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Dimethyloxalylglycine (DMOG)-Induced Hypoxia Promotes Migratory and Invasive Properties of HCT116 Colon Cancer Cell Line

(Hipoksia Aruhan Dimetiloksalilglisin (DMOG) Menggalakkan Sifat Migrasi dan Invasif Titisan Sel Kanser Kolon HCT116)

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ABSTRACT

Hypoxia, a condition characterised by low oxygen levels, leads to increased production of a protein called hypoxia-inducible factor-1 alpha (HIF-1α) in cancer cells. This protein is involved in driving processes such as vascularization, cytoskeletal reorganisation, and epithelial-to-mesenchymal transformation (EMT), which contribute to metastasis. Previous studies used hypoxic workstations, chambers, and incubators to evaluate the effects of hypoxia on colon cancer cell lines. In a cell culture model, hypoxic conditions can also be induced using dimethyloxalylglycine (DMOG) as the hypoxia-mimicking agent. This study aims to investigate the effects of DMOG-induced hypoxia on colon cancer metastasis, focusing on cell migration and invasion. HCT116 cells were subjected to hypoxic conditions by treating them with DMOG, and the expression of HIF-1α proteins was measured at various time points, followed by wound healing and invasion assays. It was found that HIF-1α protein expression increases after 6 h of DMOG induction and persists for 24 h. At 6 and 24 h, a significantly higher percentage of hypoxic cells migrated compared to normoxic cells. The invasion assay demonstrated that hypoxic cells were more invasive than normoxic cells within 24 h. Thus, the increase in migration and invasion of cells is comparable to the increase in HIF-1α expression at 6 and 24 h. These findings suggest that DMOG induces HIF-1α expression in colon cancer cells, leading to enhanced cell migration and invasiveness. The established model can be further utilised in gene knockdown or drug treatment studies to evaluate the effects of hypoxia on cancer cells.

Keywords: Colorectal cancer; DMOG; HIF-1α; hypoxia; metastasis

ABSTRAK

Hipoksia, merupakan keadaan yang dicirikan oleh paras oksigen yang rendah, membawa kepada peningkatan pengeluaran protein yang dipanggil *hypoxia-inducible factor-1 alpha* (HIF-1α) dalam sel kanser. Protein ini terlibat dalam memacu beberapa proses seperti vaskularisasi, penyusunan semula sitoskeleton dan transformasi epitelium-ke-mesenkima (EMT) yang menyumbang kepada metastasis. Kajian terdahulu menggunakan stesen kerja, kebuk dan inkubator hipoksik untuk menilai kesan hipoksia pada titisan sel kanser kolon. Dalam model kultur sel, keadaan hipoksik juga boleh diaruh menggunakan dimetiloksalilglisin (DMOG) sebagai agen memimik hipoksia. Kajian ini bertujuan untuk mengkaji kesan hipoksi yang diaruh DMOG pada metastasis kanser kolon dengan memberi tumpuan kepada migrasi dan invasi sel. Sel HCT116 menjadi hipoksik dengan merawatnya menggunakan DMOG, kemudian ekspresi protein HIF-1α diukur pada pelbagai titik masa, diikuti dengan ujian migrasi dan ujian invasi. Didapati bahawa pengekspresan protein HIF-1α meningkat selepas 6 jam aruhan DMOG dan berterusan selama 24 jam. Pada 6 dan 24 jam, peratusan migrasi sel hipoksik meningkat dengan signifikan berbanding sel normoksik. Ujian pencerobohan menunjukkan bahawa sel hipoksik lebih invasif daripada sel normoksik dalam masa 24 jam. Oleh itu,

peningkatan dalam migrasi dan pencerobohan sel adalah selari dengan peningkatan ekspresi HIF-1α pada 6 dan 24 jam. Penemuan ini menunjukkan bahawa DMOG mengaruh pengekspresan HIF-1α dalam sel kanser kolon yang membawa kepada peningkatan kadar migrasi dan pencerobohan sel. Model ini boleh digunakan selanjutnya dalam kajian berkaitan penindasan gen atau rawatan ubat untuk menilai kesan hipoksia pada sel kanser.

Kata kunci: DMOG; HIF-1α; hipoksia; kanser kolorektal; metastasis

Introduction

Cancer has been regarded as one of the primary factors contributing to the overall decline in human life expectancy (Bray et al 2021). Across the world, approximately 19.3 million new cancer cases have been reported, and nearly 10.0 million casualties eventuated in 2020 (Sung et al. 2021). Colorectal cancer (CRC) is the third most occurring cancer worldwide (10.2%) and the second leading cause of cancer death (9.2%) (GLOBOCAN 2018). In Malaysia, CRC is the second most common cancer in both males and females, accounting for 13.5% of cases (MOH 2019), with a mortality rate of 9.8 cases per 100 000 population (NCPR-CRC 2014).

Metastasis is thought to be responsible for around 90% of cancer deaths (Chaffer & Weinberg 2011). After more than 50 years, this estimate has not significantly changed. Currently, twenty percent of newly diagnosed cases of colorectal cancer have metastatic disease, and another 25% of patients with localized tumours will acquire metastases later (Biller & Schrag 2021). Metastatic cells create a microenvironment that promotes angiogenesis and proliferation, leading to the development of malignant secondary tumours (Seyfried & Huysentruyt 2013). The hypoxic microenvironment is one of the key causes of metastasis (Neophytou et al. 2021).

Hypoxia is one of the characteristics of the tumour microenvironment (TME), caused by the disparity between oxygen demand and supply. In solid tumours, the abnormal blood vessel growth brought on by the improper balance between pro- and anti-angiogenic signals severely affects oxygen transport (Rankin, Nam & Giaccia 2016). Hypoxia establishes the expression of HIF-1α at the molecular level, and stimulates the expression of genes that coordinate migration, invasion, angiogenesis and glycolytic metabolism which enables cells to become acquainted with low oxygen tension (Semenza 2012). Clinically, HIF-1 is linked to cancer progression and poor clinical outcome in a number of solid tumour types, including breast, cervical, and colon cancer (Semenza 2010). HIF-1α signalling is sufficient to increase the migration and invasion capacity of tumour cells, highlighting the importance of HIF-1α and its downstream targets in metastatic tumours (Hanna et al. 2013; Wong et al. 2011). However, the molecular mechanism responsible for the increased metastatic capacity of hypoxic colon cancer cells is not well characterized. Therefore, the present study was undertaken to determine the effects of hypoxia induction using different models on the expression of HIF-1α in HCT116 cells and investigate its relation with the migration and invasion capacity of the HCT116 colon cancer cells.

Materials and Methods

Cell lines and culture conditions

The human colorectal carcinoma cell line (HCT116) was purchased from the American Type Culture Collection (ATCC, USA) and routinely cultured in McCoy's 5A medium (Cytiva, USA) containing 10% (v/v) fetal bovine serum (FBS; Hyclone, USA) and 5% Penicillin-streptomycin solution in a humidified atmosphere of 5% CO_2 at 37 °C. When 80% confluence was reached, cells were detached by incubation with 5 mM trypsin/EDTA and harvested after centrifugation in a LC-8 LAB (Benchmark Scientific, USA) at 1500 rpm for 5 min at room temperature. Cells were re-suspended in media and viable cells counted using a hemocytometer and trypan blue staining.

Hypoxia treatment

The HCT116 cells were subjected to three different hypoxia models. The first model was using 1 mM DMOG as the hypoxia mimetic agent, incubated in the normal CO_2 incubator with 20% O_2 supply. Second model was cells treated with 1 mM DMOG and incubated in the hypoxia chamber with 1% O_2. The third model where the cells were incubated in 1% O_2 in the hypoxia chamber

without DMOG treatment. The protein lysates were extracted out from the cells in the control group and the three hypoxia models at 6, 24 and 48 h timepoints.

WESTERN BLOT

Whole cell lysates were used for immunoblotting. Briefly, cells grown in 6-well plates were washed with cold PBS, and then lysed with RIPA buffer containing protease inhibitor (Thermo Scientific, USA). Protein concentrations were determined by Bicinchoninic acid (BCA) protein assay with Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA). Equal amounts (20 µg) of protein were separated by SDS-PAGE and transferred onto the nitrocellulose membrane (Azure Biosystems, USA). Membranes were blocked in TBST containing 5% non-fat skim milk at room temperature for 1 h and probed with primary antibodies overnight at 4 °C. Then, the membranes were blotted with an appropriate horseradish peroxidase-linked secondary antibody. Chemiluminescence was performed according to the manufacturer's instructions with Western Bright ECL HRP substrate (Advansta, USA). Antibodies to anti-HIF-1α, anti-β-actin and HRP-linked goat anti-rabbit secondary antibodies were from Cell Signaling Technology (MA, USA). Densitometry analysis was performed with ImageJ analysis software and sample intensity was normalized to β-Actin intensity.

SCRATCH MIGRATION ASSAY

To investigate the migratory effect of hypoxia on HCT116, the cells (1.0×10^6) were seeded in 12-well plates. When the cells formed a confluent monolayer, they were scratched using a 200 µL pipette tip and washed in PBS to remove cell debris. The cells was subsequently maintained in 1% serum medium and photographed after 6, 24 and 48 hours of wound formation. The relative wound size at each time point was analysed using the ImageJ software and defined as migration percentage.

TRANSWELL INVASION ASSAY

The invasion assays for HCT116 cells were performed using a 24-well transwell chamber (Corning, USA) with an 8-µm-pore PET membrane. 5×10^4 cells were plated in the top chamber with Matrigel-coated membrane (Corning, USA). Cells in medium with 1% serum were plated in the upper chamber, and the medium containing 20% serum was added in the lower chamber as a chemoattractant. After 24 h of incubation at 37 °C, the cells were fixed in ice cold absolute ethanol and stained with crystal violet dye (Sigma-Aldrich, USA) and the cells that invaded through the pores to the lower surface of the filter were counted under a light microscope. The invaded cells were photographed at 100× magnification and the invasions were determined by counting cells in at least 5 different fields of each well and were expressed as the average number of cells per field.

STATISTICAL ANALYSIS

All data, expressed as means ± SEM, were from at least three separate experiments. Groups were compared by 2-way ANOVA with $P < 0.05$ was considered as significant.

RESULTS

THE EXPRESSIONS OF HIF-1α IN THE DIFFERENT HYPOXIA INDUCTION MODELS

In order to investigate the effects of three different hypoxia induction models on HCT116 colon cancer cells, the expression of HIF-1α protein was detected by Western blotting using β-actin as the internal control. A band of the expected size (β-actin~ 42 kDa; HIF-1α~ 120 kDa) for each protein was obtained with their specific primary antibodies. After 6 h of incubation under the three different hypoxia induction models, the expressions of HIF-1α were the highest in all three models. However, after 24 h, the expressions of HIF-1α reduced in the cells treated with DMOG in 1% oxygen, while HIF-1α in other hypoxia models remained high. As expected, there was no detectable HIF-1α expression observed in the normoxic cells at all observed timepoints (Figure 1). When looking at the reproducibility of HIF-1α expressions across different replicates (n = 4), cells treated with DMOG alone showed the most consistent results, while the least consistent results were obtained from the cells incubated in the hypoxia chamber with 1% oxygen without DMOG treatment. The statistical analysis using 2-Way ANOVA showed no significant difference in the expression of HIF-1α between different hypoxia induction models and different time points (Figure 2).

THE EFFECTS OF HYPOXIA INDUCTION MODELS ON THE MIGRATION OF HCT116 CELLS

As the reproducibility of HIF-1α expressions in HCT116 cells incubated in the hypoxia chamber (1% O_2) without DMOG treatment was the lowest compared to other

models, this model was omitted from the subsequent experiment. Next, the two hypoxia models were proceeded to the wound healing assay to determine the migration percentage between those two models: DMOG alone and DMOG with 1% O_2. The results showed that at 6 h, hypoxia induction by DMOG alone exhibited a higher percentage of migrating cells compared to the control and hypoxia induction by DMOG in 1% O_2 with $p<0.01$. At 24 h, the percentage of hypoxia-induced cell migration by DMOG alone is significantly higher compared to the control ($p<0.05$), but not in cells induced by DMOG in 1% oxygen (Figure 3).

THE EFFECTS OF HYPOXIA INDUCTION MODELS ON THE INVASION OF HCT116 CELLS

To reinforce our findings from the wound-healing assay, we further studied cell invasion using the transwell invasion assay method. When compared to normoxic cells, the results showed that cell invasion increased by 41% and 13% (Figure 4) in cells treated with DMOG alone and the DMOG + 1% O_2 models, respectively. These results are consistent with the wound healing assay, which showed the highest migration percentage coming from the DMOG alone model.

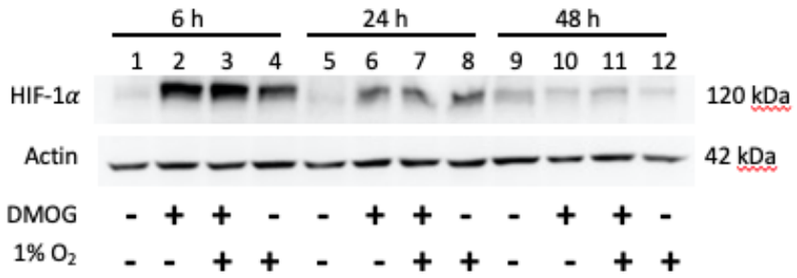

FIGURE 1. Representative image depicting HIF-1α protein levels upon hypoxia induction in the three different hypoxia models: DMOG alone, DMOG + 1% oxygen and 1% oxygen. Lane 1: Normoxia at 6 h; lane 2: DMOG at 6 h; lane 3: DMOG + 1% oxygen at 6 h; lane 4: 1% O_2 at 6 h; lane 5: Normoxia at 24 h; lane 6: DMOG at 24 h; lane 7: DMOG + 1% O_2 at 24 h; lane 8: 1% O_2 at 24 h; lane 9: normoxia at 48 h; lane 10: DMOG at 48 h; lane 11: DMOG + 1% O_2 at 48 h and lane 12: 1% O_2 at 48 h

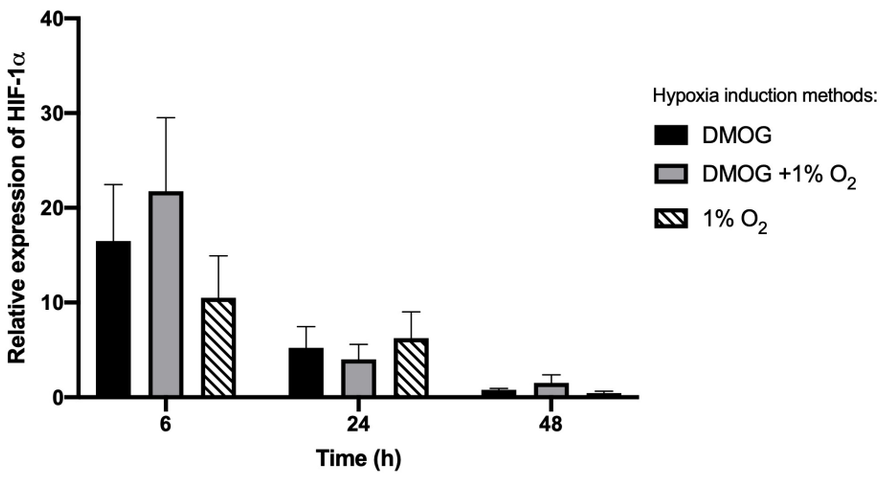

FIGURE 2. The expression of HIF-1α protein at different time point under different hypoxia induction models represented in bar graph. However, the results are not statistically significant ($p>0.05$). Error bars represent the standard error mean (±SEM) of four (n=4) independent experiments

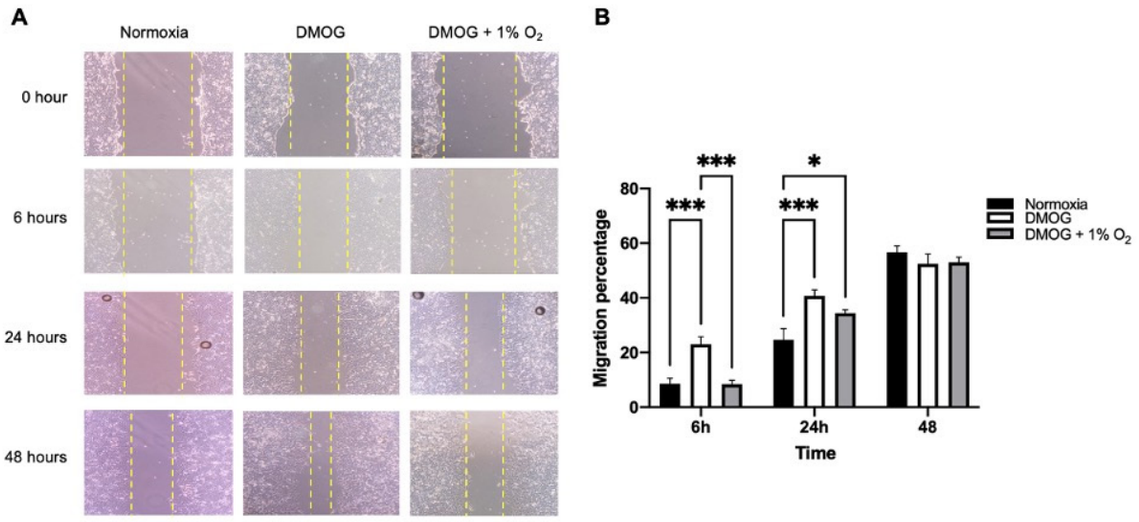

FIGURE 3. A). Representative images of scratch assays at 100x magnification at 0, 6, 24 and 48 h after hypoxia induction using two different models. Cells induced with DMOG alone exhibited a higher migratory rate compared to normoxic and cells induced with DMOG in 1% O_2, B). The percentages of cell migration are shown as the scratch gap at different hours, subtracting the value from the initial gap at 0 h ± SEM (n=3). ** $p<0.01$ and *$p<0.05$ is significantly different (1-way-ANOVA and multiple comparison – Tukey)

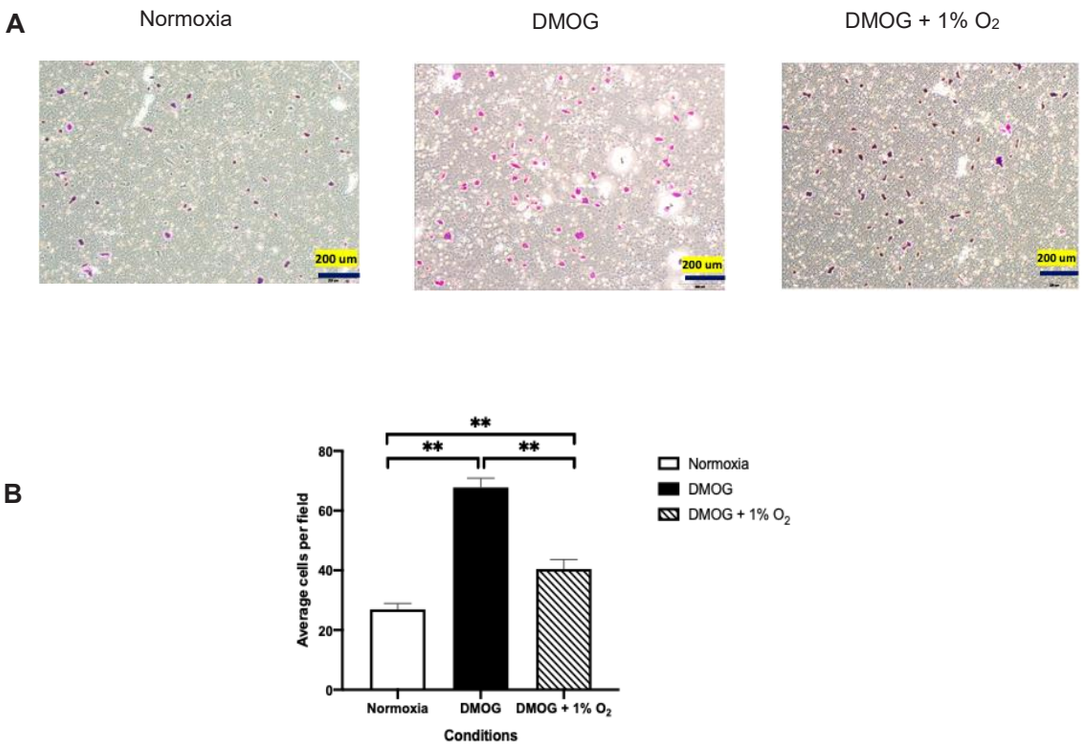

FIGURE 4. Transwell invasion assay was used to examine cell invasion. A). Cells induced with DMOG alone exhibited more invading cells than other conditions. The representative images showed invaded cells stained with crystal violet solution. Images of the transwell membrane are taken with a 20x objective using the light microscope. The cells were incubated in normoxic and hypoxic conditions (DMOG alone and DMOG + 1% O_2) for 24 h. B). The invading cells graph demonstrated that within 24 hours, hypoxic cells incubated in DMOG alone have significantly the highest invasive capabilities ($p<0.01$) than normoxic and DMOG + 1% O_2 hypoxia induction model. The error bar is ± SEM (n=3). ** $p<0.01$ and *$p<0.05$ is significantly different (1-way-ANOVA and multiple comparison – Tukey)

DISCUSSIONS

The hypoxic tumour microenvironment is critical for the development of tumours, which tend to manifest a bad prognosis and are resistant to many forms of treatment. During the development of a tumour in a hypoxic milieu, HIF-1α functions as a crucial transcriptional regulator of genes involved in ATP production in cells, proliferation, apoptosis, and blood vessel development (Rana, Singh & Koch 2019). The typical method for inducing hypoxia in *in vitro* research is to lower the oxygen content of the culture environment to 1-5% instead of 18%, which then lowers the pericellular oxygen tension (Rinderknecht et al. 2021). Yet, there are a few downsides to using hypoxic chambers or incubators. One significant drawback is that the oxygen concentration in the environment may not always match the oxygen content in cells (Pavlacky & Polak 2020). Hence, we compare the alternative methods to mimic hypoxia using DMOG, hypoxia induction using a hypoxia chamber, and a combination of DMOG and a hypoxia chamber.

In the present study, we first determine which hypoxia induction models produced the highest HIF-1α expressions in HCT116 cells within variable time points and which models give the most reproducible results across replicates. Then, we investigated the relation between the expression pattern of HIF-1α proteins in different models and the migration and invasion capacity of HCT116 cells under hypoxic conditions. Our study demonstrated that expressions of HIF-1α are increased in all models within 6 to 24 h upon hypoxia induction. However, the expression of HIF-1α is decreased in all models after 48 h of hypoxia induction. The increased expressions of HIF-1α in all models proved that the hypoxic condition has been successfully induced in all models. Nevertheless, the reproducibility of highly expressed HIF-1α was different between the three models. The cells treated with DMOG alone exhibited the most consistent expression across all replicates. Whereas hypoxia induction using 1% O_2 model was the least.

The amount of HIF that is produced by cells is subject to stringent regulation by a group of hydroxylases that consists of three prolyl hydroxylases (PHD1-3) and one asparaginyl hydroxylase. The PHDs give signals that leads to the HIF-1α degradation in normoxia (Chan et al. 2016). Since oxygen is one of the PHDs' substrates, these enzymes are inactive in hypoxia. In low oxygen levels, HIFs are stabilised and hundreds of downstream genes are upregulated. Hypoxia mimetic agents are a class of pharmacological or biological compounds that have the ability to stabilise HIF such as DMOG (Davis et al. 2019). DMOG, is an ester of N-oxalyl-glycine that enters the cells rapidly and blocks all PHDs enzymes (Jaakkola et al. 2001; Takeda, Cowan & Fong 2007). It has been demonstrated that DMOG more accurately imitates the transcriptional response to hypoxia by non-specifically stabilise HIF-1α both in the *in vitro* and *in vivo* models (Chan et al. 2016; Ogle et al. 2012; Singh et al. 2020). Our research supports those findings by demonstrating increases in the HIF-1α levels in response to DMOG treatment as well as in hypoxia chamber.

In a previous study, it was reported that DMOG-induced hypoxia occurred in HCT116 colon cancer cell, as the HIF-1 signalling pathway was found to be the top enriched pathway of the highly expressed genes shared between physiological hypoxia and DMOG-induced hypoxia. Additionally, the citric acid cycle, glucogenesis, glutamate metabolism and one-carbon metabolism are other overlapped metabolic processes (Imran Khan 2022).

To validate the effects of hypoxia, we investigated the relation between the expression pattern of HIF-1α proteins in different models with the migration and invasion capacity of HCT116 cells under hypoxic conditions, using the wound healing and transwell invasion assays. Our study showed a significant increase in the migration rate and invasion capacity of hypoxic cells induced by DMOG alone, compared to DMOG + 1% O_2 model. The timeline for these effects was mirrored by increases in HIF-1α protein at 6 and 24 h. Wang and Semenza (1993) previously reported that HIF-1α DNA-binding activity has started as early as 4 h of hypoxia exposure on Hep3B cells to transcriptionally activate the HIF-1α target gene such as erythropoietin (EPO). By contrast, the expression of HIF-1α protein was observed to be diminished after 24 hours and fell to base-line levels at 48 h of hypoxia. This result is in line with previous findings where, in A549 lung (Uchida et al. 2004) and MDA-MD-231 breast cancer cell lines (Hamad et al. 2020), downregulation of HIF-1α expression was exhibited after prolonged hypoxia exposure at 12 and 48 h. Previous study showed that the decrease in the HIF-1α protein was the consequence of negative feedback regulation. The initial rise in HIF-1α proteins increased natural antisense HIF-1α (aHIF), which in turn destabilized HIF-1α mRNA and finally decreased HIF-1α protein expression (Uchida et al. 2004).

The HIF-1 heterodimer complex, composed of two subunits, HIF-1α and HIF-1β, serves as a significant

transcription factor during hypoxia (Semenza et al. 1996). The activity of the HIF-1 heterodimer complex is governed by the oxygen-regulated subunit HIF-1α, while the HIF-1β subunit is constitutively expressed. Its expression has been observed in a variety of solid malignancies, including colorectal cancer (Chai et al. 2022; Sebestyén et al. 2021; Singh et al. 2017). Target genes involved in metastasis, invasion, and metabolism are activated by HIF-1 via binding to hypoxia-responsive regions (Majmundar, Wong & Simon 2010). HIF-1α controls extracellular matrix (ECM) remodelling-related genes, which is one of the ways it aids cancer cell migration and invasion (Wicks & Semenza 2022).

The ECM goes through major alterations as cancer progresses, which may make it easier for cancer cells to migrate and invade their surroundings. HIFs are responsible in facilitating the growth of several kinds of collagens, including basement membrane, filament-forming collagens and fibrillar. Furthermore, cancer cells keep the highly cross-linked collagen fibres, which operate as an inflexible track for efficient cell movement (Wicks & Semenza 2022). A number of genes involved in ECM remodelling, such as matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA), have been reported to have increased expression in response to HIF-1α. MMPs are enzymes that can break down ECM proteins, whereas uPA is involved in the activation of plasmin, another enzyme that can break down ECM proteins (Paul, Mistriotis & Konstantopoulos 2017). HIF-1α can promote the breakdown of the ECM by boosting the activity of these enzymes, which makes it simpler for cancer cells to travel and infiltrate nearby tissues.

The epithelial-mesenchymal transition (EMT) play a vital role in the metastasis of cancer. As cells undergo EMT, the stiff cytoskeleton and numerous cell-cell interactions responsible for the immobile epithelial phenotype are replaced by the motile mesenchymal phenotype. The mesenchymal phenotype was characterised by the loss of cell-cell interactions, cytoskeleton fluidity, and ECM remodelling (Wicks & Semenza 2022). Multiple HIF target genes have an impact on the alterations associated with EMT. In numerous malignancies, the expression of epithelial cell-specific genes, including E-cadherin, is decreased, and HIF plays a role in regulating one or more of the transcriptional repressors that control EMT, such as SNAIL, SLUG, TWIST, ZEB1, and ZEB2 as reported by Zeisberg and Neilson (2009).

Conclusion

In summary, the findings from this study suggest that the use of DMOG, a hypoxia mimicking agent, has effectively induced the expression of HIF-1α and has had a notable impact on the migration and invasion of HCT116 colon cancer cells. This indicates that the hypoxia induction method using DMOG can produce similar effects to physiological hypoxia. Therefore, it can serve as a reliable model to study the effects of hypoxia on cancer cells in conjunction with other studies, such as gene knockdown or drug treatment studies. Along with that, the findings of this study suggest that the DMOG-induced hypoxia model may have important implications for cancer research. By examining the effects of hypoxia on cancer cells, researchers may be able to develop novel therapies or identify potential therapeutic targets. In addition, the use of the DMOG-induced hypoxia model may shed light on the underlying mechanisms of cancer metastasis, which could contribute to the development of more effective cancer therapies. Overall, this study demonstrates the potential significance of the DMOG-induced hypoxia model in cancer research and suggests that further study is warranted.

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