice was injected subcutaneously at the lower right backside. After 24 h of implantation, the mice were fed with drinking water containing 0.0% (group I), 0.2% (group II) or 0.4% (group III) 2-DG (w/v) until the termination of the study. Similarly, for RIA, 6- to 8-week-old athymic Balb C nu/nu female mice (N = 7/group) were randomised into 4 groups, i.e. group I: normal (no radiation and no 2-DG), group II: radiation alone (0.3 Gy), group III: radiation and fed with 0.2% of 2-DG w/v and group IV: radiation and fed with 0.4% 2-DG w/v. A radiation dose of 0.3 Gy was given locally on the lower right backside (2×2 cm^2 area) for the radiation treatment groups. 2-DG was administered to mice of groups III and IV daily in their drinking water at specified doses mentioned previously, after implantation of Matrigel and continued until the termination of the study. In brief, 500 μL of unpolymerised Matrigel (~10 mg/mL) was injected subcutaneously 24 h after irradiation beneath the irradiated skin. Later, 3 mice per group from the RIA experiment were sacrificed on day 3, and the Matrigel plugs were dissected out, photographed and fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E) for histology. The sections were examined by light microscopy and photographed. At day 7, the remaining mice from the RIA experiment and all the mice from the TAA experiment were injected intravenously (tail vein) with 200 μL of FITC-dextran (25 mg/mL) and then sacrificed after 20 min. FITC-dextran quantification within the plugs was performed by incubation of the plugs in 200 μL of Dispase overnight, followed by homogenisation and centrifugation for 1 min at 20,000 g. Fluorescence from the supernatant was read at 485/510 nm using a fluorescence plate reader (BioTek Flx 800). In an earlier experiment, 0.3 Gy of focal irradiation was given at the lower back of mice to show that low-dose radiation can induce angiogenesis as assessed by neovascularisation beneath the irradiated skin.

2.11. Immunohistochemistry

Matrigel specimens containing LLC tumours were fixed in 10% phosphate-buffered formalin for 24 h. The specimens were then embedded in paraffin and sectioned into 5-μm-thick slices, which were placed on egg albumin–coated glass slides. The sections were processed for immunohistochemistry using the Mouse Immunocruz™ staining kit and protocol provided by the manufacturer (Santa Cruz Biotechnology) for the endothelial marker, factor VIII.

2.12. Spontaneous and experimental metastases

C57BL/6 mice were implanted with LLC cells (0.5×10^6) subcutaneously in the hind leg for spontaneous metastases and intravenously (0.1×10^6 cells) for experimental metastases. Twenty-four hours after the tumour cell injection, the animals were randomly divided into the control and 2-DG groups where the treatment groups were administered with 0.2 and 0.4% 2-DG (w/v) in drinking water for the remainder of the time. Four weeks later, the mice were euthanised, and the primary tumours and lungs were removed. The lungs were fixed in Bouin's solution (SRL), and metastasis was quantified by manual counting of the lung surface colonies under a dissecting microscope.

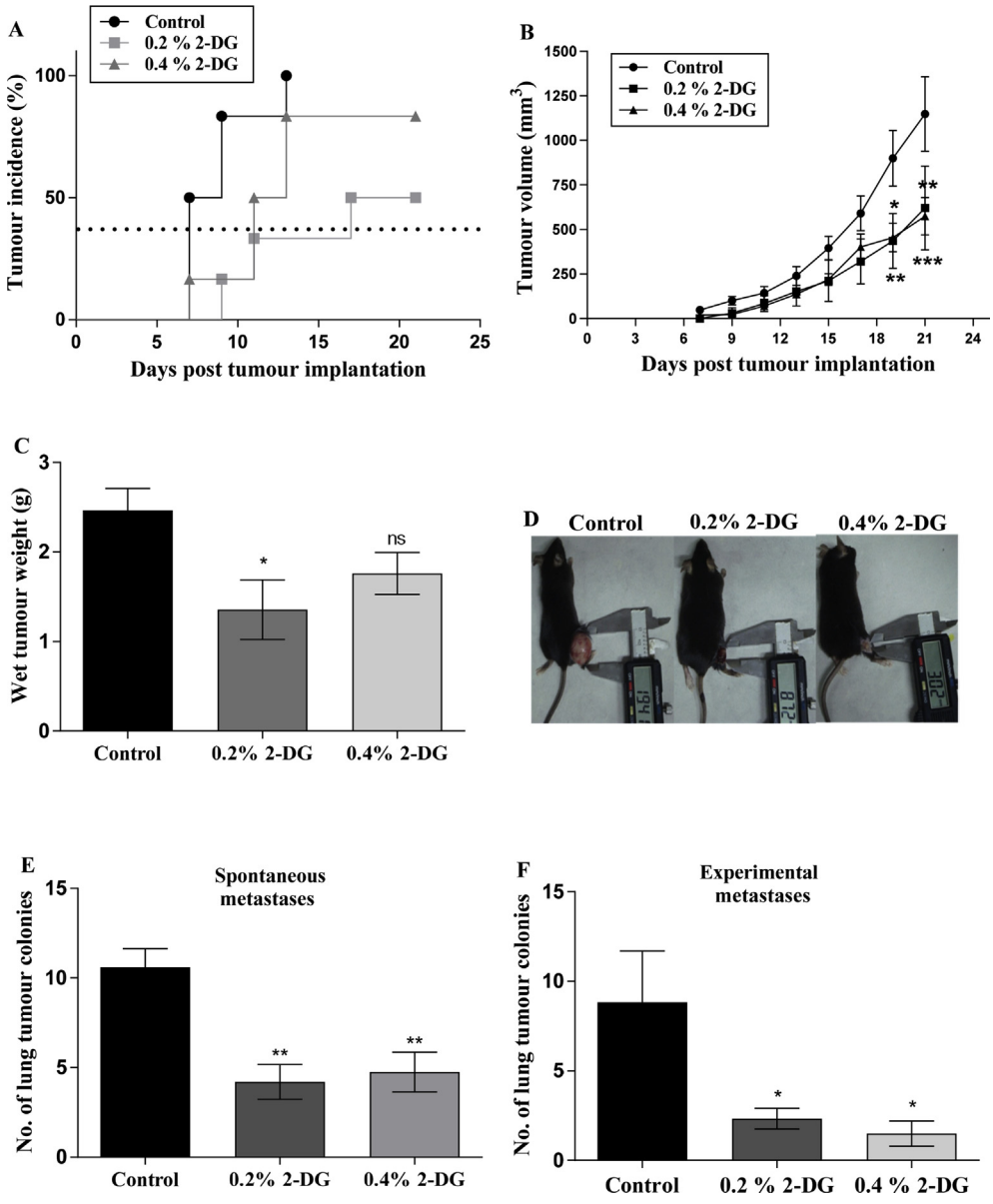

Fig. 1. **Dietary 2-DG inhibited the process of tumourigenesis in the transplanted Lewis lung carcinoma (LLC) tumour model and reduced the number of spontaneous and experimental lung metastases.** (A) Tumour latency (palpable) and incidence; (B) tumour volume; (C) tumour weight (day 28); (D) representative photographs of animals demonstrating differences in the tumour burden (day 21); (E) average ± SE of spontaneous lung metastases; (F) average ± SE of experimental lung metastases. *, $p < 0.05$; **, $p < 0.01$. SE , Standard error; 2-DG, 2-deoxy-D-glucose.

2.13. Measurement of VEGF

Blood was directly drawn on anaesthesia using ketamine/xylazine intra-peritoneal *(i.p)* from the retro-orbital plexus on day 7 after implantation from the Matrigel LLC–implanted C57BL/6 mice. The serum samples were evaluated for VEGF levels following the manufacturer's instructions for the mouse VEGF-ELISA kit (RayBiotech).

2.14. Statistical analysis

All the data were analysed using GraphPad Prism (version 5.01). For statistical significance analysis, Student's t-test or one-way or two-way analysis of variance was used with Tukey's, Dunnett's or Bonferroni's multiple comparison post-test.

3. Results

3.1. Dietary 2-DG administration after tumour implantation impairs growth of LLC

Our earlier study had shown that dietary 2-DG administration initiated 3 months before tumour implantation was effective in inhibiting tumourigenesis in the EAC tumour model [30]. To evaluate the effectiveness of dietary 2-DG in impairing tumourigenesis when initiated

after tumour implantation, we used the LLC tumour model in C57BL/6 mice. The tumour incidence was reduced by 50% and 17% in 0.2% w/v and 0.4% w/v 2-DG, respectively, with a delayed latency (median days) of 11 days (19 vs 8 days) and 4 days (12 vs 8 days) (Fig. 1A). In addition, the rate of growth of subcutaneous tumour was lower in the 2-DG–fed animals, with a tumour volume of $621 \pm 236\,mm^3$ on day 21 in 0.2% w/v 2-DG ($p < 0.01$) and $574.4 \pm 234\ mm^3$ in 0.4% w/v 2-DG ($p < 0.001$) in comparison with $1148 \pm 208\ mm^3$ in the control group (Fig. 1B). The average tumour weight was also lower at both the doses of 2-DG than in the untreated group with the mean tumour weight of 1.35 ± 0.33 g ($p < 0.05$) and 1.76 ± 0.23 g ($p = 0.08$) in 0.2% and 0.4% w/v 2-DG, respectively, versus 2.46 ± 0.24 g in the control group (day 21; Fig. 1C). A marked reduction in the tumour vascularity of the LLC tumours was also observed in the 2-DG–fed mice (day 21, Fig. 1D), similar to our earlier observations in the EAC tumour model [30].

3.2. Dietary 2-DG reduces spontaneous and experimental lung metastases

Previously, we have shown that systemic 2-DG administration could significantly inhibit the proteolytic activity of Matrix metalloproteinase (MMP)-9, thereby suggesting inhibition of vascular invasion and spread through the extracellular matrix (ECM) and metastases [30]. To strengthen this further, in the present study, we examined the lung tumour colonies to determine whether dietary 2-DG could inhibit the metastatic spread of the disease. 2-DG significantly diminished both spontaneous and experimental metastases, evidenced by the reduction in the number of tumour nodules in the lung. Nearly twofold decrease in the number of tumour colonies was observed in spontaneous lung metastases (Fig. 1E), 10.6 ± 1.03 vs 4.2 ± 0.96 in 0.2% and 4.7 ± 1.1 in 0.4% w/v 2-DG groups ($p < 0.01$ for both the groups). In addition, about fourfold decrease was noted in experimental metastases (Fig. 1F), 2.3 ± 0.33 and 1.5 ± 0.5 at 0.2% ($p < 0.01$) and 0.4% w/v 2-DG ($p < 0.05$) doses, respectively, compared with 8.8 ± 1.16 in the control animals ($p < 0.05$). This suggests that dietary 2-DG effectively compromises the process of metastases, besides inhibiting the primary tumour growth.

3.3. Dietary 2-DG inhibits tumour-associated angiogenesis in the implanted tumour model

Because neovascularisation is vital to the formation and growth of solid tumours and metastases, we examined whether dietary 2-DG impedes tumour growth and metastasis by inhibiting angiogenesis (*in vivo*) using the tumour Matrigel plug implant in mice. 2-DG was administered in daily drinking water of mice, starting a day after implantation of Matrigel with tumour cells (0.5×10^6 cells). 2-DG at both the doses (0.2% and 0.4% w/v) significantly reduced neovascularisation in the Matrigel plugs that was visually obvious by day 7. A marked decrease in vascular density and network along with reduced tumour growth was observed (Fig. 2A). Quantification of tumour angiogenesis using the FITC-dextran method showed a decrease of 1.8 ($p < 0.05$) and a 2-fold decrease ($p < 0.01$) in the 0.2% and 0.4% w/v 2-DG groups, respectively (Fig. 2B). In addition, factor VIII staining (indicated by black arrows) was noticeably reduced in the 2-DG–fed mice, indicating a reduction in the microvessel density (Fig. 2C). Furthermore, we evaluated the effect of dietary 2-DG on circulating VEGF in tumour/Matrigel mice, where the concentration of VEGF in the serum of tumour-bearing 2-DG–treated mice was found to be lower in 0.2% and 0.4% 2-DG groups (0.063 ± 0.004 and 0.060 ± 0.003 pg/ml, respectively) than in the control animals (0.096 ± 0.016 pg/ml; Fig. 2D). These results suggests the *in vivo* antiangiogenic potential of dietary 2-DG in addition to tumour growth inhibition.

3.4. Dietary 2-DG inhibits radiation-induced angiogenesis in vivo

Exposure of tumour stroma and tissue adjacent to the irradiated area has been shown to be a potential contributing factor in the regrowth of local tumour after radiotherapy [17,18]. To investigate if 2-DG impairs the process of angiogenesis irrespective of the nature of stimulation of angiogenesis, we used an *in vivo* model of low-dose radiation-induced angiogenesis where a dose of 0.3 Gy was given to the normal tissue (skin in this case), which could activate the process of angiogenesis (Fig. S1). Furthermore, to test whether dietary 2-DG reduces low-dose IR-stimulated angiogenesis in mice, we used *in vivo* Matrigel plug angiogenesis assay. Matrigel plugs were analysed on days 3 and 7 after implantation, a time point where neovascularisation was clearly visible (in the radiation group). The network of capillaries (degree of angiogenesis) was greatly reduced in 2-DG–fed animals (Fig. 3A). The level of FITC-dextran fluorescence in Matrigel plug homogenates (Fig. 3B) was significantly higher (2.3-fold; $p < 0.001$) in the irradiated animals, whereas that of fluorescence in the plugs in 2-DG–fed animals was ~ 2-fold lower than that in the plugs in irradiated animals. These differences were also observed histologically by H&E staining of the Matrigel plugs, depicting a significant increase in the red blood cell–containing capillaries in the irradiated mice (Fig. 3C), with a marked reduction in the capillary formation in the 2-DG–fed mice.

3.5. 2-DG inhibits growth and proliferation of HUVECs by inhibiting glycolysis

Stimulation of the ECs by VEGF has been shown to be associated with enhanced glycolysis [20,22]. To

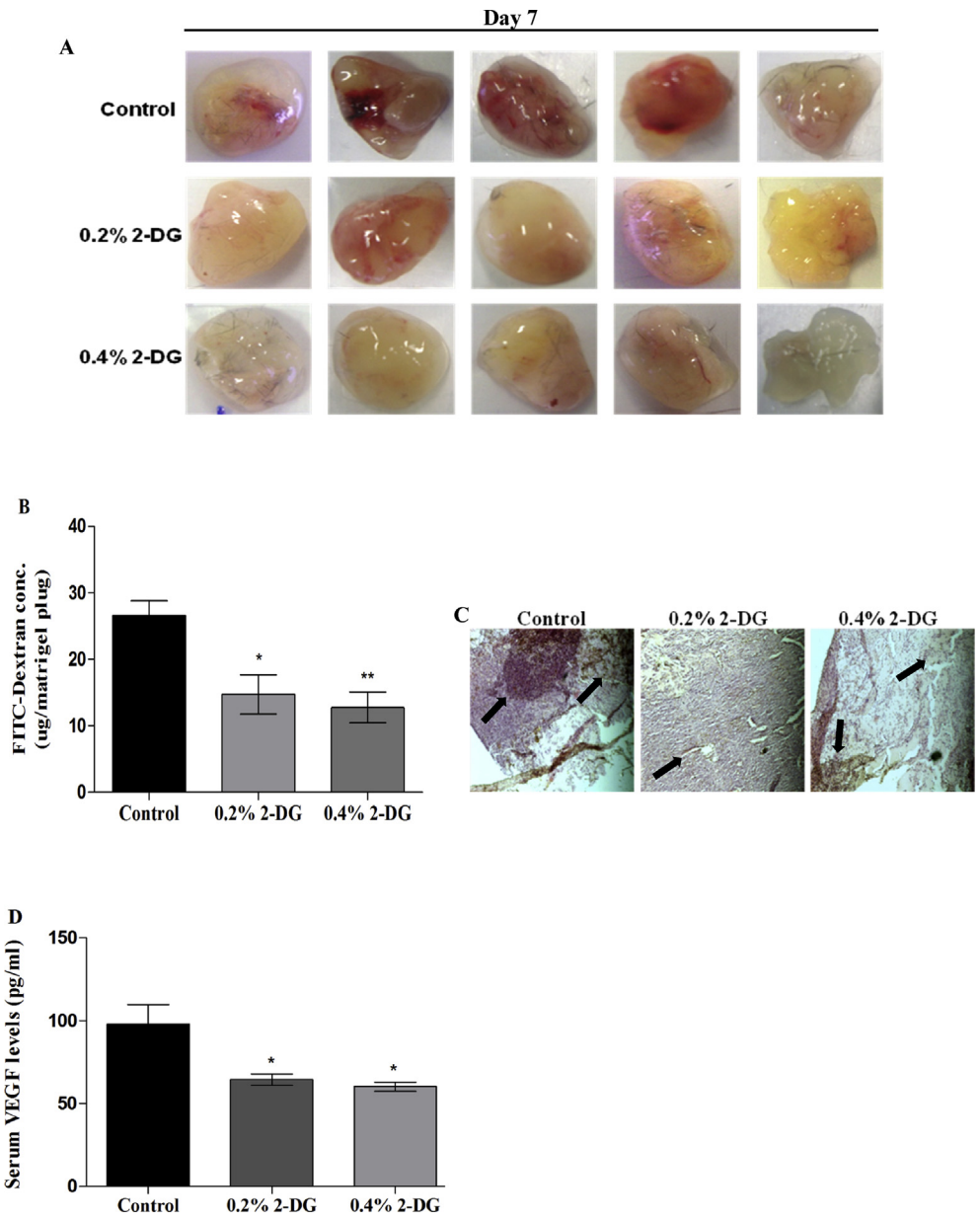

Fig. 2. **Dietary 2-DG reduced intratumour microvessel density and VEGF levels in tumour (LLC)-induced angiogenesis studied by the *in vivo* Matrigel plug assay.** (A) Representative photographs of the Matrigel plugs; (B) angiogenic index quantified by determining the FITC-dextran fluorescence in the Matrigel plugs; (C) factor VIII immunostaining in the Matrigel plug sections; (D) serum VEGF (day 7; after implantation). Values presented are means ± SE. *, $p < 0.05$; **, $p < 0.01$. SE , Standard error; FITC , Fluorescein isothiocyanate; 2-DG, 2-deoxy-D-glucose; VEGF, vascular endothelial growth factor.

determine the effects of 2-DG on the cells of endothelial origin, cytotoxicity and growth were assessed on HUVECs *in vitro*. Furthermore, to assess the impact of metabolic targeting of the endothelial compartment, effects of 2-DG on the glucose usage/lactate production and ATP generation were also analysed. The MTT assay showed a significant ($P < 0.001$) time- and concentration-dependent inhibition in the metabolic viability, with nearly 90% decrease at 72 h in cells treated with 25 mM 2-DG (Fig. 4A). The cell growth analysed by enumerating the cell number under these conditions showed a 2-DG concentration–dependent decrease in the cell number with a 50% growth inhibition at 25 mM (Fig. 4B). Furthermore, the fraction of non-viable (trypan blue–positive) cells did not increase considerably in 2-DG–treated HUVECs (data not shown), suggesting that it mainly exerted a growth-inhibitory effect, but was non-toxic. Glucose usage and lactate production studied at the end of 24-h incubation showed inhibition of glucose usage and lactate production by 2-DG at concentrations beyond 6.25 mM, with a 2-fold decrease in glucose consumption

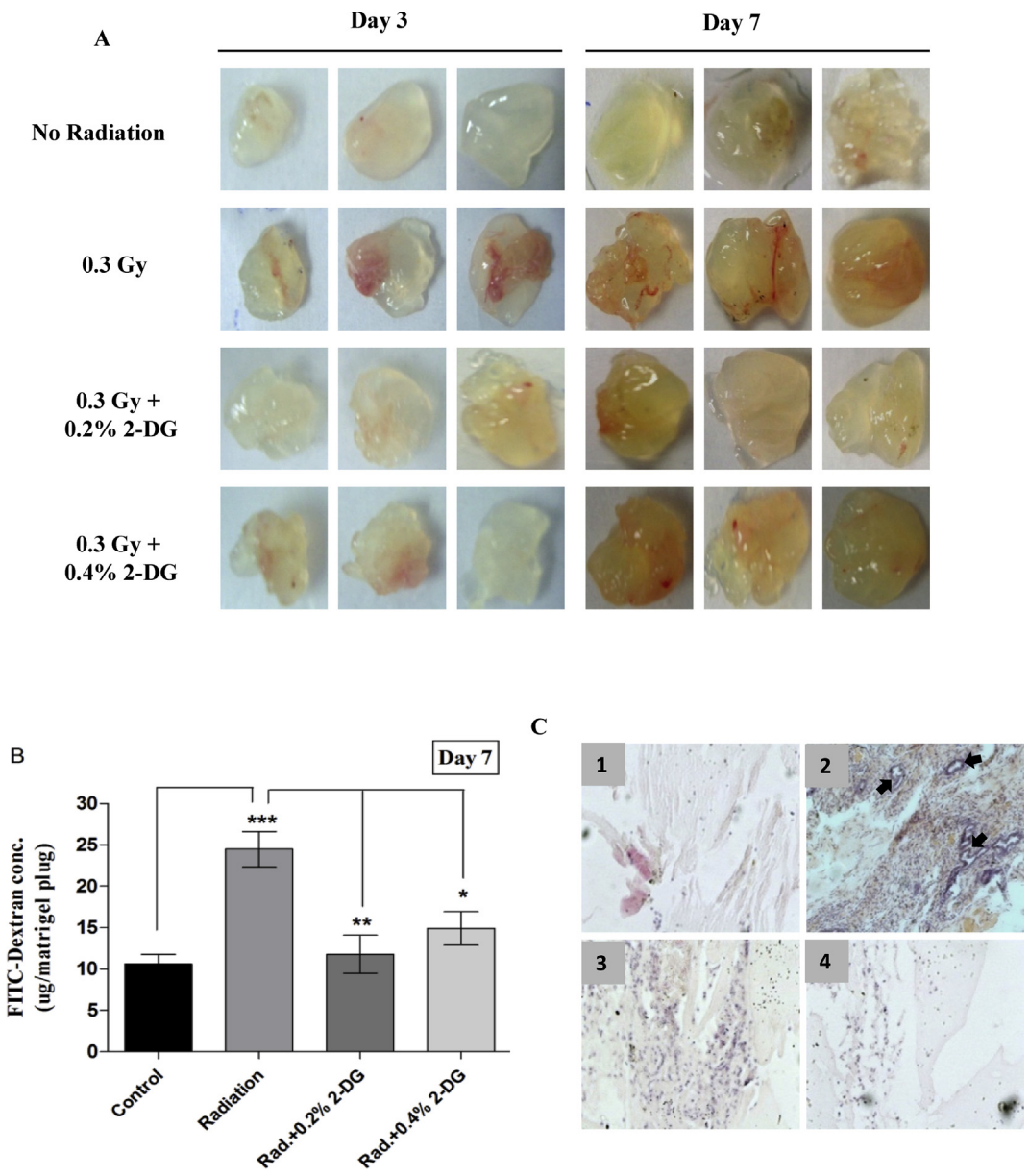

Fig. 3. **Matrigel plug assay showing reduction in radiation (low-dose IR)-induced angiogenesis by dietary 2-DG *in vivo* and** (injection of Matrigel subcutaneously beneath the irradiated area). (A) Representative photographs of Matrigel plugs at days 3 and 7; (B) angiogenic index quantitation measured by the FITC-dextran fluorescence in the Matrigel plugs; (C) representative photomicrographs of histological analysis (H&E staining) showing intraplug vessel morphology. Arrows indicate regions of microvessel density. IR, ionising radiation; FITC, Fluorescein isothiocyanate; 2-DG, 2-deoxy-D-glucose; H&E, haematoxylin and eosin.

at 12.5 mM ($p < 0.05$) and 7-fold decrease at 25 mM 2-DG ($p < 0.01$), while a 1.7- and 2.6-fold decrease was observed in the lactate production (Fig. 4C). Surprisingly, the lowest dose of 2-DG (e.g. 0.625 mM) showed an increase in glucose consumption and lactate production. Under these conditions, the ATP levels showed a significant decrease at all 2-DG concentrations (Fig. 4D, overall $p < 0.05$). These observations indicated that the inhibition of glycolysis resulting in the depletion of ATP may be partly responsible for the growth inhibition, especially at higher concentrations (beyond 6.25 mM).

3.6. 2-DG inhibits the migration and invasion potential of HUVECs

The angiogenic cascade during tumour development consists of energy-requiring processes upon EC activation such as degradation of the basement membrane in the ECM by proteases and migration and proliferation of ECs. The effect of 2-DG on HUVEC migration was evaluated by the scratch assay. 2-DG alone and in presence of $CoCl_2$ (simulating hypoxic signalling) significantly inhibited the migration of HUVECs in a dose-dependent manner observed at 24 h (Fig. 5A), with

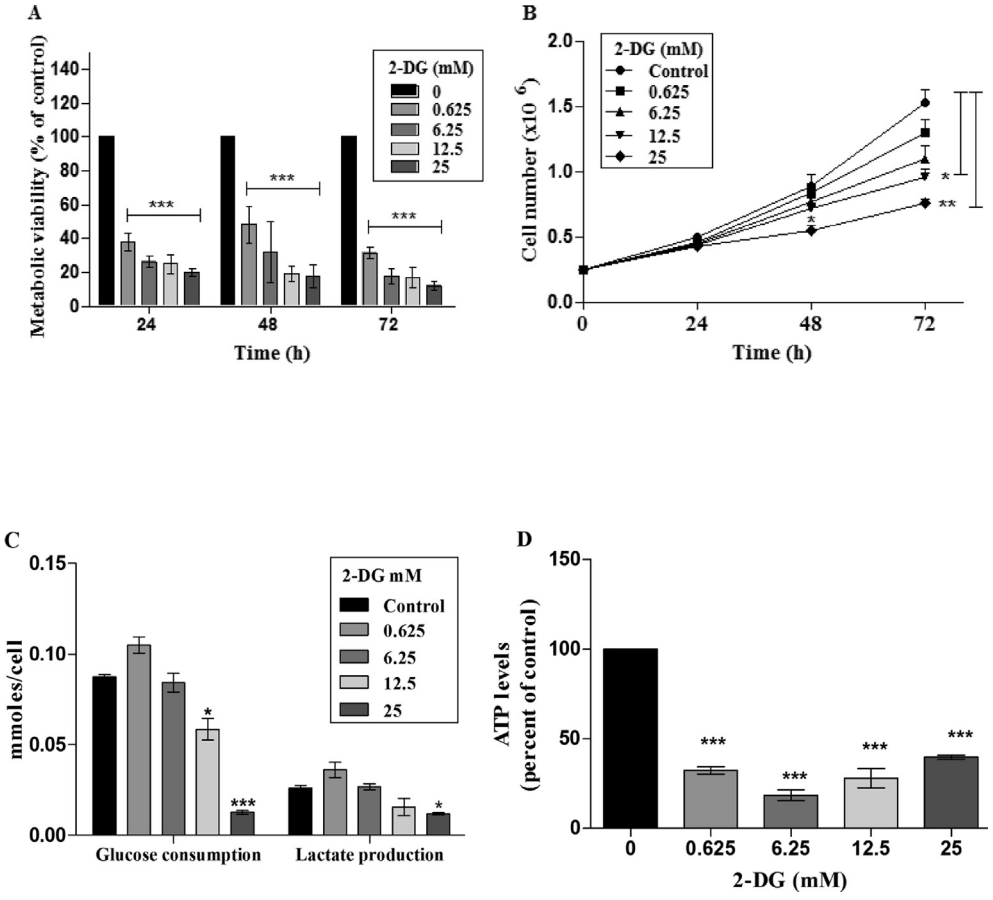

Fig. 4. **2-DG compromised the metabolic viability and impaired the growth of HUVECs by inhibiting glycolysis.** (A) Time- and 2-DG concentration–dependent changes in the metabolic viability; (B) growth kinetics of HUVECs; (C) glucose consumption and lactate production; (D) intracellular ATP levels. Values presented are means ± SE. *, $p < 0.05$; ***, $p < 0.001$. SE , Standard error; 2-DG, 2-deoxy-D-glucose; HUVEC, human umbilical vein endothelial cell.

95% inhibition at the highest concentration (25 mM) under normoxia, compared with 100% recovery of the wound (scratch) in the control group (Fig. 5B). Hypoxia in solid tumours has been shown to significantly increase EC expression of GLUT-1 and glucose uptake [21], suggesting enhanced sensitivity on metabolic targeting. As predicted, HUVECs exposed to $CoCl_2$ and simultaneously treated with 2-DG showed a greater increase in migration inhibitory activity than cells treated with 2-DG alone (Fig. 5A). In the presence of $CoCl_2$, 2-DG was relatively more effective in blocking migration compared with normoxia with 62–80% inhibition ($p < 0.001$) in the concentration range studied (0.625–25 mM). The inhibitory effects of 2-DG on invasion was evaluated by the *in vitro* transwell invasion assay, which showed nearly 50% ($p < 0.01$) decrease in the number of invading cells at all concentrations of 2-DG (Fig. 5C and D). These results indicate that the migration and invasion ability of HUVECs is significantly suppressed by 2-DG, suggesting a functional impairment in the endothelial compartment.

3.7. 2-DG impairs capillary formation by HUVECs (in vitro tubulogenesis)

Effects of 2-DG on the angiogenic activity were assessed by examining the ability of capillary tube formation by HUVECs *in vitro*. 2-DG caused a significant reduction in capillary tube and network formation, both under normoxic and hypoxic conditions (Fig. 6A). Quantification of the total number of tubes (Fig. 6B), tube length (Fig. 6C), covered area (Fig. 6D) and branching points (Fig. 6E) showed marked inhibition of tube formation by 2-DG under normoxia and hypoxia ($CoCl_2$), except at 25 mM under normoxic conditions.

3.8. 2-DG inhibited low-dose radiation-induced migration of HUVECs

To ascertain whether low-dose IR can enhance angiogenic potential, we also analysed the migration of HUVECs exposed to low-dose IR (0.5 Gy) by the scratch wound healing assay. Here, the media for

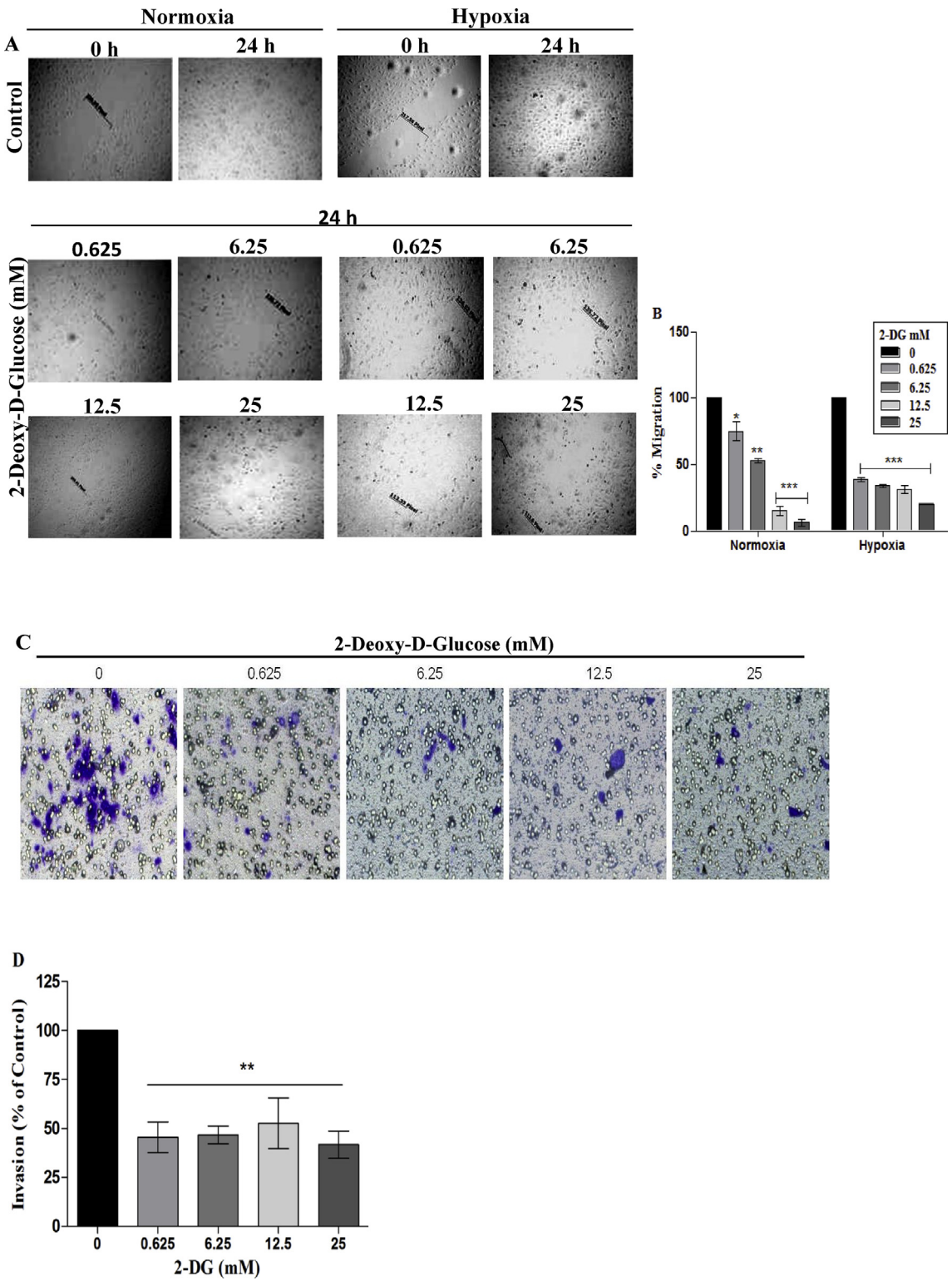

Fig. 5. **Inhibitory effects of 2-DG on the migration and invasion of HUVECs.** (A) Representative images depicting migration by HUVECs after exposure to 2-DG for 24 h (with or without hypoxia) in the wound-healing assay; (B) normalised values of cell migration. (C) Representative photographs showing the invasion of HUVECs in transwell assay; (D) normalised values of invasion. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. SE , Standard error; 2-DG, 2-deoxy-D-glucose; HUVEC, human umbilical vein endothelial cell.

HUVECs did not contain ECGF to keep the cells unstimulated so that they are stimulated only upon irradiation. A radiation dose of 0.5 Gy stimulated faster (by 16 h) HUVEC migration and wound recovery (100%) than that (by 24 h) in the aforementioned study. On the other hand, cells irradiated and then treated with

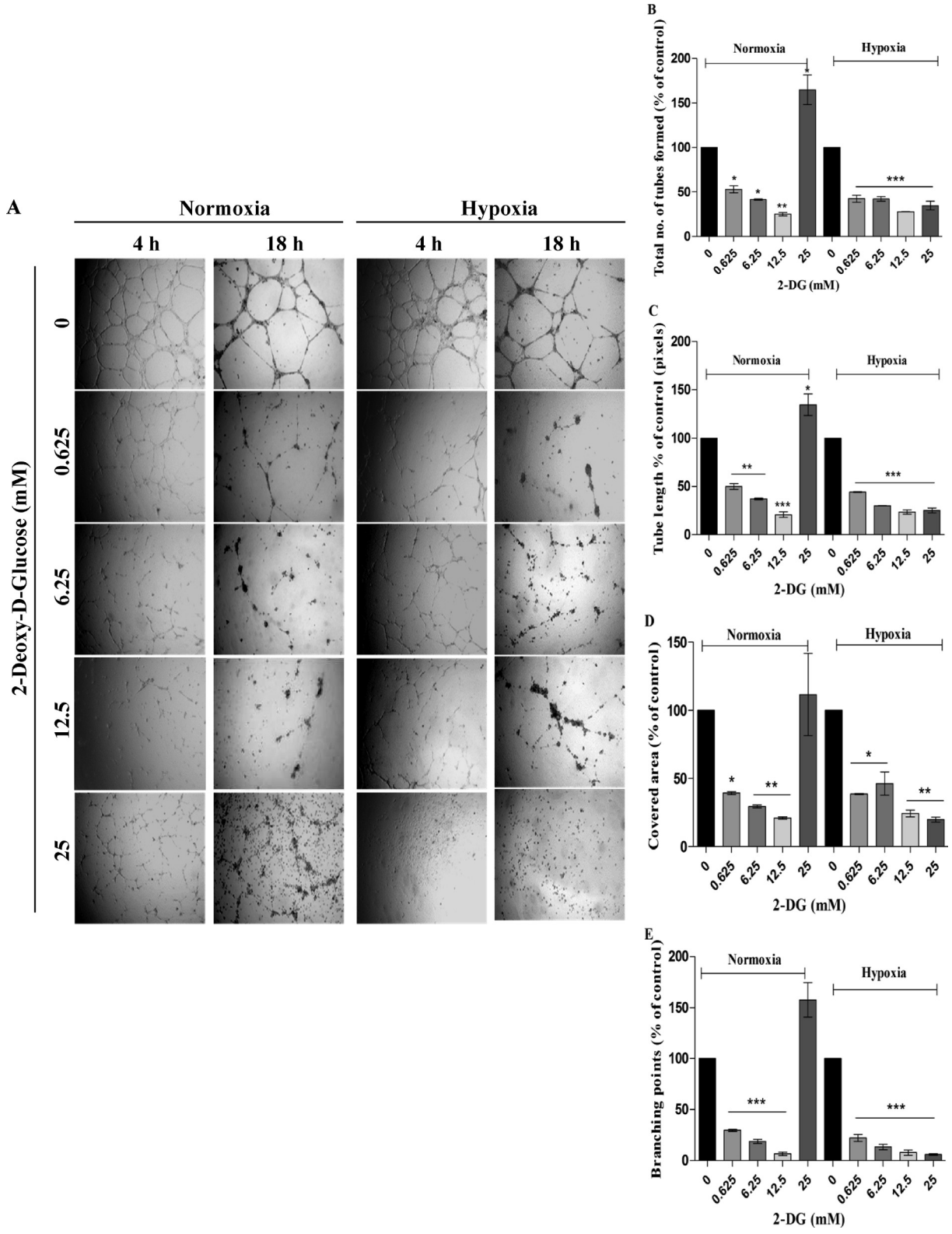

Fig. 6. **2-DG inhibited capillary-like structure formation of HUVECs *in vitro*.** (A) Representative microphotographs showing the effects of 2-DG on tubulogenesis under normoxic and hypoxic conditions. Relative changes in (B) the number of tubes formed; (C) tube length; (D) covered area and (E) branching points. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. 2-DG, 2-deoxy-D-glucose; HUVEC, human umbilical vein endothelial cell.

2-DG showed no or significantly reduced migration compared with the irradiated controls (Figure S2 A). The inhibition under normoxic conditions was concentration dependent and varied from 30% to 95% (Figure S2 B). Inhibition under hypoxic conditions was more profound, with >90% ($p < 0.001$) observed at all concentrations (Figure S2 B). These data indicated that the low dose of IR enhances HUVEC migration, which is accentuated under hypoxic conditions. This migration is significantly inhibited by 2-DG, which is more prominent under hypoxia.

4. Discussion

Growth and metastasis of solid tumours rely heavily on the angiogenic potential of the tumour cell causing proangiogenic changes in the TME, facilitating the neovascularisation in the tumour stroma [33,34]. The activated endothelium is generally associated with enhanced VEGF levels and/or hypoxia-induced expression of glucose transporters, resulting in enhanced glycolytic flux of ECs [20–23]. Because the activated endothelium is an important component of the TME [35], approaches that suppress angiogenesis besides inhibiting the tumour growth directly will be effective in retarding tumour growth and metastasis. The inhibition of angiogenesis observed here and reported earlier [36] establishes the potential of 2-DG to impair the process of metastases. Thus, radiosensitising effects of 2-DG with direct effects on tumour cells and immune modulation reported earlier [37–41] establishes this glucose analogue as an ideal adjuvant in the radiotherapy and chemotherapy of tumours.

Angiogenic and the tumour matrix–associated factors influence the growth and the process of metastasis. Inhibition of tumour (LLC)-associated and radiation (IR)-induced neovascularisation by dietary administration of 2-DG observed here (Fig. 2 and 3) suggests that it impairs both these processes. Reduction in the serum levels of VEGF (an important angiogenic factor) in LLC-bearing mice and inhibition of angiogenesis observed in the *in vivo* Matrigel studies (Fig. 2A–D) coupled with impaired primary tumour growth (Fig. 1A–D) and metastases (Fig. 1E and F) in mice fed with 2-DG supports this notion. This impairment appears to be linked to the reduced MMP-9 activity we reported (Saurabh *et al.*, 2015) as MMP-9 is known to stimulate VEGF, supporting tumour invasion, angiogenesis and metastases [30,42]. In addition, the inhibitory effects of 2-DG on growth and proliferation (Fig. 4A and B) as well as suppression of cell migration (scratch assay; Fig. 5A and B), invasion (trans-well assay; Fig. 5C and D) and Matrigel tube formation (tubulogenesis; Fig. 6A–E) under normoxic and hypoxic conditions suggest that a direct effect of 2-DG on the ECs could also contribute to the impaired angiogenesis. Indeed, ECs are known to depend on glycolysis for their function both *in vitro* and *in vivo* [43,44]. Reduced glycolytic energy (ATP) production (Fig. 4C and D) appears to be one of the important contributing factors for the compromised functional status of HUVECs, although alterations in gene expressions, oxidative stress and Unfolded protein response (UPR) cannot be excluded [37,45].

Earlier studies have shown that 2-DG impairs the growth and *in vitro* equivalent of angiogenesis (migration and honeycomb formation) in HUVECs at concentrations that do not affect tumour cells directly, but elicits an UPR response by interfering with N-linked glycosylation, a process that is glycolysis independent, whereas *in vivo* studies have also shown the anti-angiogenic effect in the $LH_{BETA}T_{AG}$ mouse model of retinoblastoma [36,46]. Consistent with these observations, the present study for the first time demonstrates the antiangiogenic effect of dietary 2-DG administration (in drinking water), besides the growth inhibition reported earlier [30]. Profound reduction in the tumour vascular networks that could be correlated with reduction in levels of VEGF and a significant reduction in tumour dissemination were observed in spontaneous and experimental metastases in mice that were on dietary 2-DG (Figs. 2 and 3). The VEGF-VEGFR1 signalling pathway has been implicated in carcinogenesis and appears crucial for tumour invasion, metastasis and angiogenesis [47]. Abrogation of radiation (low-dose IR)-induced angiogenesis (Fig. 3) and impairment of tumour-induced angiogenesis (Fig. 2) suggests that dietary 2-DG may be useful in fractionated radiotherapy, where low doses of radiation received by the tumour (and normal) tissue surrounding the treatment volume has been shown to induce angiogenesis and considered as one of the reasons for recurrence and relapse [18,19].

2-DG has been well established as an adjuvant to radiotherapy and chemotherapy, besides few attempts to use it as a primary anticancer agent [48–50]. Our recent studies with the glycolytic inhibitor 2-DG, as a potential ERM agent, establishes the use of dietary administration of 2-DG as a safe antitumour strategy, without any adverse effects on general physiology along with preserved cognitive, affective and sensory-motor functions [30,51]. Suppression of tumour- and radiation-induced neovascularisation shown here is consistent with our hypothesis that the antitumour effects of 2-DG may not only be mediated entirely through its effect on the tumour cells but also be contributed by its effects on the ECs, a part of the host system, which is in line with our observations on the modifications of host-tumour interactions contributing to the radiosensitisation by 2-DG in Ehrlich ascites tumour in mice [40].

In conclusion, the results of the present study provide new insights into the targeting of metabolic reprogramming of tumours that involves the antiangiogenic effects of dietary 2-DG that may cause a shift in the

TME from a proangiogenic to an antiangiogenic state through multiple mechanisms, thereby limiting intratumoral vascularisation and RIA in the surrounding the tumour bed during radiation therapy. These results are consistent with the those of earlier studies, which show that dietary energy restriction inhibits the growth of activated ECs and thus angiogenesis [24,27,28] that could prevent regrowth of tumours after therapy [18]. Impairment of both tumour- and radiation-induced angiogenesis suggests that 2-DG has an effect on the process of angiogenesis itself that can impair growth and metastasis of tumours, which merits additional investigations.

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Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2019.09.005.

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