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Original investigation

Repositioning of Metformin: Anticancer Agent for Hypoxic Neuroblastoma Cells

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Abstract

Objectives: Neuroblastoma is an extracranial solid tumor of early childhood that has a hypoxic environment. VEGF and HIFs molecules play a role in adaptation to this microenvironment. Hypoxic microenvironment leads to poor prognosis and inadequate treatment of neuroblastoma. Metformin has been shown to inhibit tumor growth, might be a potential chemotherapeutic agent. The anti-cancer activity of Metformin on SH-SY5Y cells are not fully elucidated. The aim of this study is to determine the anti-cancer effect of Metformin on SH-SY5Y cells and to elucidate its molecular action mechanism in hypoxia/normoxia.

Patients and Methods:SH-SY5Y cells were exposed to increasing doses of Metformin. The viability of SH-SY5Y cells was evaluated using the real time xCELLigence RTCA system. Migration of SH-SY5Y cells was determined using wound healing. The effect of metformin on mRNA and protein expression levels was evaluated using Real-time PCR and Western Blot, respectively.

Results: Metformin was observed to significantly reduce the viability of SH-SY5Y cells. Metformin treatment reduced migration of SH-SY5Y cells. In addition, Metformin treatment significantly reduced mRNA expression of HIF-1α, PDK-1 and VEGF-A in SH-SY5Y cells under normoxia and hypoxia. In hypoxia condition, protein expression of HIF-1α and VEGF-A decreased after Metformin administration on SH-SY5Y cells. Protein expression of PDK-1 was observed to decrease in both normoxia and hypoxia conditions on SH-SY5Y cells.

Conclusion: In this study, the anti-cancer effect of Metformin on SH-SY5Y cells was determined. Metformin has been observed as inhibitor of migration in SH-SY5Y. Metformin treatment has been shown to reduce gene and protein levels of HIF-1α and target molecules in neuroblastoma cells.

Keywords: neuroblastoma, metformin, hypoxia, HIF-1α, VEGF-A, PDK-1

INTRODUCTION

Neuroblastoma (NB) is widely referred to as a neuroblastic tumor spectrum (consist of ganglioNBs, NBs, and ganglioneuromas) which stem from primitive sympathetic ganglion cells. NBs are 97 percent of all neuroblastic tumors, are very heterogeneous, differing in terms of histopathologic appearance, location, and biologic characteristics (1) NBs are the most impressive since their wide variation of clinical behavior, which could range from aggressive disease with metastatic spreading which led to death or spontaneous regression to maturation to a benign ganglioneuroma (2). The variations of clinical outcomes associated with several biological or clinical factors such as tumor stage and histology, patient age. Childhood tumors arise in <10 years of age children with a 90% rate. The most frequent tumor in the first year of life of children is an extracranial solid tumor, NB. The mentioned disease represents approximately 8% of all childhood cancers. NB diagnosed patients have a 15% mortality rate. The average age of diagnosis for NB is 1.5 years. (3)

Solid tumors are poorly oxygenated and adjusted to a hypoxic microenvironment which results in activation of the transcription and synthesis of vascular endothelial growth factor (VEGF), also hypoxia-inducible factors (HIFs). (4) HIFs have crucial effects in the cancer hallmarks which are immune responses, apoptosis, proliferation, genomic stability, invasion, metastasis,

and vascularization. Additionally, HIFs cause to chemotherapy resistance via various pathways. HIFs are a significant marker of poor prognosis and treatment relapse. Thus, HIFs could be important molecular targets that could be utilized to develop enhanced treatment options of treatment resistance and metastatic cancers. (5)

Chemotherapy is one of the important treatment options and has been successful in terms of long term survival outcomes in patients with refractory, metastatic, and advanced primary NBs. In children with intermediate-risk NB, the suggested therapy option is moderately intensive multi-agent neoadjuvant chemotherapy or its combination with surgical resection. However, chemotherapy-treated advanced stage NB failed to effectively abolish the disease. Thus, new combination of chemotherapy options need to be researched. (7,8)

Insulin levels are exhibited to be increased and also, the associated signalling pathway of receptor tyrosine kinase is triggered in many cancer types. Insulin causes the induction of these receptors that lead to survival and growth signals to the cells via the PI3/AKT pathway. (9,10) Together with all information above, metformin, an antidiabetic which decreases hepatic gluconeogenesis, is recently introduced as a new potential chemotherapeutic agent. This linkage between metformin and the oncological field arises from clinical outcomes in metformin-treated diabetes patient has lower cancer incidence and improved prognosis. (11)

The goal of the study is to evaluate the anticancer effects, also underline Metformin molecular mechanism on SH-SY5Y NB cells under normoxic and hypoxic conditions. In this context, hypoxia related molecules such as hypoxia-inducible factor 1-alpha (HIF-1α), vascular endothelial growth factor A (VEGF-A), pyruvate dehydrogenase kinase 1 (PDK-1) and glucose transporter 1 (GLUT-1) have been demonstrated in terms of gene and protein levels.

METHODS

Cell Culture

Human NB SH-SY5Y cell line was purchased from the ATCC (ATCC® CRL-2266, USA). The cells were cultured in DMEM/Ham's F12 (Biochrom Berlin, Germany), supplemented with 10% FBS (Biochrom, Berlin, Germany), 1% non-essential amino acids (Cegrogen, Stadtallendorf, Germany) 2mM L-glutamine (x Biochrom, Berlin, Germany), and 100 U/ml/100 µg/ml penicillin-streptomycin (Cegrogen, Stadtallendorf, Germany) and were maintained at 37°C in 5% CO_2 humidified incubator with 20% O_2 and 1% O_2 for normoxia and hypoxia, respectively.

Real Time Cell Viability Assay

SH-SY5Y cells were seeded into 96 E-plate at a density of 1×10^4 cells/well and incubated at 37°C and 5% CO_2 overnight. After incubation, the cells were exposed to different concentrations of Metformin for 48-72 hours. The cell viability was monitored by using the xCELLigence RTCA system (Real-Time Cell Analyzer System, Acea Biosciences Inc., San Diego, CA). The cell index value which presents cell viability was analyzed by reflecting impedance change.

Wound Healing Assay

For migration assays, 6x104 cells were plated in a 6-well plate under normoxic and hypoxic conditions after undergoing serum starvation with 1% FBS overnight. The cells were scratched with a sterile 100µl pipette tip to generate a wound on a cell monolayer and washed with PBS to remove debris. After washing, cells were exposed to 5, 10, and 20mM Metformin for 24 hours and captured at 0, 6, and 12 hours using the inverted microscope (Carl Zeiss Suzhou Co., Ltd, Axio Vert. A1). The marked area of each wound was measured with Image J ver. 1.149 software program (NIH).

Real-time PCR

Total RNA was extracted from SH-SY5Y cells treated with 10, and 20 mM Metformin for 48 hours under normoxic and hypoxic conditions using the RNeasy® kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. The quality and quantity of total RNA were detected by NanoDrop Spectrophotometer (Mastrogen, Taiwan). After extraction, cDNA was synthesised from 1 µg of RNA per each samples by using the RT^2 First strand kit (Qiagen, Maryland, USA) in accordance with the manufacturer's protocol. Quantitative Real-time PCR was performed on an Rotor gene Q (Corbett Research, Qiagen, Germany) using the SYBR Green PCR MasterMix (Qiagen, Maryland, USA) containing 10 µM primers (Table 1). To normalise the relative levels of mRNA, β-actin was used as an internal control. After all amplifications, the identity and purity of the amplified products were checked by analyzing the melting curve. The relative mRNA expression levels of all samples were calculated using the $2^{-\Delta\Delta Ct}$ method with the Ct values normalized. All reactions were amplified using the following parameters: initial denaturation at 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds (data acquisition). Melting curve analysis was performed ramping from 60 °C to 95 °C and rising by 1 °C every 5 s.

Table 1. Primers used in current study

Gene	Primer Sequence	Amplicon (bp)
HIF-1α	(F) 5'AAGAACAAAACACACAGCGAAGC-3'	199
	(R) 5'-TCACAAATCAGCACCAAGCAGG-3'	
PDK-1	(F) 5′- CAACAGAGGTGTTTACCCCC-3′	105
	(R) 5′-ATTTTCCTCAAAGGAACGCC-3′	
VEGF-A	Qiagen RT^2 qPCR Primer Assays (cat no. PPH00251C)	71
GLUT-1	Qiagen RT^2 qPCR Primer Assays (cat no. PPH02043C)	96
ACTB (β-ACTIN)	Qiagen RT^2 qPCR Primer Assays (cat no. PPH00073G)	154

Western Blot

SH-SY5Y cells were seeded in T25 flasks at a density of 1×10^6 and exposed to Metformin for 48 hours. The cells were lysed with ice-cold 1X RIPA buffer (Cell Signalling, Leiden, The Netherlands) containing 1mM PMSF for 5 minutes on ice and sonicated briefly. Cell lysate was centrifuged at 14.000 × *g* for 15 minutes at 4°C. The protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) as described by the manufacturer. Equal amounts of protein samples (20 µg/well) were loaded

and separated electrophoretically by SDS-PAGE. The gel was transferred to PVDF membranes (Roche, Mannheim, Germany) and the membranes were blocked with 5% non-fat dried skim milk powder in 1x TBS-T for 1 hour. Thereafter, the membranes were incubated with primary antibodies against anti-rabbit HIF-1α (1:500, 14179S), anti-rabbit PDK-1 (1:2000, ab207450), anti-rabbit VEGF-A (1:500, ab52917), anti-mouse GLUT-1 (1:500, ab40084) and alpha actinin (1:3000, 6487S) as a housekeeping which were prepared in 5% BSA or 5% non-fat dried skim milk in 1X TBS-T buffer at 4°C overnight as described by the manufacturer. The blots were washed in 1xTBS-T and incubated with horseradish peroxidase conjugated secondary antibodies. The images were captured by UVP bioimaging system (UVP Inc., Upland, CA) and the band intensity was quantified by UVP LabWorks 4.6 Image Acquisition software. The relative protein levels were calculated as the ratio to the level of α-actinin.

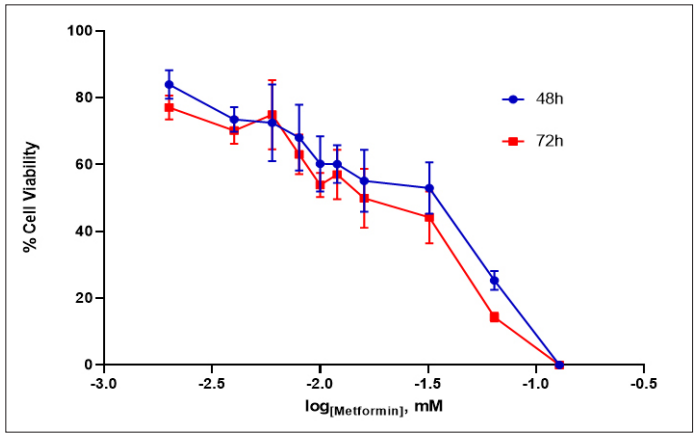

Figure 1. The percentage of cell viability of Metformin treated SH-SY5Y cells.

Statistical Analysis

The half maximal inhibitory concentrations (IC_{50}) of Metformin was analyzed with GraphPad Prism 8.4.3 (GraphPad Software, Inc., CA, USA). All data were expressed as the means ± SEM of at least three independent experiments. Group comparisons were evaluated using One-way ANOVA test with Dunnett's Post Hoc and Kruskal Wallis test. All p-values <0.05 were considered statistically significant.

RESULTS

Effects of Metformin on cell viability

To determine the effects of Metformin on cell viability, cells were treated with Metformin (2-128 mM) for 72 hours. We found a dose-dependent reduction with an IC_{50} value of 20mM and 14mM of Metformin for 48 and 72 hours, respectively in SH-SY5Y cells (Figure 1).

Metformin Suppresses on SH-SY5Y Cell Migration

Wound healing assay was performed to examine the effects of Metformin on cell migration (Figure 2) showed that Metformin

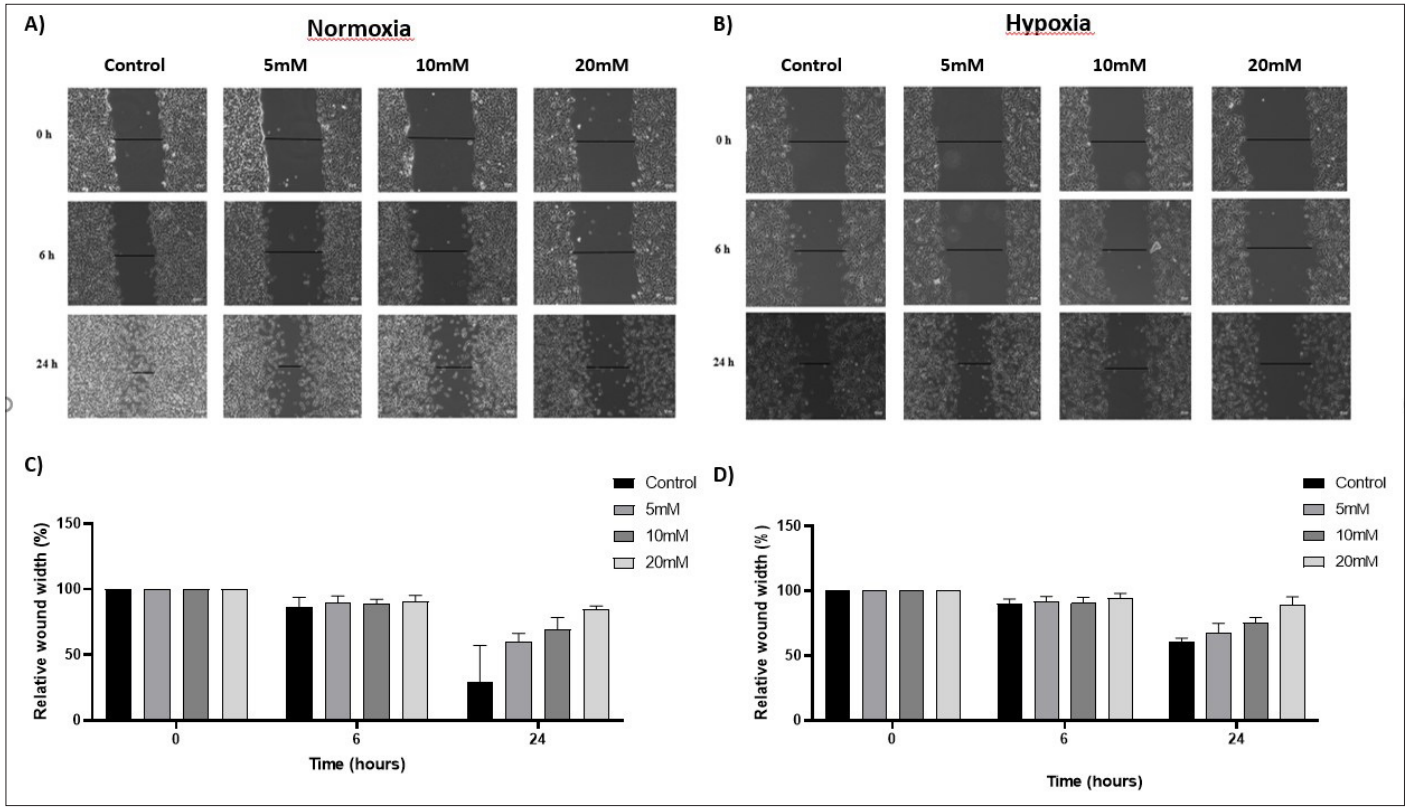

Figure 2. Cell migration was analyzed by wound-healing assay under normoxia **(A and C)** and hypoxia **(B and D)**. SH-SY5Y cells were cultured in different concentration of Metformin (5, 10, and 20mM). The cell migration was observed by microscopy and documented by photography at 0, 6, and 12 hours. There were no statistically significant differences between normoxia and hypoxia groups.

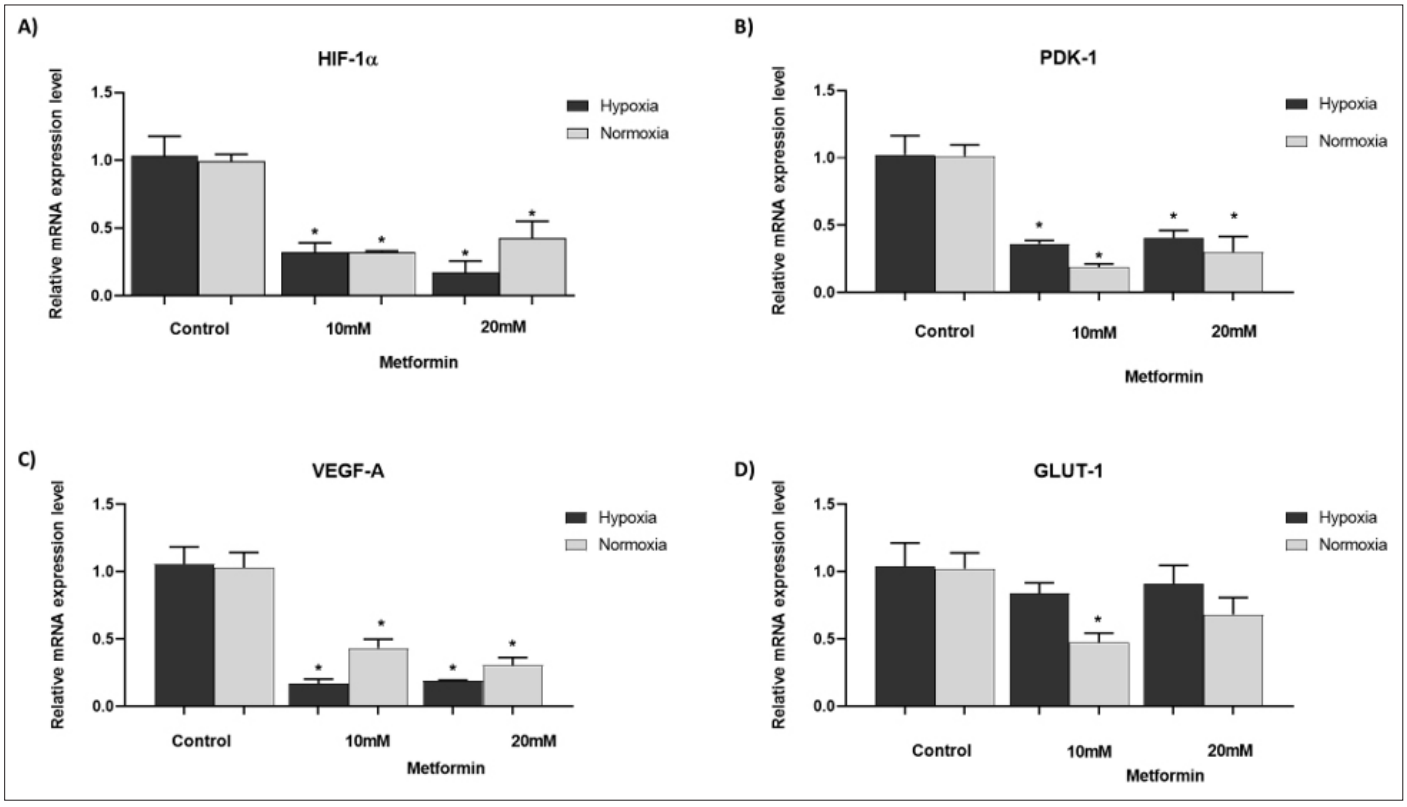

Figure 3. Effects of Metformin on mRNA expression level of HIF-1α **(A)**, PDK-1 **(B)**, VEGF-A **(C)** and GLUT-1 **(D)** under normoxia and hypoxia. Cells were exposed to different concentration of Metformin for 48 hours of hypoxia (1% oxygen) or normoxia (20% oxygen), the total RNA was collected and used for qRT-PCR analysis of HIF-1α, PDK-1, VEGF-A and GLUT-1 mRNA expression.

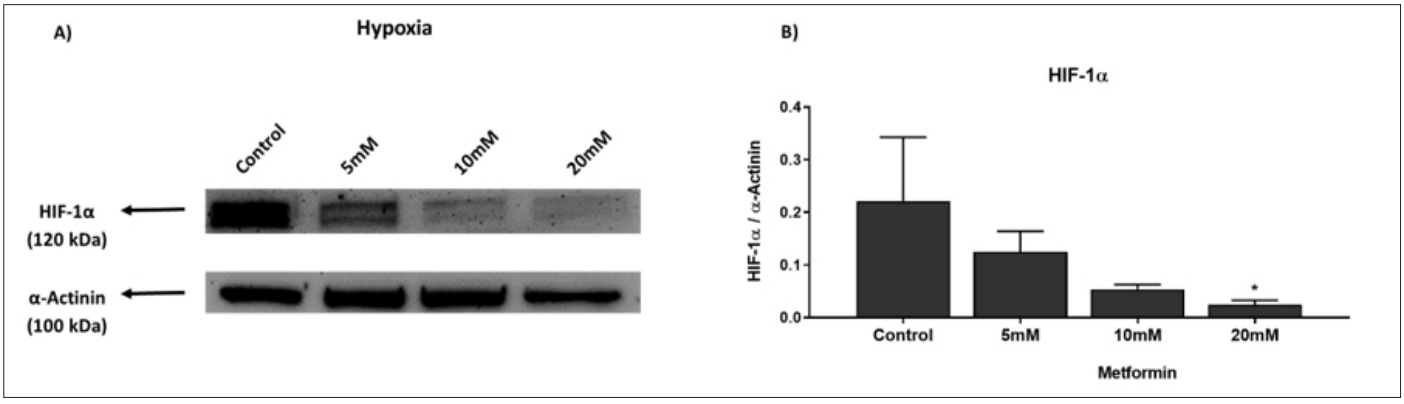

Figure 4. The expression level of HIF-1α in cell lysate were detected by western blotting. Cells were treated with Metformin for 48 hours under hypoxic condition. Whole-cell lysates (20μg) were loaded and subjected to western blotting analysis with an anti-HIF-1α under hypoxia shown in **(A)** and **(B)**.

considerably reduced the migration of SH-SY5Y cells compare with the control in a time and dose-dependent manner from 0 to 24 h under both normoxic and hypoxic conditions.

Metformin inhibits the transcription activity of HIF-1α and its target genes.

Real-time qPCR was used for the quantification of HIF-1α, PDK-1, VEGF-A, and GLUT-1 mRNA expression levels under normoxia and hypoxia for the various concentrations of Metformin (10 and 20mM). We have shown that Metformin treatment significantly decreased HIF-1α, PDK-1 and VEGF-A mRNA expression compare with the non-treated controls in SH-SY5Y NB cells under normoxia and hypoxia ($p<0.05$, Figure 3). In contrast, we did not find a statistically different GLUT-1 mRNA expression under normoxia and hypoxia except for 10mM under normoxia.

Metformin supresses the protein levels of HIF-1α and its target molecules.

The protein levels of HIF-1α, PDK-1, VEGF-A and GLUT-1 were determined by western blotting. We confirmed that Metformin (5, 10, and 20mM) significantly inhibited the HIF-1 protein

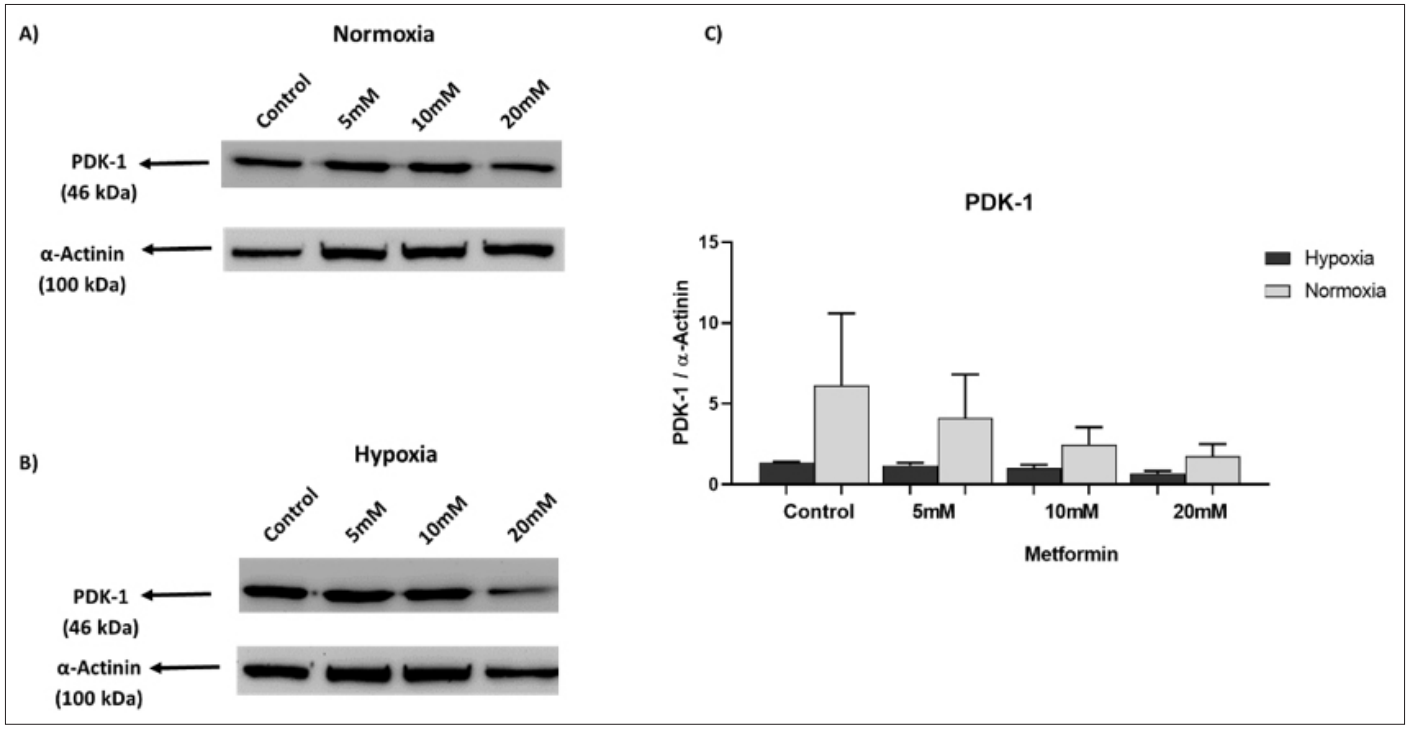

Figure 5. Western blot was performed to evaluate the expression of PDK-1 protein level in SH-SY5Y cells under normoxia and hypoxia shown in **(A)**, **(B)**, and **(C)**.

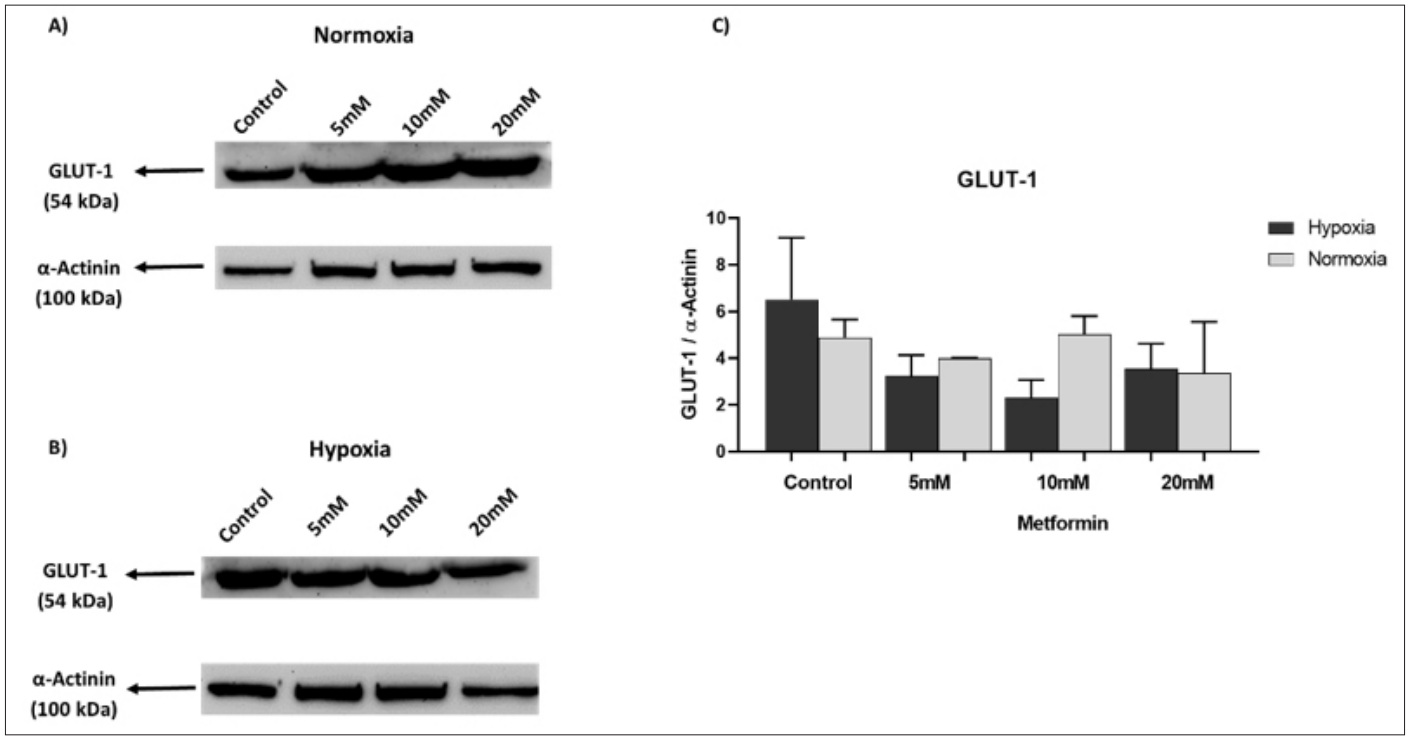

Figure 6. Effects of Metformin on GLUT-1 protein expression levels in NB SH-SY5Y cells under normoxia and hypoxia shown in **(A)**, **(B)** and **(C)**.

expression in a dose-dependent manner as compare to control in hypoxia (Figure 4(A), 4(B)). PDK-1 protein level was reduced after Metformin treatment under normoxia and hypoxia (Figure 5(A), 5(B) and 5(C)). To determine whether this inhibition of HIF-1α with Metformin affects downstream proteins, VEGF-A and GLUT-1, were checked by western blotting under normoxia and hypoxia (Figure 6, 7). We shown that Metformin inhibited VEGF-A expression in SH-SY5Y cells after 5mM (Figure 7(B)) and GLUT-1 protein level was reduced with metformin under hypoxia (Figure 6 (B) and (C)).

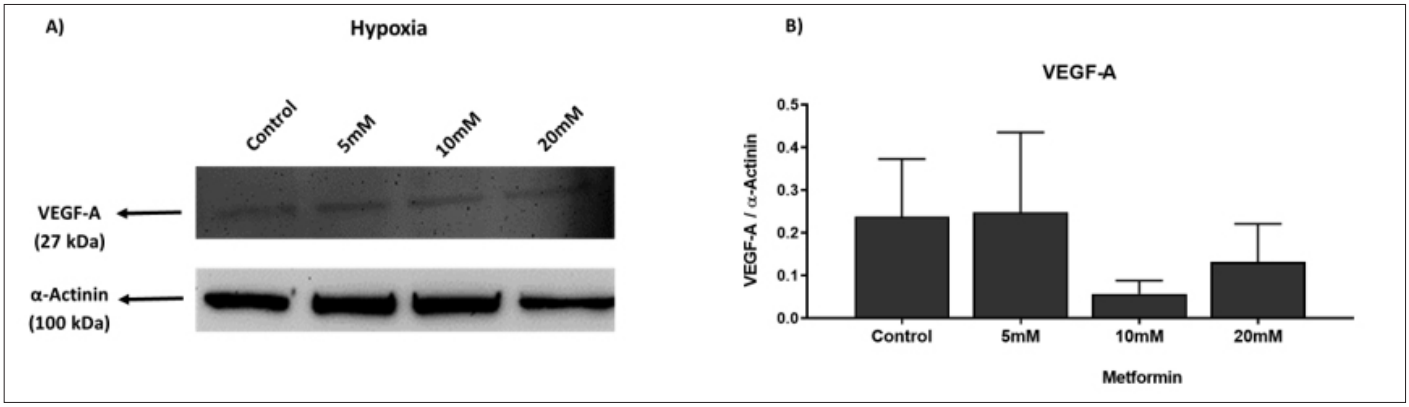

Figure 7. VEGF-A protein was analysed by western blotting with metformin treated SH-SY5Y cells under hypoxia show in **(A)** and **(B)**.

DISCUSSION

Neuroblastoma is noteworthy in terms of a variety of clinical behaviors. For several decades, specified therapies, located on the absence or presence of specific biological and clinical aspects, have been applied for patients classified as a low or intermediate risk which gives rise in survival outcomes. Treatment improvements for high-risk neuroblastoma, even though the results for these patients yet remain poor, with long-term survival <50%.[12] Neuroblastic tumors that appear in childhood have biologic heterogeneity cause separated therapeutic strategies. Favorable biological featured tumors are treated with reduced therapeutic intensity is a clear trend. Currently, researchers have been trying to discover new treatments that are based on the microenvironment and oncogenic characteristics in the tumor cells (13). Hypoxic microenvironment has a critical role for solid tumors such as neuroblastoma which have a large hypoxic microenvironment. The main regulator factor of hypoxia is HIF-1α, which has been reported to be upregulated in neuroblastoma (14). Metformin, an antidiabetic agent, inhibits the complex-1 of mitochondria, and recent studies have shown that metformin inhibited HIF-1α levels in different cancer types such as colorectal cancer, breast cancer, and osteosarcoma (15).

In the current study, we indicated that Metformin decreases the expression levels of HIF-1α and its downstream molecules as PDK-1, GLUT-1, and VEGF-A on the SH-SY5Y cell line. We also evaluated the antitumor effects of Metformin on cell viability and migration. We found that reduction in Metformin treated cells on cell viability in a dose-dependent manner.

The proliferation of the inhibitory dose of metformin has been detected as compatible with other studies. In a study, Costa D. et al, have been investigated that 20 mM Metformin treatment causes a statistically significant reduction of proliferation rate after 48 hours, also in SH-SY5Y and SKNBE2 cell lines. (16) In another investigation Mouhieddine T.H. et al showed that metformin inhibited the proliferative activity of SH-SY5Y and U251 cells. The inhibitory effect of approximately 50% at 72 h at 10 mM for SH-SY5Y. (17)

In the current study, migration characteristic evaluation of the SH-SY5Y cell line was found consistent with the other studies. Metformin non-treated SH-SY5Y cell line was not capable to close completely the wound at 48 h. which illustrates that Metformin significantly decreased the migration of treated cells. In a study, metformin non-treated SH-SY5Y cells are not capable to close totally the wound at 48 hours which illustrates that Metformin significantly decreased the migration of the treated cells. (17) In another study, SH-SY5Y cells were significantly inhibited by 33% in knockdown of HIF-1α SH-SY5Y cell line at hypoxia. (18)

In this study HIF-1α in terms of protein and gene are reduced by Metformin treatment in a dose-dependent manner on SH-SY5Y cells which are correlated with the other investigations. VEGF-A expression levels are reduced by Metformin. The results are convenient with the other studies. In several studies, Metformin is related to the inhibition of HIF-1α stability in cancer cells, reducing HIF1-targeted genes expression levels, including VEGF-A, thus causing growth inhibition. (19, 20 21), commonly identified by decreased microvessel density and small tumor vessel size (22, 23).

The current study showed that the PDK-1 gene and protein expression levels decreased by Metformin in a dose-dependent manner in hypoxic and normoxic microenvironment on SH-SY5Y cells. HIF1-α association with PDK-1 also demonstrated in our results which is convenient compared to other studies. In a study, HIF-1α induces the transcription of genes encoding many glycolytic enzymes, glucose transporters, and activates PDKs, which reduces the flow of glucose-derived pyruvate into the tricarboxylic acid (TCA) cycle. (24)

In this investigation, GLUT-1 gene and protein expression levels are not found affected by Metformin nor in hypoxia neither normoxia. There are several studies that showed that the effect of Metformin is on GLUT-1 expression levels in SH-SY5Y cells differs. In a study, the effect of metformin in elevating protein levels of crucial molecules that is one of the glycolytic molecules as GLUT1 in cancer cells were observed by Chaube et al. (25) Additionally, in another study indicated that Metformin is not effective on total protein amount, gene expression or localization of this carrier of plasma membrane, thus excluding drug effects on GLUTs in cancers. (26)

To conclude, Metformin has a crucial effect on HIF-1α, PDK-1, and also VEGF-A which are key molecules that indicate that there is a relation between the microenvironment and metabolism of cancer cells. Metformin, as a novel promising agent, has potential effects and could be used as a chemotherapeutic agent.

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