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Comparison of Nickel and Cobalt Induced Hypoxic Cell Models using Cell Proliferation Assay

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by

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Thank you to my lab mates for their constant support, reassurance and friendships. Thank you to Jennifer Babcock for always being present, constantly encouraging me and for sharing her immense knowledge. Thank you to the other students and special friends I have made at Seton Hall University for the constant support and for being such great study buddies.

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TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

Hypoxia is an imbalance in oxygen delivery and oxygen consumption, ultimately affecting cell survival. Low levels of oxygen diminish adenosine triphosphate synthesis resulting from a decline in oxidative phosphorylation in the mitochondria, therefore inducing apoptosis and cell death. To create a hypoxia mimicked environment, we used hypoxia mimetic compounds cobalt and nickel to treat human neuroblastoma (NMB) cells. Using hypoxic mimic human neuronal cell models, we examined and compared the effects of compound-induced hypoxia on NMB cell proliferation. The cells were treated with 100μM and 300μM concentrations of each compound at 24- and 48-hour intervals. To investigate cell proliferation, the thymidine analog EdU was used, which incorporates into DNA during DNA synthesis. Flow Cytometry was used to measure and analyze the fluorescence of EdU incorporation. Results showed the inhibition of cell proliferation using both nickel and cobalt treated cells as compared to the control cells with no treatment. Furthermore, the inhibition of cell proliferation was dependent upon the exposure time and concentration dose. Interestingly under the same concentration, cobalt produced greater inhibition of cell proliferation than nickel. Although the causes of differences are unclear currently, possible reasons are discussed and hypothesized. In conclusion, this study demonstrated differences in the inhibition of cell proliferation using nickel or cobalt hypoxic mimetic compounds in human neuronal cells. Further research to determine mechanisms behind different cell models will provide additional information and understanding in regard to hypoxia related diseases.

INTRODUCTION

Oxygen is essential for all eukaryotic survival and is a key element for the production of adenosine triphosphate (ATP). ATP is the main energy source for biological functions, such as homeostasis, self-repair and tissue sustenance (Guzy & Schumacker, 2006). To meet the supply-demand of ATP, oxidative phosphorylation utilizes metabolic substrates NADH and $NADH_2$ to synthesize ATP from ADP and inorganic phosphates. In mitochondria, oxidative phosphorylation requires oxygen consumption to synthesize ATP. There are several intricate pathways that allow cells to sense oxygen level fluctuations leading to the development of adaptations that maintain oxygen tension. When cells encounter insufficient ATP production with the loss of oxygen, the survival of the cell is threatened. This disadvantage leads to cells evolving different responses to preserve the required oxygen for survival. Different tissues have adapted various defense mechanisms under low oxygen conditions known as hypoxia. For example, arterial chemoreceptors can monitor blood oxygen levels, or cells that secrete hormones to regulate adequate supply of tissue oxygenation (Guzy & Schumacker, 2006). Upon hypoxia, some cells have the ability to decrease oxygen utilization, allowing for an increase in survival rate.

Hypoxia is the condition of low levels of oxygen and is around 3-5% O_2 within the cellular environment (Humar et al., 2002). Pathologically, hypoxia occurs during acute and chronic vascular disease, pulmonary disease, and cancer, ultimately leading to cell death if severe and/or prolonged. Further, different tissues require specific amounts of oxygen, when altered these changes are critical in the pathogenesis of diseases such as stroke, myocardial infarction and chronic lung disease (Lopez-Barneo et al., 2001). Hypoxia threatens cell survival because it is a precursor to anoxia; a cellular environment that contains zero oxygen. The

physiological responses to hypoxia can be categorized as acute or chronic. Chronic hypoxia responses can take hours, days or even weeks, whereas acute response times are seconds to minutes. Examples of acute responses are hyperventilation (arterial and airway chemoreceptors), activation of glucose uptake (cardiac and skeletal muscle, fat tissue), or increase heart output (peripheral chemoreceptors, heart muscle). Chronic responses occur at the cellular and/or gene level, such as activation of glucose metabolism and transport, erythropoiesis (bone marrow), angiogenesis and neovascularization (vascular endothelium, hypoxic or ischemic tissues), production of vasodilators (vascular endothelium and smooth muscle), and the changes of various gene expression (Semenza, 2000). Acute responses depend upon excitable cells that have oxygen sensitive ion channels, allowing for quick responses. Prolonged hypoxia leads to the changes in gene expression for transporters, enzymes and growth factors that aid against oxygen deficiency (Humar et al, 2002). Key regulators of transcriptional responses to oxygen deprivation are proteins known as hypoxia-inducible factors, which sense instabilities in oxygen levels. To maintain oxygen homeostasis, developmental and physiological pathways are triggered to upregulate oxygen delivery (Huang et al., 2014).

Ultimately, hypoxia will lead to non-oxidative synthesis of ATP, neovascularization, and an increase in oxygen carrying capacity of blood. Cellular response to hypoxia leads to the upregulation of various genes that aid in glucose uptake, glucose metabolism, apoptosis, glycolysis, glycolytic enzymes, and vascular endothelial growth factor (VEGF) (Humar et al., 2002; Guzy & Schumacker, 2006; Diguilio et al., 2018). Cellular response is not immediate because upregulation of genes can include transcription and translation, which requires hours or days.

HIF TRANSCRIPTION FACTORS AND ISOFORMS

Hypoxia-inducible factors (HIF) belong to a family of transcription factors, including HIF-1, HIF-2, and HIF-3, which can regulate several hypoxia response genes. While differential regulation of the HIF isoforms isn't completely understood, they all depend upon the hydroxylation of proline and asparaginyl residues conserved in the N and C termini (Ratcliffe, et al., 2017). Of the isoforms, HIF-1 and HIF-2 are the most well studied. HIF-1 and HIF-2 have similar DNA-binding and heterodimerization domains but different sequences in their transactivating domains, suggesting that they have unique target genes. Studies have suggested that HIF-1α induces the expression of several glycolytic genes, while HIF-2 is cell-specific and tissue limited (Hu et al., 2003; Raval et al., 2005; Wigpur et al., 2016). Mole and colleagues compared HIF-1α and HIF-2α DNA binding discovering many loci bound both HIF-α isoforms, however, substantially more bound to HIF-1α than HIF-2α. Additionally, further analyses found distinct patterns of binding occurred for HIF-1α (Mole et al., 2009).

In addition to the transcriptional regulation, the α-subunits of HIF-1 and HIF-2 are differentially regulated at the transcriptional, translation, and post-translation modification levels. Although there are overlaps of binding sites between HIF-1 and HIF-2, studies conducted by Mole et al. showed HIF-2 did not offer transcriptional changes during hypoxic responses but translational changes in MCF-7 cells. HIF-2 creates a hypoxia-regulated translation initiation complex between RNA binding protein RBM4 and cap-binding protein eIF4E2. This complex recruits a wide variety of mRNAs to promote active translation at different polysomes (Wigerup et al., 2016).

Furthermore, Wigpur and collaborators utilized experimental and clinical data to show HIFs regulated metastasis and treatment resistance in various tumors. Leading to the hypothesis

that HIF inhibitor are likely to target important oncogenic processes or pathways. Interestingly, the two isoforms tend to have contrasting effects on tumor growth, specifically in cell cycle and apoptotic regulators c-MYC and p53. In summary, HIF-2 and HIF-1 can have opposite effects, such is the case in essential cell cycle regulator and oncogene, c-Myc. HIF-1α inhibits c-Myc ultimately suppressing proliferation and leads to c-Myc degradation. Contritely, HIF-2α enhances c-Myc activity and leads to cell cycle progression in renal carcinoma cells, NIH3T3 cells, HEK293 cells, and embryonic ECs (Wigerup et al., 2016; Gordan et al., 2007). Additional studies by Holmquist-Mengelbier et al. exhibited HIF-1α under chronic hypoxia degrades over time, while HIF-2α accumulates continuously in select neuroblastoma cells. This implicated that HIF-1α mediates quick responses while HIF-2α mediates late responses for hypoxia (Holmquist-Mengelbier et al., 2006).

Both HIFs are co-expressed in cancers. HIF-1α is primarily active initially under acute hypoxia, while HIF-2α dominates later under chronic hypoxia, as well as normoxic conditions (Holmquist-Mengelbier et al., 2006). The most notable difference among the two are tissue specific differences with few similarities as tumor suppressor proteins in glioblastoma and teratoma models. Hypoxia inducible factors-1 and 2 overexpression have been associated with poor clinical outcomes in patients with various cancers. In addition, the HIF-1α protein is overexpressed in various common solid malignant tumors including breast, colon, gastric, lung, skin, ovarian, pancreatic, prostate, and renal carcinomas compared to their respective normal tissues. While the two isoforms have poor prognosis' when upregulated in cancer, the cancer type varied. HIF-1α overexpression led to poor survival in cervical cancer, endometrial carcinoma, oligodendroglioma, ovarian cancer, and different breast cancer subtypes. HIF-2α overexpression led to poor prognosis in breast cancer, glioblastoma, head and neck squamous

carcinoma, and non-small cell lung cancer. Conversely, HIF-1α overexpression showed an improvement of diagnoses in head and neck cancer, and non-small cell lung cancer (Wigerup et al., 2016).

Of the HIF isoforms, HIF-1 is well studied. The structure of HIF-1 is a basic helix-loop-helix(bHLH)/PAS heterodimeric transcription factor, containing the dimerization of two subunits, 120-kDa HIF-1α and 91-94kDa HIF-1β (Chanel et al., 1998; Brunelle et al., 2005). This specific hypoxia-inducible transcription factor is ubiquitously expressed and is an important factor during hypoxia cellular responses. Of the two subunits, HIF-1α is oxygen-regulated and required for cellular response to hypoxia. The β subunit is unique to HIF-1, also referred as the aryl hydrocarbon receptor nuclear translocator or ARNT, is constitutively expressed and has the ability to dimerize with the arylhydrocarbon receptor (Semenza, 2000). Notably, HIF is regulated through enzymatic post-translational hydroxylation of the α subunit.

Hypoxia-inducible factor-1α is constitutively transcribed, and translated, but readily degraded under normoxic conditions. This protein has 826 amino acids with bHLH and PAS domains located on the N-terminal, which are critical for both dimerization and DNA binding (Lopez-Barneo et al., 2001). There are two transactivation domains and one protein stability region located on the C-terminal of HIF-1α. The domain fundamental for stabilization is located between residues 401 and 603.

After stabilization, HIF-1α translocates to the nucleus, where it dimerizes with HIF-1β. The transcription factor will bind to the A/GCGTG consensus motif in the promoter regions of target genes known as hypoxia response elements (HREs) (Wigerup et al., 2016; Urrutia and Aragones, 2018). When the monomers form the heterodimeric transcription factor, the genes transcribed will favor tumor aggression, as well as the cell's survival under hypoxia (Diguilio et

al., 2018). For HIF-1α stabilization, the α-subunit activates through the detection of low oxygen levels (around 5% O_2), which is sensed within 30 mins. HIF-1α accumulation begins to steadily increase until it reaches full accumulation near 0.5% O_2 roughly 4-8 hours later (Lopez-Barneo et al., 2001; Jiang et al., 1996; Brunelle et al., 2005).

With the decrease in oxygen tension, HIF-1 binds to HREs on the 3' flanking region of the erythropoietin gene, the 5' flanking region of the VEGF gene and the regulatory elements encoding glycolytic enzymes (Chanel et al., 1998). There is a HIF-binding region on all the genes that are hypoxia-sensitive. Hypoxia potentiates HIF-1 activity through HIF-1α transcriptional activation, mRNA stabilization, nuclear translocation, and the prevention of HIF-1α degradation (Semenza, 2000). There are cis-acting DNA sequences that are required and a trans-acting factor that allows binding to HIF-1 (Wang et al, 1995).

There are multiple factors required for degradation of HIF-1α, including proteins, enzymes, oxygen, and other cofactors. Prolyl hydroxylases (PHDs) are a family of enzymes with oxidative properties. PHD's are tetramers containing 2 hydroxylase units and 2 protein disulfide isomerase subunits that covalently modify HIF-1α (Harris, 2002). The family includes PHD1, PHD2, and PHD3. PHDs hydroxylate specific proline residues (Pro402 and Pro564) within the oxygen-dependent degradation (ODD) domain and facilitate interactions with ubiquitin ligase (Brunelle et al., 2005). These enzymes require 2-oxoglutarate (α-ketoglutarate), oxygen as substrates and iron as a cofactor (Guzy & Schumacker, 2006; Urrutia and Aragones, 2018). Therefore, PHDs sense deviations in the required substrates and cofactors. With a decline in oxygen tension, PHD ability to hydroxylate is limited due to the restricted oxygen available.

The α subunit's ability to sense a decline in oxygen tension is due to its ODD domain. Located within the ODD domain are highly conserved proline residues which are required for

degradation via the PHD/pVHL pathway. This pathway includes the ubiquitination and degradation of HIF-1α by prolyl hydroxylases and ubiquitin ligase. PHDs are essential for post-translational modification signals to properly degrade HIF-1α. Proline hydroxylation modifies the α subunit required for the von Hippel Lindau (pVHL) tumor suppressor protein to bind to the modified unit. This protein, also known as the E3 recognition component of the ubiquitin ligase, is required for ubiquitin labeling and the proteasomal degradation of HIF-1α.

There are stimuli that regulate HIFs such as growth factors, cytokines, hormones, and other various stressors. Many growth factors have cognate receptors that interact and signal the phosphoinositide 3-kinase (PI3K) or Ras/MAPK pathways which express HIF. Further, PI3K pathway inhibition will lead to basal and mitogen-induced HIF inhibition (Wigerup et al., 2016). Although these non-hypoxia driven stimuli can normalize hypoxic environments, there are elements that induce hypoxia.

HYPOXIA MIMETICS

Chemical compounds that can stabilize HIF-1α under normoxic conditions are known as hypoxia mimetics. These compounds artificially induce hypoxia by stabilizing HIF-1α and/or inhibiting PHD enzymes both directly and indirectly. Further, directly targeting PHDs can lead to inhibition of activity altogether. Many mimetics will indirectly target PHDs by depleting the availability of iron, increasing oxygen species production, or creating competition. There are several compounds commonly used as hypoxia mimetics, such as Cobalt and Nickel.

Cobalt (II) Chloride hexahydrate ($CoCl_2$) is a water-soluble chemical mediator of hypoxia, also known as, a hypoxic mimetic (Diguilio et al., 2018). Cobalt treatment can increase the cellular content of HIF-1α through the direct inhibition of prolyl hydroxylases, as well as,

increasing ROS generation (Chandel et al., 1998). Therefore, cobalt induces gene expression, specifically RNAs encoding glycolytic enzymes to combat against hypoxia.

Water-soluble and water-insoluble nickel differ in carcinogen potency as well as entrance into the cell. As a hypoxic mimetic $NiCl_2$ blocks iron uptake, while simultaneously competing against iron dependent enzymes leading to iron depletion, as well as, HIF-1α stabilization (Costa et al., 2005). Prolyl hydroxylases are indirectly inhibited due to their dependence upon iron as a co-factor. This inhibition allows HIF-1α to stabilize, and activate the hypoxia signaling cascade.

HYPOXIA AND CANCER

A tumor microenvironment includes blood and lymphatic vessels, fibroblasts, immune cells, and an extracellular matrix. Upon progression, cancer cells have limited access to oxygen and nutrients, causing hypoxic regions. These regions allow for aberrant vascularization which is related to cancer progression. Cancer cells will undergo genetic and adaptive changes to promote survival and proliferation under oxidative stress. These adaptions stimulate malignant phenotypes promoting aggressive tumor behavior. Continual proliferation will lead tumors to outgrow blood supply, resulting in chronic hypoxia.

Peter Vaupel and colleagues utilized oxygen electrodes to study tumor oxygen supply for squamous tumors of head and neck, cervical and breast cancers. The results of the study show low oxygen tension in tumors led to an increase in metastasis, decreasing patient survival in all cancers studied. There are reports about VHL mutations and HIF-2α upregulation inducing a hypoxic environment in renal cancer, and cerebellar hemangioblastomas (Harris, 2002). Additionally, the overexpression of HIF-1α was reported in colon, breast, gastric, lung, skin, ovarian, pancreatic, prostate and renal carcinomas (Zhong, H. et al, 1999).

PURPOSE OF EXPERIMENT

Based on the information above, this study examined and compared the effects of hypoxic mimetic compounds, cobalt and nickel, on NMB cell proliferation using a human neuroblastoma NMB cell model. The proliferation rates of surviving cells under challenges of the hypoxic mimic compound at various exposure time and concentrations were investigated. A hypoxia induced cell model was created using cobalt, nickel, and human neuroblastoma cells. To measure the cell proliferation, cells were treated with EdU then examined and analyzed with a flow cytometer.

FLOW CYTOMETRY

The MASQUANT analyzer is a highly sensitive multi-parameter flow cytometer that utilizes fluidic and optical components for cell analysis. The machine allows for the rapid analysis of multiple characteristics of different cells supplying both qualitative and quantitative data. Possible characteristics that can be measured include cell size, cytoplasmic complexity, DNA or RNA content, and a range of membrane-bound and intracellular proteins (Brown and Wittwer, 2000).

Forward scatter (FSC) measures physical properties such as size, while side scatter (SSC) measures the granularity of the cell. Flow cytometry utilizes fluorescent dyes to bind and intercalate with cellular components, including DNA or RNA. Additionally, antibodies can be employed to promote the binding of specific proteins and fluorescent dyes.

Flow cytometry utilizes lasers to excite the fluorescent molecules to higher energy states. When the molecules come down to their resting state, the fluorochromes will emit light energy at a higher or different wavelength. Using multiple fluorochromes will allow the simultaneous

measurement of several different cell properties due to the different emission wavelengths. In this study, fluorescent 488 is the dye used to measure EdU incorporation.

Within the machine, cells are sheathed in an isotonic fluid to create a single cell line or laminar flow, thus allowing each individual cell to pass through the light emission. Each cell will pass through a beam of monochromatic light that intersects the cell. The beams then bounce off in different directions depending on the properties of each cell. Optics collect the lights emitted into various filters and dichroic mirrors which allow specific wavelengths to be isolated from the rest (Brown and Wittwer, 2000). These signals can then be detected by photomultiplier tubes allowing for digitization for computer analysis, via MASQUANT analyzer.

MATERIALS AND METHODS

CELL CULTURE

Human Neuroblastoma Cells (NMB) were cultured in Roswell Park Memorial Institute (RPMI) (Invitrogen, Carlsbad, CA, USA), supplemented with 10% Fetal Bovine Serum (FBS) (Atlanta Biologicals, Flowery Branch, GA, USA) and treated with antibiotics penicillin and gentamicin. The NMBs were grown in culture flasks at 37°C in 5% CO_2 incubator.

TREATMENT

Cells were seeded into six well plates at $7x10^5$ cells concentration per well. Cells were incubated overnight, harvested and then treated with 100μM and 300μM $NiCl_2$ and $CoCl_2$ concentrations, separately. Cells were incubated for 24- and 48-hour exposure times. Additionally, each well was treated with 1μM EdU for 16 hours to track proliferation.

CELL PROLIFERATION ASSAY

The neuroblastoma cells were harvested using PBS EDTA, then centrifuged at 1000 RPMs for five minutes. Cells resuspended in 1% BSA in PBS then centrifuged and fixed using the click-it fixative, 4% paraformaldehyde. The NMB cells were centrifuged and resuspended in 1% BSA in PBS to quench any unreacted fixative. Cells were permeabilized using click-it permeabilization wash and reagent. EdU buffer additive was added to permeabilized NMB cells to allow for the detection of EdU under dye fluorescent 488. The fluorescent signals of control and treated cells were examined using Flow cytometry. Parameters used were: FSC 207V, SSC 322V and B1 316V.

CLICK- IT REACTION

To detect cell proliferation, cells were incubated with 5-ethynyl-2'-deoxyuridine (EdU) which is a nucleoside analog of thymidine. EdU directly measures active DNA synthesis of the cell cycle. It is cell permeable and is incorporated into the DNA during Synthesis (S) phase. Further, EdU is an easier, faster option that doesn't require a denaturation step. Click-it chemistry was used to detect the presence of EdU by a copper-catalyzed covalent reaction between a fluorescent azide and the alkyne on the EdU molecule. This reaction forms a stable triazole ring, allowing Flow cytometry to detect and measure the fluorescence of EdU. The fluorescent dyes are required for measurement because the dyes will intercalate into the helical structure of DNA. This fluorescence is then directionally proportional to the amount of DNA in the nucleus. Utilizing this fluorescence to amount for any increase or decrease in DNA, this can show cell proliferation (increase in DNA) or the inhibition of proliferation (decrease in DNA). Flow results were analyzed using MASQUANT Analysis and depicted via histograms.

RESULTS

Cells developing adaptive and/or genetic changes to survive under a low oxygen environment can lead to tumorigenesis. Therefore, hypoxia is an important element for cancer research. These adaptations promote malignant phenotypes, ultimately leading to aggressive tumor behavior **(**Harris, 2002). Many advanced cancers, such as fibroblasts, neck tumors, cervical cancer, and soft tissue sarcoma, are known to induce the overexpression of HIF-1α which is associated with vascularity, and tumor aggression, thus combating hypoxia and encouraging cell survival (Wigerup et al., 2016).

Nickel and Cobalt are two commonly used hypoxic mimetics. Therefore, we utilized these compounds to induce a hypoxic environment, then examined and compared how the metals affected the cell proliferation of surviving neuroblastoma cells under various exposure times and doses. Cells were then incubated with the thymidine analog EdU which is incorporated into the DNA during DNA synthesis. Incorporation of EdU was measured and analyzed using flow cytometry and MASQUANT.

Human NMB neuronal cells were treated with concentrations of 100μM and 300μM of $CoCl_2$ and $NiCl_2$ to create a hypoxic mimic neuronal cell model. All cells were treated with 1μM of EdU for 16 hours. Control cells were treated with only EdU. Prior to this study, we conducted experiments with three different concentrations of EdU at 24- and 48-hour intervals, to find a concentration without producing cytotoxicity. Of the three concentrations, 1μM EdU yielded the results we required with minimal cell death.

The graphs are measuring EdU incorporation which occurs during S Phase. The higher the FITC signal indicates more nucleotide incorporation, suggesting cell proliferation. The large

second peak is the fluorescence of EdU assimilation into the cells, after DNA synthesis. The first and smaller peak is indicative of G1 phase. S phase is the dip between the peaks, which is the area EdU begins incorporation into the DNA. If there is a decrease in EdU incorporation, then there will be a shift to the left or a decrease in the second peak.

DETERMINATION OF EDU INCORPORATION OF NICKEL TREATMENT

The results of EdU incorporation of cells treated with Nickel for 24 hours are shown in Figures 1a-c. At 24 hours, Nickel treated cells showed slight inhibition of cell proliferation from the graph's shift to the left, best depicted in Figure 2 the overlay of graphs from Figures 1a-c. Figure 1 shows a slight variation from the control peaks (1a), but there isn't a significant difference. Even between the variance in concentrations there isn't a substantial disparity. For better understanding, the overlay of Figure 2 shows a clear shift to the left and a dip at 1e1. This confirms more cells have stayed in G1 phase and have not proceeded through S phase verifying a decrease in cell proliferation.

Conversely, the higher exposure time shows a significant effect on cellular proliferation. Figures 3a-c represent EdU incorporation of cells treated with Nickel for 48 hours. There is clear inhibition of proliferation at the longer exposure time and at the higher concentration seen in Figure 3c. There is a noteworthy increase in the first peak, and a clear shift to the left. These distinctions indicate a decrease in cellular proliferation based on EdU incorporation. Again, the control (3a) shows the second peak is the fluorescence of EdU incorporation. Inhibition is better depicted in Figure 4, the overlay of graphs from Figures 3a-c. This overlay further confirms the significance of an exposure time of 48 hours and 300μM concentration. There is a slight shift to the left at the 24-hour exposure, however, cellular proliferation is clearly stalled at the longer exposure time. Any shifts to the left or increase in the first peak verify the lack of EdU

incorporation. No integration means cells are not undergoing synthesis, due to proliferation inhibition.

DETERMINATION OF EDU INCORPORATION OF COBALT TREATMENT

The results of the cobalt treatments revealed a greater effect on cell proliferation than nickel. At 24-hour exposure, the cells treated with cobalt shifted immensely as seen in Figures 5a-c. The histograms of Figure 5a and Figure 5b show cells treated with cobalt shifted to the left, as well as, the first peak increases in size. This clear shift represents cell proliferation inhibition because EdU is not being incorporated due to cells not transitioning through S phase. Further, Figure 5c characterizes the stark contrast at the higher concentration of cobalt exposure. Figure 6 illustrates the overlay of the histograms in Figure 5, demonstrating the inhibition in cell proliferation. The 300μM cobalt treatment is completely void of cells within the second peak. The lack of the second peak shows that the cells did not transition past S phase, therefore no EdU was incorporated during synthesis. Although the cells were treated with the same concentration and exposure times, Cobalt yielded significant results compared to Nickel. Inhibition of cell proliferation occurred quicker, even at the lower dosages.

The most significant inhibition of proliferation is in Figures 7a-c. These graphs depict EdU incorporation of cells treated with Cobalt for 48 hours. The histogram of Figure 7c are cells treated with 300μM of Cobalt for 48 hours, which show a single peak demonstrating that cell proliferation was indeed inhibited. The treated cells did not transition into S phase but were completely fixed in G1. Figure 7b yielded a larger difference than the same concentration at a shorter exposure time (Figure 5b). Further observation of full inhibition is portrayed in the overlay of Figures 7a-c as shown in Figure 8; all cells shifted to the left and are only in the first peak confirming no EdU incorporation. For both hypoxic mimetics, the results showed that cell

inhibition is time and concentration dependent. The longer the exposure time the greater the inhibition, and correspondingly for a higher concentration of each compound. Of the two metals, cobalt inhibited proliferation quicker and at a smaller dose, further, the highest inhibition was seen at the longest exposure time along with the highest dosage.

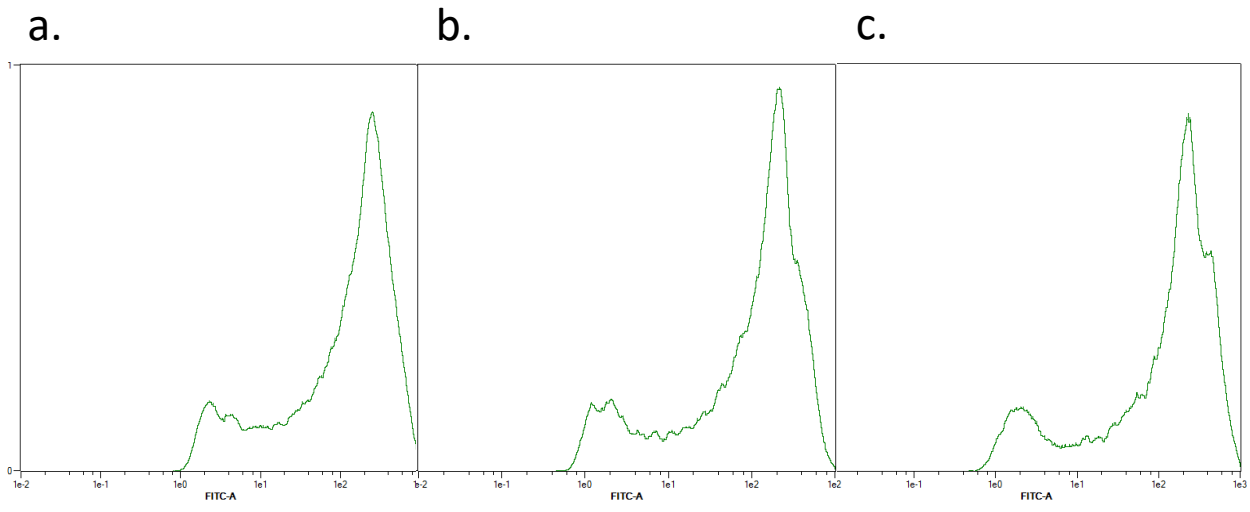

Figure 1: 24-hour Nickel Treatment Results

Figure 1a represents the control with no nickel treatment. All controls were treated with only 1μM of EdU for 16 hours. The next two graphs are both 24-hour treatments of $NiCl_2$. Figure 1b shows the results of cells treated with 100μM $NiCl_2$ and 1μM of EdU. Figure 1c depicts the results of cells treated with 300μM $NiCl_2$ and 1μM EdU.

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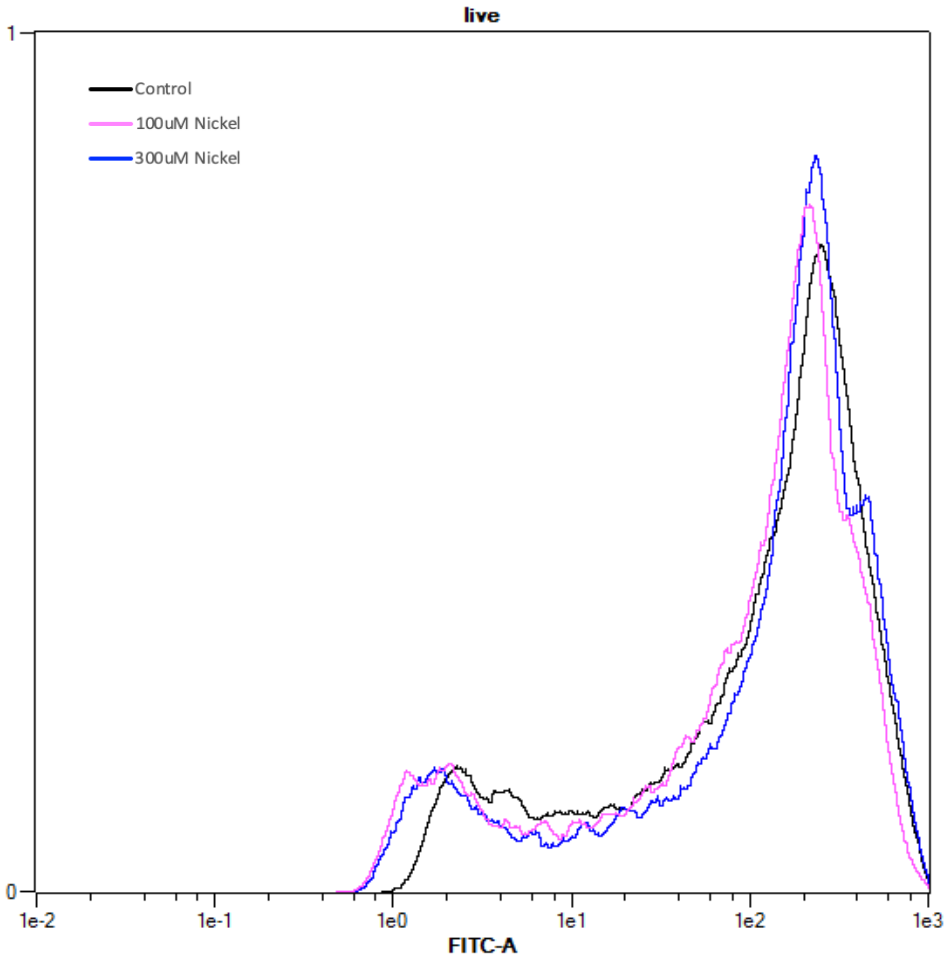

Figure 2: Overlay of 24-hour Nickel Treatments

The control is represented by black. Pink is the 24-hour 100µM Nickel treatment and blue is the 24-hour 300µM Nickel treatment, all were treated with 1µM EdU for 16 hours. The shift to the left is indicative of proliferation inhibition due to more cells stuck in the G1 phase (peak one) as opposed to transitioning through S phase. There is a decrease in the second peak, as further verification of the decrease in proliferation.

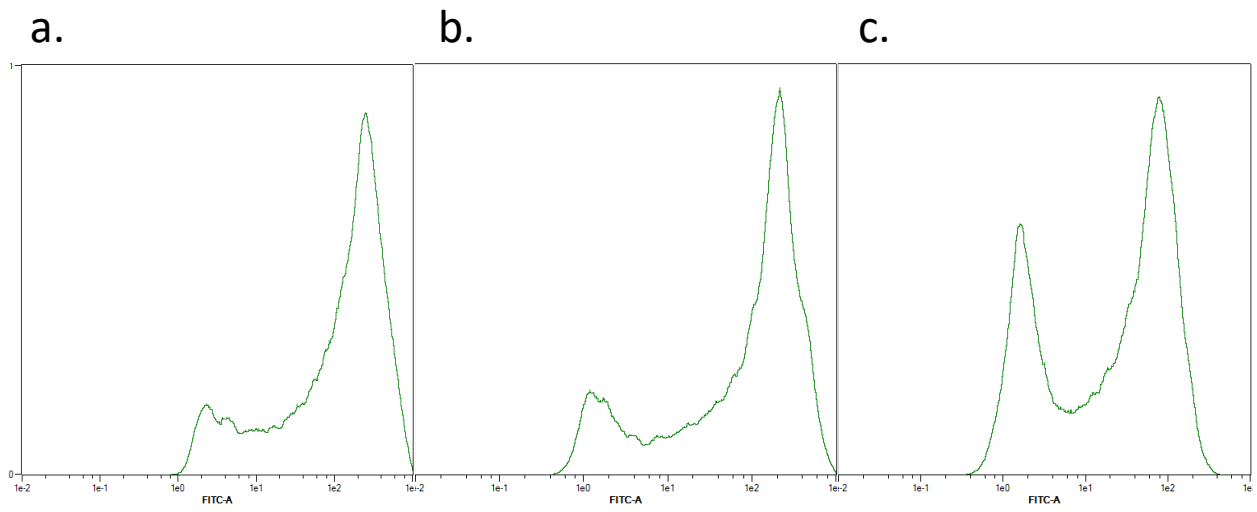

Figure 3: Results for 48-hour Nickel treatment

Figure 3a is the control, which was only treated with 1μM EdU for 16 hours. Figure 3b is the results of cells treated at 48 hours with 100μM $NiCl_2$ and 1μM EdU 16-hour treatment. Figure 3c is the results of the cells treated at 48-hour with 300μM Nickel and 1μM EdU 16-hour treatment, which shows a clear increase in the first peak. Comparing Figures 3a and 3b,, there is a shift to the left, as well as an increase in the first peak, which is indicative of a decrease in cellular proliferation due to cells being stuck in G1 phase. The most significance is seen in 3c. The first peak has a large increase, as well as a shift to the left. This shows proliferation inhibition because EdU is not getting incorporated into the cells because they are not passing through S phase.

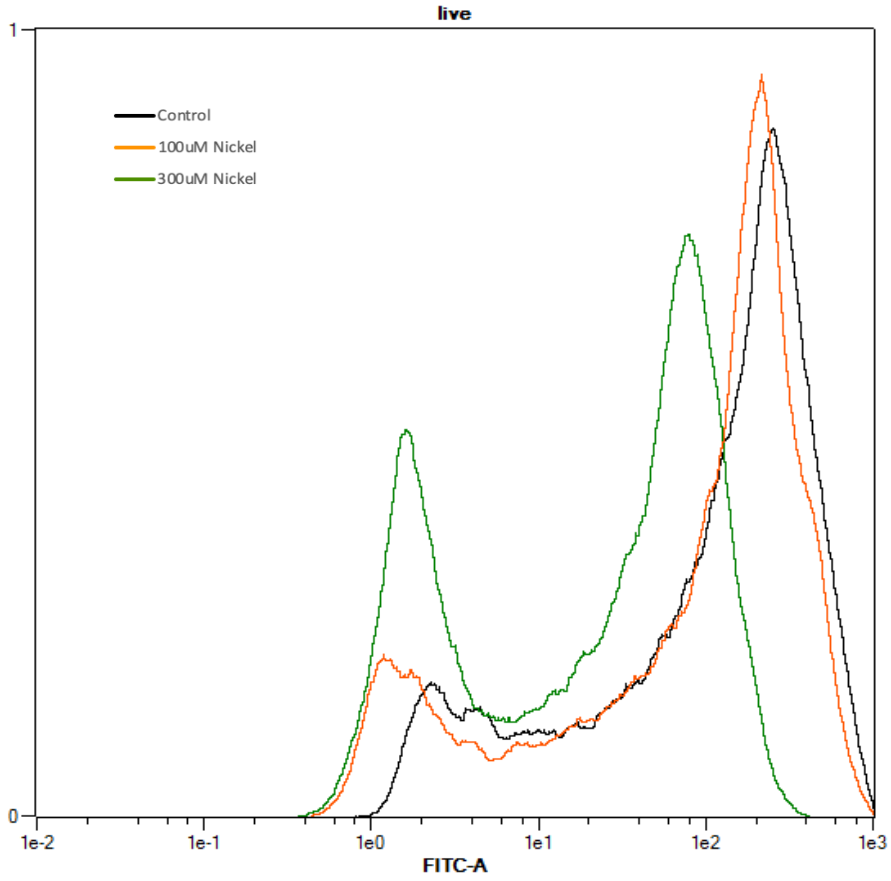

Figure 4: Overlay of 48-hour Nickel treatment

Black represents the control which was treated with 1µM EdU for 16 hours. Orange signifies the cells treated with 100µM Nickel and 1µM EdU at a 48-hour and 16-hour exposure times, respectively. Green denotes the cells treated with 300µM Nickel and 1µM EdU at a 48- and 16-hour intervals, respectively. At 100µM Nickel there is a clear shift to the left, showing more cells are in the first peak. However, at 300µM Nickel there is a large shift to the left and a large increase in the first peak because cells are stuck in the G1 phase and the EdU is not being detected.

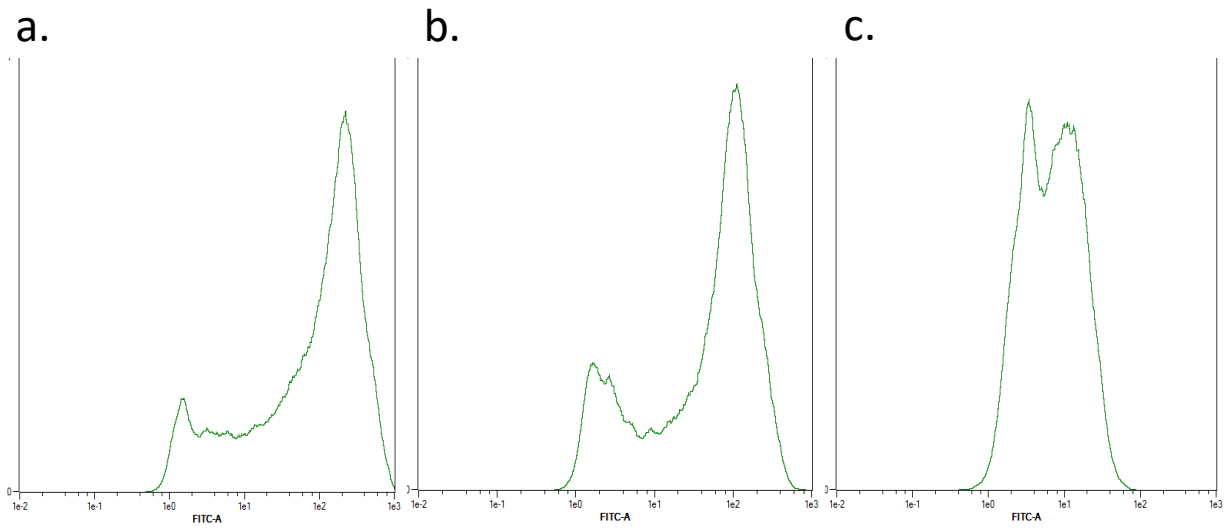

Figure 5: Results of 24-hour Cobalt Treatments

Figure 5a represents the control treated with 1µM EdU for 16 hours. Figure 5b is the 24-hour 100µM Cobalt treatment with 16 hours of 1µM EdU. Lastly, Figure 5c is a 24-hour 300µM Cobalt treatment with 16 hours of 1µM EdU. The first peak in the 5b shows an increase compared to the control. While, 5c shows a clear shift to the left and changes in both peaks. Both are indicative of cell proliferation inhibition because the EdU fluorescence is not being detected, meaning the cells are not transitioning into S phase.

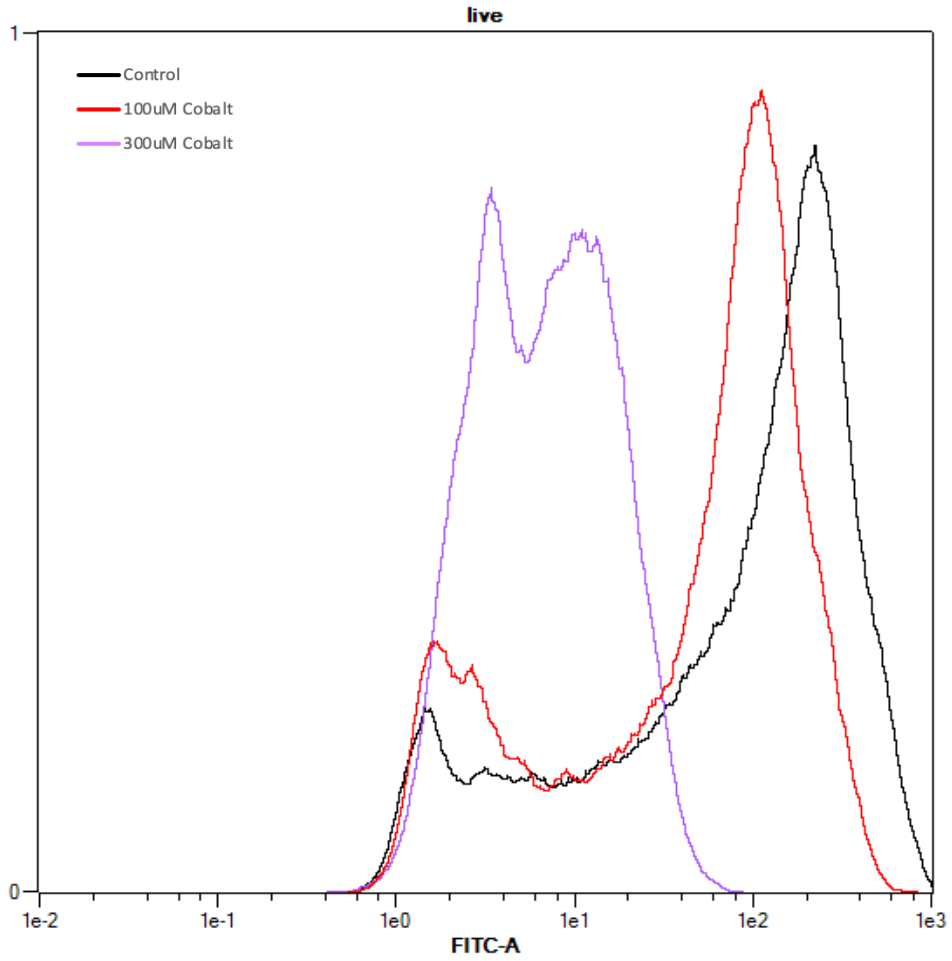

Figure 6: Overlay of 24-hour Cobalt Treatments

Black represents our control; the red indicates the 24-hour 100µM Cobalt with 16-hour 1µM EdU treatment and the purple is the 24-hour 300µM Cobalt with 16-hour 1µM EdU treatment. Looking at the overlay, there is a clear shift to the left in both concentrations. The 300µM cobalt treatment shows a greater effect on the neuroblastoma cells than the 100µM and Nickel treatments. The black line shows where EdU is incorporated, while the purple line shows a complete lack of the second peak, indicating no cells in that area. This confirms that EdU was not incorporated into the DNA because the cells never passed through the S phase.

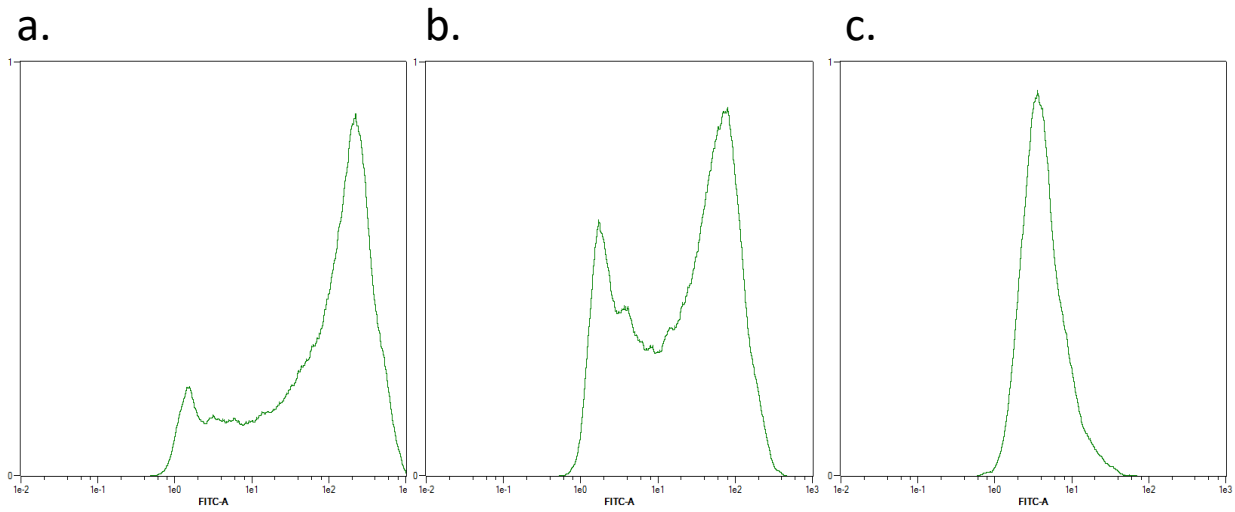

Figure 7: Results of 48-hour Cobalt Treatments

Figure 7a is the control treated with 1µM EdU for 16 hours. Figure 7b represents the results of cells treated with 48-hour 100µM cobalt and 16-hour 1µM EdU. Figure 7c depicts the results of cells treated with 48-hour 300µM cobalt and 16-hour 1µM EdU. The 48-hour 300µM cobalt treatment only has one peak due to cells being fixed in G1 phase. This peak shows that cell proliferation was fully inhibited due to a lack of cells transitioning.

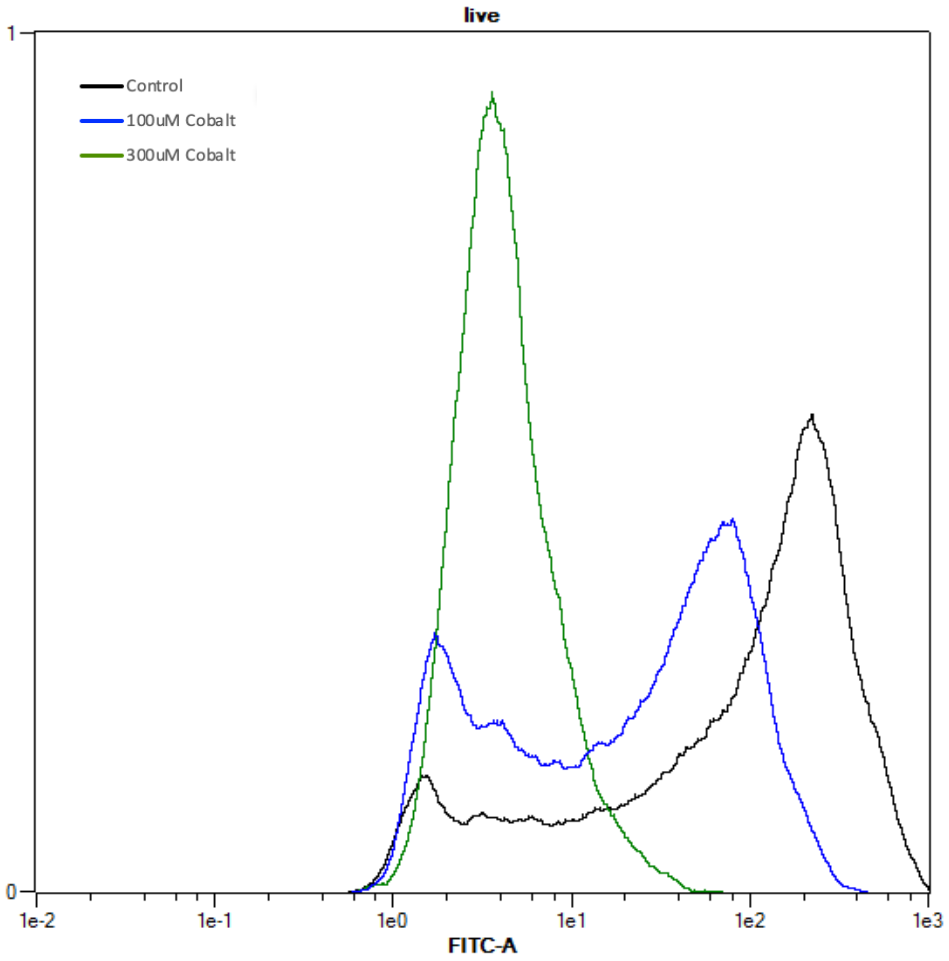

Figure 8: Overlay of 48-hour Cobalt Treatment

The control is signified by black, and blue indicates cells treated at 48 hours with 100µM cobalt and 16-hour 1µM EdU. Green characterizes the cells treated at 48 hours with 300µM cobalt and 1µM EdU. There is a clear shift to the left for both treated cells. Green shifted more than all other treatments, showing that 300µM of cobalt yielded the greatest effect on cell proliferation of neuroblastoma cells. At this exposure time, all cells were prevented from entering S phase.

DISCUSSION

Hypoxia has no conclusive definition, except that it is a condition where oxygen levels are below the requirement for cell survival. Scientists and doctors cannot agree on definitive percentages to claim a tissue or cell is hypoxic due to the varying oxygen levels in diverse tissues (Hockel and Vaupel, 2001). Conversely, scientists agree understanding the effects of hypoxia on cells and the human body is important because of the possibilities to discover benefits to help treat other diseases, such as cancer. Normally, hypoxia leads to a decrease in ATP levels, an increase in oxygen related stress and ultimately, cell death. In this study, human neuroblastoma (NMB) cells were treated with hypoxic mimetic compounds $CoCl_2$ and $NiCl_2$ to create a hypoxic mimetic cell model system. Results showed that nickel and cobalt-induced hypoxic mimic condition can cause cell death which corroborates the cobalt results from "Human Neuroblastoma Adaptation to Cobalt Chloride-Induced Hypoxia" (McAuliffe., 2017). However, there were still surviving cells under these hypoxic mimic conditions. Likely they have developed the adaptive responses. As compared to the non-treated control cells, these surviving cells showed a significant decrease in EdU incorporation as the concentrations and exposure time increased. Although the compounds are similar, the results were significantly incongruent. In fact, the results from nickel treated cells were not as substantial as the cobalt treatments. Both mimetics induced a hypoxic environment through the stabilization and accumulation of HIF-1α, resulting in inhibition of cell proliferation.

In this study, cobalt chloride and nickel chloride were used to induce a hypoxic environment. Nickel showed slight inhibition of proliferation at all concentrations and exposure, however, the longest exposure with the highest dose yielded the most significance. In fact, the 24-hour treatments at both concentrations showed trivial variability. Though there was a slight

shift between Figures 1b and 1c the difference isn't as significant as the longer exposure time. The shift can be better analyzed in the overlay shown in Figure 2. Moreover, the 48-hour 100μM exposure did not show a substantial disparity as displayed in Figures 3a and 3b. The 48-hour 300μM treatment in Figure 3c exhibited significant inhibition with a sizeable increase in the first peak and a large shift to the left. The overlay of Figure 4 are the graphs from Figures 3a-c, and a shift for the 100μM treatment can be seen, while the treatment with 300μM shows a decrease in the second peak and an increase in the first peak. These changes denote inhibition of cell proliferation at the 48-hour exposure for both 100μM and 300μM nickel treatments because EdU was not incorporated as seen in the nonexistent second peak. Conversely, cobalt significantly inhibited cell proliferation at 24-hour 100μM treatment, and amplified inhibition as exposure and concentration increased.

Cobalt yielded greater influence on cell proliferation than nickel, even at the lowest dose and least exposure time. At the shortest exposure, cobalt inhibited proliferation more than nickel at the highest concentration and longest exposure time. The 24-hour 100μM treatment showed a shift and an increase in the first peak, similar to nickel exposure, as presented in Figure 5b, compared to the control in Figure 5a. However, the 24-hour 300μM treatment yielded a complete change, with nearly all the cells shifting out of the second peak as displayed in Figure 5c. The large variations can be fully analyzed in the overlay of Figure 6, where the complete shift of Figure 5c confirms inhibition of cell proliferation. As the exposure and concentration increased, inhibition of proliferation grew greater as well. The 48-hour 100μM treatment shows a severe increase in the first peak and a large shift to the left. The 100μM concentration at both 24-hour (Figure 5b) and 48-hour (Figure 7b) yielded increases in the first peak and shifts, however, the longer exposure produced greater effects. Comparably, the 300μM generated the largest

inhibition at both exposure times, with highest inhibition at 48-hour as seen in Figure 7c. The control shows the second peak is 1e2 to 1e3 on the x-axis, this signifies EdU incorporation. Utilizing this information to further analyze Figure 7c, there is no peak between 1e2 and 1e3, due to the lack of EdU incorporation. All cells are stuck in G1 phase, concluding full inhibition of cell proliferation. To fully analyze the effects of cobalt at 48-hour exposure, the overlay in Figure 8 illuminates the complete shift for 300μM and the large increase of cells stuck in G1 for 100μM.

Cells could not incorporate EdU due to inhibition, preventing the transition into/out of synthesis phase. Possible explanations could be the mechanism behind hypoxia induction, the amount of ROS produced or cobalt toxicity. Littman et al. conducted a study to examine chondrogenesis and compare cobalt induced hypoxia with a cellular environment living in low oxygen levels in human mesenchymal stem cells treated for 24 hours, 4 days and 7 days. From their results, cobalt treated cells resulted in cell death more than cells treated within a hypoxic environment. They theorized cobalt may induce apoptosis pathways, resulting in higher levels of cell death. Comparing these results to the results in this study, cobalt may inhibit cell proliferation more than Nickel because it induces pathways leading to cell death. Further hypothesis includes the different mechanism of prolyl hydroxylase inhibition. Cobalt directly inhibits PHDs, while nickel indirectly inhibits PHDs through blocking iron uptake, and competing against iron dependent enzymes (Chandel et al., 1998; Costa et al., 2005). Comparing and examining the difference between direct and indirect inhibition may further aid in understanding key differences of cobalt and nickel as hypoxic mimetics.

Both metals belong to group VII in the periodic chart, confirming similar chemical properties. Further, each compound produces reactive oxygen species and allows for the

accumulation of HIF-1α. Long term exposure to $NiCl_2$ as well as $CoCl_2$ has shown detrimental effects on the human body (Yao et al., 2014). Extensive exposure to $NiCl_2$ will lead to an accumulation of nickel intracellularly. In a study conducted by Costa et al., at 24 hours of exposure nickel ions reside in the cytoplasm, however, when increased to 72 hours, the ions translocate to the nucleus. In the same study, Cobalt is proven to have harsh consequences on embryonic stem cells. Nickel compounds are water-insoluble and water-soluble. The water-insoluble nickel are readily phagocytized while the water-soluble diffuses through the cell membrane (NTP Toxicology and Carcinogenesis Studies of Ni Sulfate Hexahydrate, 1996). Combining the results from previous studies, we hypothesized that the translocation of nickel will increase the harmful effects on the cell, including cell proliferation. This hypothesis was proven correct when we saw inhibition of proliferation due to prolonged $NiCl_2$ exposure.

Due to the severity and possible detrimental effects, cells have adapted defense mechanisms to fight hypoxia and alleviate oxygen tension. As described by Hockel and Vaupel, pathological causes of hypoxia include low oxygen partial pressure/oxygen tension in arterial blood due to pulmonary diseases or high altitudes (hypoxemic hypoxia), reduced ability of blood to carry oxygen, reduced tissue perfusion, deterioration of the diffusion of geometry and inability of cells to use oxygen due to intoxication caused from poisoning. While the underlying mechanism behind sensing oxygen change is not wholly understood, a family of proteins known as hypoxia inducible factors has an oxygen sensitive subunit to mediate hypoxia. Most notably, HIF-1α is oxygen sensitive, however, it is also important for tumor microenvironment (Diguilio et al., 2018).

Hypoxia-regulated pathways that overlap with oncogenic signaling pathways include cellular proliferation, angiogenesis, metabolism, apoptosis, immortalization, and migration

(Harris, 2002). Further, cellular proliferation induces growth factors that promote cellular proliferation, migration, and regeneration, as well as, transforms growth factor-β and platelet-derived growth factor glioblastoma. Hypoxia induced angiogenesis via growth factors, such as VEGF, can be blocked by inhibitors of oncogene signaling pathways. The tumor microenvironment includes blood vessels, lymphatic vessels, fibroblasts, immune cells and chemicophysical components which leads to restricted oxygen and nutrients. This limited access leads to hypoxic regions which allows for aberrant vascularization which is considered a feature of cancer and promotes cancer progression. Further, hypoxia induces differentiation of non-specific CD4+ T cells into regulatory T cells or T helpers (Petrova et al., 2018). Additionally, Gatenby and colleagues used oxygen electrodes to study tumor oxygen supply; their results showed low oxygen tension in tumors lead to an increase in metastasis and poor survival in patients for squamous tumors of head and neck, cervical and breast cancer.

Under hypoxia, cells switch from glucose metabolism and oxygen-dependent tricarboxylic acid to glycolysis, the oxygen-independent metabolic pathway. Kim et al. provided the first evidence that HIF-1 actively participates in metabolic reprogramming from the TCA cycle to glycolysis by showing that HIF-1 directly targets the gene encoding pyruvate dehydrogenase kinase 1 (PDK1). This led to the inactivation of pyruvate dehydrogenase (PDH), which catalyzes pyruvate into acetyl-CoA. Cancer cells can utilize glycolysis as its primary mechanism of ATP production. The utilization of glycolysis instead of the TCA cycle permits cancer cells to consume less energy. Further, studies show that telomerase increases when cancer cells are within a hypoxic environment, which leads to cellular immortalization (Harris, 2002).

From the analysis of our results, we conclude that the inhibition of cell proliferation is dose-dependent and time-dependent. Paralleling the compounds, we conclude cobalt has a

greater effect on cell proliferation than nickel. For future studies, we can further compare other hypoxic mimetic compounds, such as desferrioxamine (DFO), and its effects on cell proliferation. More experimental analysis can lead to a better understanding of the mechanisms behind cobalt and nickel induced hypoxia, as well as, future possibilities of utilizing these understandings to treat cancer.

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