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Effect of DMOG on Growth and Activity of NIH/3T3, BJ, L929, HaCaT Cells in Hypoxic Environment

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Abstract. Hypoxia, an oxygen-deficient environment, plays a critical role in promoting various biological processes and serves as a key regulator of cellular physiology, driving diverse biological processes through oxygen-sensing mechanisms. Therefore, the impacts of the hypoxia-mimicking molecule DMOG (dimethyloxalylglycine) need to be fully understood. This study aims to investigate the effects of DMOG on the growth and activity of NIH/3T3 fibroblasts and HaCaT keratinocytes in a hypoxic environment. After conducting a dual-timepoint experiment with 24-hour and 48-hour exposures to DMOG, we employed the CCK-8 assay to quantitatively assess cellular viability across a DMOG concentration gradient. Our findings suggest that as the concentration of DMOG increases, HaCaT cells exhibit an initial rise followed by a subsequent, monotonic decrease in cell growth rate, whereas NIH/3T3, BJ, and L929 cells show a more pronounced decreasing trend in cell growth with rising DMOG concentration. These results establish a concentration-dependent inverse relationship between DMOG administration and cellular proliferation, highlighting potential cytotoxic limitations for therapeutic applications of hypoxia mimetics in epithelial and stromal cell populations. The differential response patterns between cell lineages suggest tissue-specific susceptibility to HIF (hypoxia-inducible factor) pathway modulation, providing crucial insights for optimizing hypoxia-targeted interventions in regenerative medicine.

Keywords: DMOG, Hypoxia, Cell viability, CCK-8 assay, HaCaT cells

1. Introduction

Hypoxia is an effective micro-environment factor that drives the development of metastatic tumour. It directly raises the expression of genes relevant to angiogenesis, glucose transportation, anaerobic metabolism, cell survival etc. In the field of clinical applications, hypoxia is closely linked to mestasis and low survival rate of patients suffering from cancer [1].

The transduction of hypoxic signal is majorly mediated by hypoxia-inducible transcription factors HIF1 and HIF2. In an aerobic environment, HIF-1α on two conserved proline residue are constitutively hydroxylated by PHD, and identified by E3 ubiquitin ligase substrate recognition component VHL [2]. After identification, 26s proteasome will ubiquitinate and subsequently degrade the alpha subunits [3]. Whereas in hypoxia, the HIF are stabilized and therefore activate gene expression containing hypoxia response elements. Degradation of HIF is determined by

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whether PHD can effectively perform its functions. Oxygen, iron, 2-oxoglutarate, and ascorbate are required for PHD to perform hydroxylation reaction and signal HIF alpha degradation; therefore, any competition or depletion of such elements, such as an absence of oxygen, will cause PHD to fail in emitting signal and further degrade HIF. Targeting this mechanism, multiple drugs have been discovered to mimic an hypoxic environment, including dimethyloxalylglycine (DMOG) [4]. DMOG is a cell-permeable inhibitor of prolyl-4-hydroxylases and specifically functions on enzymes belonging to the 2-oxoglutarate (2-OG) dioxygenase family, including prolyl hydroxylases (PHD1, PHD2, PHD3) [5].

The impact of DMOG differs depending on cell types. NIH/3T3 and L929 cells both originated from mouse and belong to fibroblast cell line. They are widely used in studies involving DNA transformation, transfection and toxicity assessments [6]. While BJ and HaCaT cells are catogorized into human stromal cell line and human epithilial cell line respectively. Previous study has found that low-concentrated DMOG has the ability to promote cell proliferation, migration in HaCaT cells [7]. Whereas regarding L929 cells, a hypoxic environment might reduce the synthesis of collagen and further the viability [8]. However, despite the profound depth of current research towards hypoxia, there are still few study that constructs an in vitro model comparing the effects of hypoxia on different types of cells. Therefore, this study aims to provide an insight on the effect of hypoxia by comparing the changes in viability of NIH/3T3, BJ, L929, HaCaT cells after treatment of DMOG.

2. Methodology

Cell preparation:

The growth medium was first warmed in a 37 °C water bath. Cells were then removed from liquid nitrogen and immediately placed into a 37 °C water bath, with gentle stirring for uniform melting. After melting, the outer surface of the tube was cleaned with 70% ethanol and placed into a clean bench. Cell suspension was then transferred into a 15 mL tube and centrifuged at 1200 rpm for 4 min. The supernatant was discarded, and the pellet was resuspended in the prepared medium using a pipette. Next, cell suspension was transferred into a culture dish and evenly distributed by crosswise shaking. Cells were examined under an inverted microscope to ensure adhesion to wall and cell morphology. The cultures were then incubated at 37 °C in a humidified incubator with 5% CO_2until recovery was complete, with approximately 95% of the cells adhering and showing normal morphology. Culture dish was subsequently removed from the incubator, and cell attachment and morphology were observed again under an inverted microscope. The medium was then completely discarded, and 1 mL of 0.25% trypsin was added to cover the surface and digest the cells. Once the cells detached, 5 mL of complete medium (at least twice the volume of trypsin) was added to neutralize the enzyme. Pipetting was repeated to obtain a single-cell suspension, for further transfer into a centrifuge tube and centrifugation at 1200 rpm for 5 min at 4 °C. After centrifugation, the supernatant was carefully removed, and the pellet was resuspended in fresh culture medium for subculture.

3. Cell plating

3T3, BJ, L929, and HaCaT cells in the logarithmic growth phase were collected and digested with trypsin, and the digestion was terminated by adding complete medium. The supernatant containing trypsin was discarded, and an appropriate amount of complete medium was added to resuspend the cells by gentle pipetting. The cells were then counted using a hemocytometer, and the concentration

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was adjusted to an appropriate range. The prepared cell suspension was inoculated into 96-well plates at a density of 5,000–10,000 cells per well, depending on the experimental requirements. The seeded 96-well plates were placed in a 37 °C incubator with 5% $CO_2$to allow the cells to adhere and grow.

4. Addition of DMOG

After cell adherence, the culture medium in the 96-well plate was carefully removed to avoid damaging or removing the cells. According to the experimental design, different concentrations of DMOG solution were added to different types of cells, with multiple replicates for each concentration. The DMOG concentrations used were as follows:

Table 1. DMOG concentration levels set for NIH/3T3, HaCaT, BJ and L929 cells

cell type	DMOG concentrations (mM)
NIH/3T3	0, 0.4, 0.6, 0.8, 1, 2, 4, 8, 10
HaCaT	0, 0.02, 0.04, 0.05, 0.1, 0.2, 0.4, 0.8, 1.0
BJ	0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.0, 1.5, 2, 3
L929	0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0

A control group was included, in which an equal volume of complete medium was added. The 96-well plate containing DMOG was then incubated at 37 °C with 5% $CO_2$for 48 hours.

Observation and evaluation of cell viability:

After 48 hours of culture, the 96-well plate was removed from the incubator. The supernatant was discarded, and 100 μL of culture medium was added to each well. Subsequently, 10 μL of CCK-8 reagent was added to each well, and the plate was gently shaken before being incubated again at 37 °C with 5% CO_2 for 45 min to 2 hours. The absorbance (OD value) of each well was measured at 450 nm using a microplate reader. The relative cell proliferation rate was then calculated based on the obtained OD values.

5. Results

The growth rate of NIH/3T3 cells was evaluated at 24 h and 48 h across a range of DMOG concentrations (0 mM, 0.4 mM, 0.6 mM, 0.8 mM, 1 mM, 2 mM, 4 mM, 8 mM, and 10 mM). The results indicate that DMOG exerts both a concentration and time dependent effect on cell growth. At both time periods, the growth rate relative to the 0 mM control exhibits distinct patterns: lower concentrations (0.4–1 mM) may slightly modulate cell growth, while higher concentrations (2 mM and above) likely induce a more pronounced inhibition, with the suppression increasing along with both concentration and incubation time. A significant decrease in growth rate takes place as the concentration increases from 1mM to 2mM and from 2mM to 4 mM. The maximum growth rate observed is approximately 100% of the control, while the minimum drops to around 7% at the highest concentration and longest incubation period.

Microscopic observations also showed that fewer cells were present in the high-concentration DMOG groups at both time points, especially in Fig. 2F–I and Fig. 3O–R. In addition, cell density was generally higher at 24 h than at 48 h.

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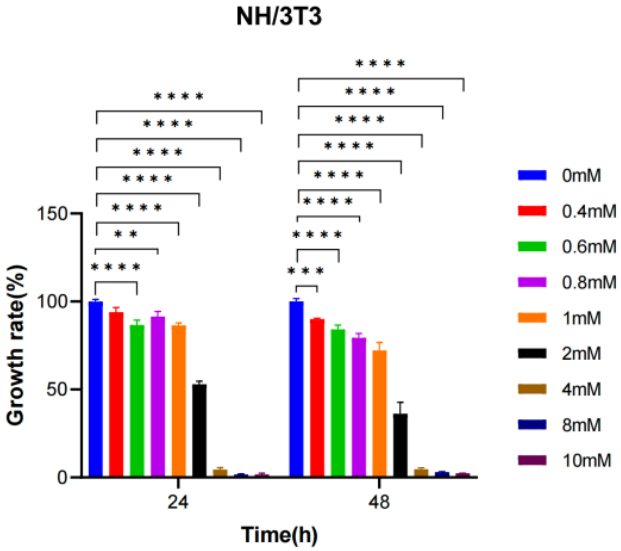


Figure 1. Effect of DMOG on NIH/3T3 cell growth rate at 24 h and 48 h

Note: NIH/3T3 cells were treated with DMOG at concentrations of 0, 0.4, 0.6, 0.8, 1, 2, 4, 8, and 10 mM, and the growth rate was measured at 24 h and 48 h.

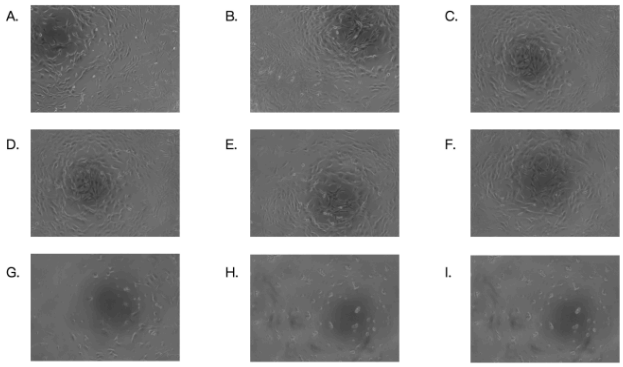


Figure 2. Morphological changes of NIH/3T3 cells after 24 h DMOG treatment

Note: A: 0 mM; B: 0.4 mM; C: 0.6 mM; D: 0.8 mM; E: 1 mM; F: 2 mM; G: 4 mM; H: 8 mM; I: 10 mM.

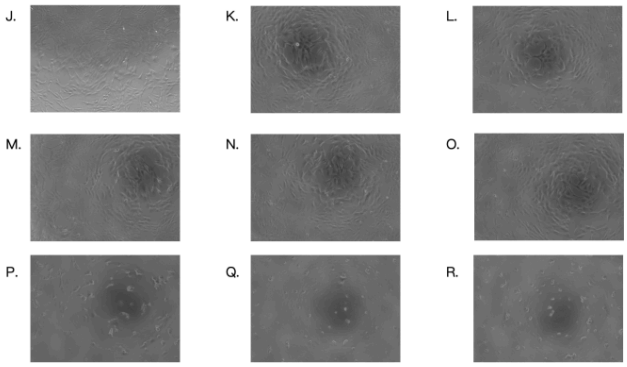


Figure 3. Morphological changes of NIH/3T3 cells after 48 h DMOG treatment

Note: J: 0 mM; K: 0.4 mM; L: 0.6 mM; M: 0.8 mM; N: 1 mM; O: 2 mM; P: 4 mM; Q: 8 mM; R: 10 mM.

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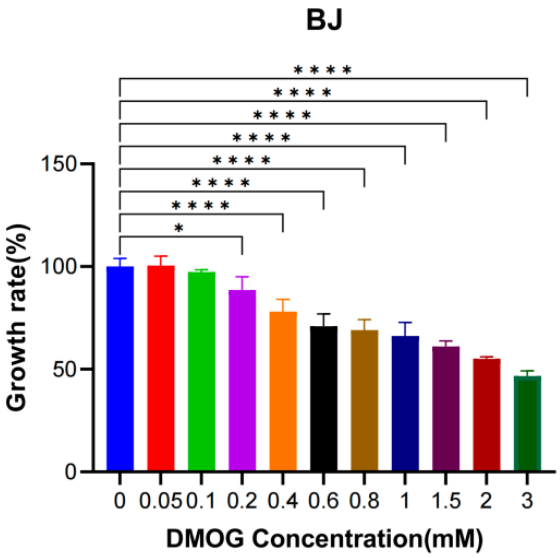


Figure 4. Effect of DMOG on BJ cell growth rate

Note: growth rate of BJ cells under concentrations of 0 mM, 0.05 mM, 0.1 mM, 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM, 1.0 mM, 1.5 mM, 2.0 mM, 3.0 mM.

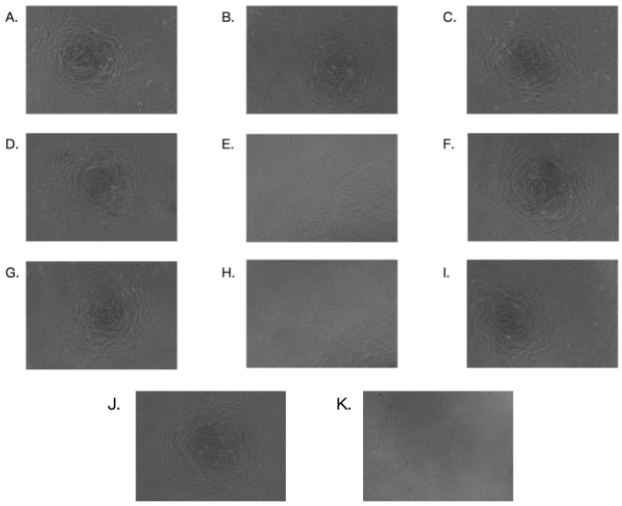


Figure 5. Morphological changes of BJ cells after DMOG treatment

Note: growth condition of BJ cells under microscope: A: 0 mM, B: 0.05 mM, C: 0.1 mM, D: 0.2 mM, E: 0.4 mM, F: 0.6 mM, G: 0.8 mM, H: 1.0 mM, I: 1.5 mM, J: 2.0 mM, K: 3.0 mM

For BJ cells,the concentration of DMOG was set starting from 0 mM to 3 mM (0, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 1.5, 2, 3 mM). The data overall reveals a negative correlation between DMOG concentration and cell growth rate. However, unlike NIH/3T3, the effect of rising concentration of DMOG on BJ cells was relatively mild. A gradual decrease in growth rate was observed with no dramatic increase or decrease. Lower concentrations (0.05–0.2 mM) have a mild effect, while higher concentrations (greater or equal to 0.4 mM) tend to suppress growth progressively. The growth rate decreases from nearly 100% to approximately 50% when the DMOG concentration reaches 3 mM, as shown in Fig. 4; the corresponding cell morphology at 3 mM is shown in Fig. 5K. Interestingly,

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0.05 mM of DMOG is found to exhibit a weak stimulative effect on cell growth, with the growth rate rising to about 102%.

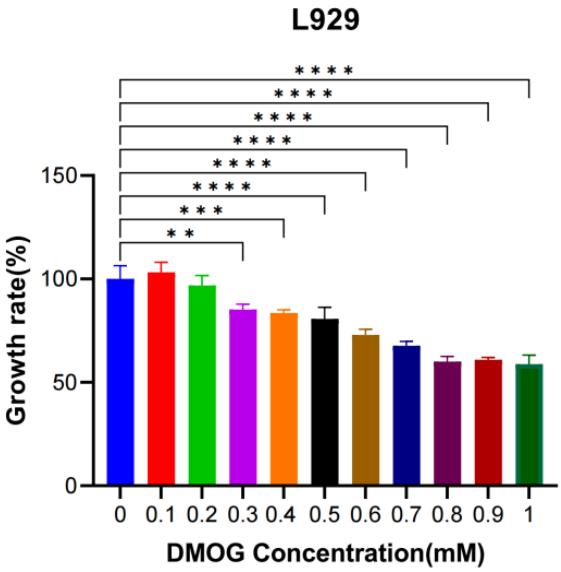


Figure 6. Effect of DMOG on L929 cell growth rate

Note: growth rate of L929 cells under concentrations of 0 mM, 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, 0.5 mM, 0.6 mM, 0.7 mM, 0.8 mM, 0.9 mM, 1.0 mM.

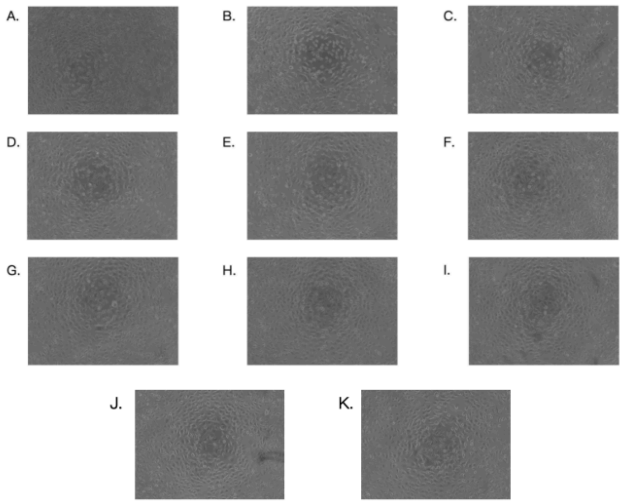


Figure 7. Morphological changes of L929 cells after DMOG treatment

Note: growth condition of L929 cells under microscope: A: 0 mM, B: 0.1 mM, C: 0.2 mM, D: 0.3 mM, E: 0.4 mM, F: 0.5 mM, G: 0.6 mM, H: 0.7 mM, I: 0.8 mM, J: 0.9 mM, K: 1.0 mM

L929 cell growth was treated with 0 mM to 1 mM DMOG (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1 mM). The cell growth rate was then measured. Similarly, the results generally reflects a continuous decrease in growth rate as the concentration rises. At lower concentrations (0–0.2 mM), the growth rate remains relatively stable and even exhibit small increases in cell growth rate. However, as the concentration reaches 0.4 mM, DMOG is more likely to induce a gradual decrease on rate of growth. By 1 mM DMOG, the growth rate is further reduced, dropping to 50% from

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100%, reflecting an evident inhibitory effect of high DMOG concentrations on L929 cell proliferation.

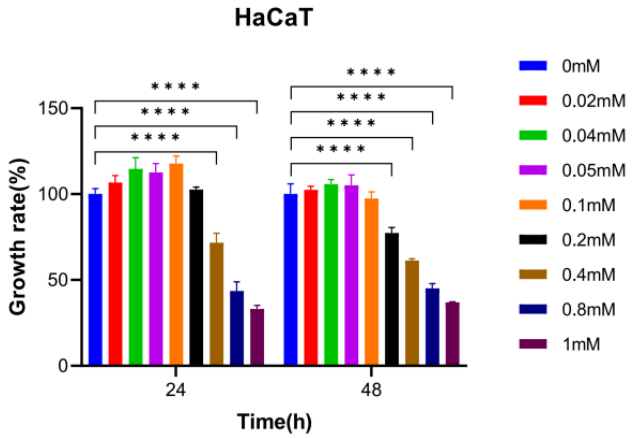


Figure 8. Effect of DMOG on HaCaT cell growth rate at 24 h and 48 h

Note: growth rate of HaCaT cells under concentrations of 0 mM, 0.02 mM, 0.04 mM, 0.05 mM, 0.1 mM, 0.2 mM, 0.4 mM, 0.8 mM, 1.0 mM.

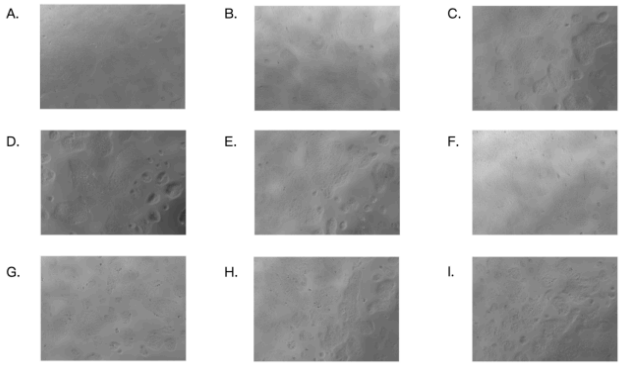


Figure 9. Morphological changes of HaCaT cells after 24 h DMOG treatment

Note: growth condition of HaCaT cells under microscope at 24 hours: A: 0 mM, B: 0.02 mM, C: 0.04 mM, D: 0.05 mM, E: 0.1 mM, F: 0.2 mM, G: 0.4 mM, H: 0.8 mM, I: 1.0 mM.

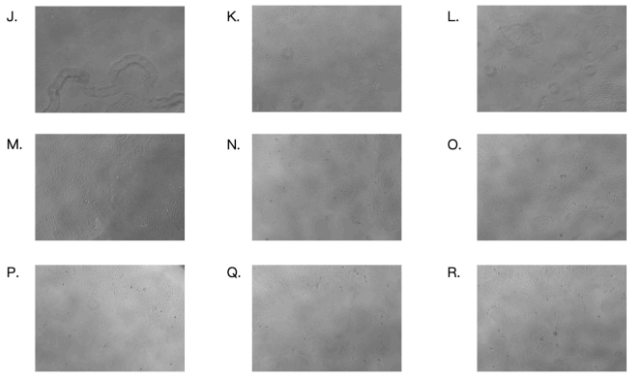


Figure 10. Morphological changes of HaCaT cells after 48 h DMOG treatment

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Note: growth condition of HaCaT cells under microscope at 48 hours: J: 0 mM, K: 0.02 mM, L: 0.04 mM, M: 0.05 mM, N: 0.1 mM, O: 0.2 mM, P: 0.4 mM, Q: 0.8 mM, R: 1.0 mM

HaCaT cell growth was analyzed at both 24 h and 48 h with DMOG concentrations of 0 mM, 0.02 mM, 0.04 mM, 0.05 mM, 0.1 mM, 0.2 mM, 0.4 mM, 0.8 mM, and 1 mM. The effects of DMOG on HaCaT cells are relatively different to the other three types of cells. At both time points, low and medium concentrations (from 0 mM to 1 mM) are witnessed to promote cell growth of HaCaT cells, with the highest growth rate measured reaching about 120%. However, when the concentration exceeds 1 mM, growth rate drops dramatically as the concentration continues to rise, eventually ending at around 40%. The inhibitory effect is generally stronger at 48 h compared to 24 h for the same concentration. The growth rate declines from near 150% (at lower concentrations) to near 0% at 1 mM, highlighting heightened sensitivity to DMOG at higher doses and longer incubation times.

Overall, all four cell lines (NIH/3T3, BJ, L929, HaCaT) exhibit a similar concentration-dependent responses to DMOG at higher concentrations. However, the effects of DMOG of low concentrations are slightly different, cell growth rate of HaCaT and L929 experienced relatively significant increases, while that of NIH/3T3 and BJ cells are more likely to be inhibited. Time-dependent effects are more prominent in NIH/3T3 and HaCaT cells, with 48 h incubation period resulting in a stronger inhibitory impact of DMOG on cell growth.

6. Discussion

The results of this experiment are mainly categorized into two types: cell growth rate of fibroblasts cells (NIH/3T3 and BJ) are continuously inhibited by the addition of DMOG, while epithilial cells (HaCaT) exhibit obvious increases in growth rate at low and medium concentrations of DMOG. However, the results obtained for L929 was an exception. Being categorized into the fabroblast cell line, its cell growth rate also witnessed minor increases at low DMOG concentrations.

In terms of the results obtained for fibroblasts cells, it is known that cells activate a series of adaptive mechanism during a hypoxia, depending on the cell type these mechanism might include slowing down processes that are highly energy-consuming such as cell proliferation etc. In fibroblast cells such as NIH/3T3 and BJ, this is achieved by regulating retino-blastoma protein (pRB) with the phosphorylation of cyclin-dependent kinases (CDKs). pRB in hyperphosphorylated state allows cell cycle to enter S phase. However, the accumulation of hyperphosphorylated pRB impedes the progress of cell cycle and blocks it at G1 phase, further stopping cell proliferation [9]. This finding copes with the graph of NIH/3T3 in this experiment, in which the cell growth rate of NIH/3T3 is significantly reduced by DMOG.

For epithilial cells (HaCaT), the rises in cell growth rate may be due to stabilizing and enhancing the expression level of factors such as highly expressed HIF-1α, HIF-2α, VEGF-A, TNF-α, IL-6, MCP-1 and IL-8. In human body system, tissues such as heart, kidneys and dermis, being surrounded by more blood vessels, have an oxygen pressure between 5% to 10%. In comparison, human keratinocytes possess a poorer blood transport system. Relatively less oxygen supply results in a much lower oxygen pressure in these cells (usually around 0.2% to 8%). Therefore, a characteristic of keratinocytes is the universality and severity of hypoxia [10]. Also, according to previous study, HIF-1α is highly expressed in these cells [11]. HaCaT cells are a type of human keratinicytes and hence share the characteristics.

Therefore in an hypoxic environment, where factors including HIF-1α, HIF-2α and so on are stabalized and even enhanced, which are relevant to increasing cell proliferation [12] [13]. This

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conclusion is also supported by the results obtained in this experiment where DMOG significantly increased the growth rate of HaCaT cells at low concentrations.

However, the results of the experiment can still be improved on the comprehensiveness and so on. For example, a notable limitation of this study is the absence of direct validation of HIF-1α protein stabilization, as Western blot analysis was not performed. Without confirming HIF-1α expression, the relationship between DMOG treatment and the observed changes in cell proliferation remains incomplete. Furthermore, apoptosis and cell cycle alterations were not investigated by flow cytometry, leaving the reason that causes reduced cell growth rate unclear. These limitations emphasizes the need for additional improvements to strengthen the comprehensiveness of our results.

Future studies can focus more on certain genetic approaches, such as Western Blotting to directly evaluate the necessity of HIF-1α in mediating DMOG's effects. Based on the limitations, the method adopted to measure DMOG's effects can also be improved. Flow cytometry can be applied to better explain the mechanism that leads to lower cell proliferation and growth rate.

To conclude, our findings have dual implications. On one hand, the proliferative effect of DMOG on keratinocytes (HaCaT) suggests potential ability in wound healing and epithelial regeneration, where enhanced epithelial cell growth is beneficial. On the other hand, the pronounced inhibitory effect observed in normal fibroblasts, particularly BJ cells, also raises concerns about potential cytotoxicity in tissue engineering contexts. Such opposing effects also highlights the necessity of application of hypoxia in clinical medicine based on cell types.

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