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DURATION OF HYPOXIA: EFFECTS ON CELL PROLIFERATION AND CELLULAR CHOLESTEROL LEVELS IN HCT-116 COLON CANCER CELLS

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ABSTRACT

Cancer cells are exposed to a harsh microenvironment that is characterized by oxygen and nutrient deprivation. Hypoxic cancer cells are known to accumulate large quantities of lipids, particularly of triglycerides (TGs). Formation of lipid droplets –that contain triglycerides and cholesterol esters– is a hallmark of hypoxic cancer cells and is positively linked to the ability of cells to survive under oxidative stress. It has been shown that cancer cells have deregulated cholesterol metabolism. Some types of cancer cells have been shown to display increased cholesterol uptake, while others display increased de novo cholesterol synthesis. The effect of hypoxia on cholesterol accumulation has not been investigated in detail. A recent study has shown that hypoxia in combination with serum-deprivation induces overall decrease in cholesterol ester levels in colon and lung cancer cell.However, hypoxia (48hrs) alone was not able to induce any changes. It has been speculated that increasing the duration of hypoxia may have some impact on cholesterol accumulation in cancer cells. The presented work aimed to study the impact of varying durations of hypoxia on cholesterol content and cell proliferation rates in cancer cells. It was observed that cholesterol-load wasslightly decreased after 48hours of hypoxia however this difference did not reach statistical significance. After 72 hour of hypoxia the cholesterol-load was same as under normoxic conditions. The cellular lipid-load was also assessed by Oil Red-O (ORO) staining which showed no visible differences between normoxic and hypoxic cells. To assess the underlying molecular mechanism the expression of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) –the rate- limiting enzyme of mevalonate pathway– was also assessed. Again no significant difference was observed in HMGCR expression between normoxic and hypoxic cells. Further studies are required to understand the hypoxic regulation of cholesterol metabolism in cancer cells.

1. INTRODUCTION

Tumour's development, progression and invasion depend greatly on its microenvironment. Nutrient deprivation, hypoxia, pH change and reduced vascularization are different stressful conditions that are potentially able to act as growth limiting factors for tumour cells. Solid tumours typically develop hostile microenvironment characterized by irregular vascularization and pooroxygen and nutrient supply [1]. Proliferation and physiology of cancer cells is largely affected by changes in supply of oxygen and nutrients [2]. Hypoxia is a potent micro-environmental factor promoting aggressive malignancy, and is known for its association with poor survival in a variety of tumor types [3-5]. Cell growth rate, metastasis, neovascularization, and response to treatment arenumber of tumor linked factors that are provoked by hypoxia. HIF-1 α is an important transcriptional factor and regulator of gene products in hypoxia. Oxygen homeostasis is maintainedby hypoxia inducible factors (HIFs) that mediate multiple protective pathways for reducing oxidative mechanism[5].

It has been shown that cancer cells have deregulated cholesterol metabolism. Some types of cancer cells have been shown to display increased cholesterol uptake, while others display increased de novo cholesterol synthesis. The inhibitors of both of these pathways have been shown anti- neoplastic effects. Number of cancer cells have been shown to accumulate greater amounts of cholesterol in comparison to the normal cells [6]. Cellular cholesterol deposits are known to support cancer cells proliferation, migration, and invasion [7]. Multiple studies have shown that hypoxia induces triglyceride accumulation in cancer cells. The effect of hypoxia on cholesterol- load has not been investigated in detail. Normal cellular mechanisms do require cholesterol; however, these requirements are elevated in cancer cells. . This enhanced level of cholesterol in cancer cells associated with their increase proliferation rates. Moreover, cellular proliferation rates were affected by regulation of cholesterol synthesis pathway. Mevalonate derivatives may be distracted towards isoprenoid formation instead of cholesterol synthesis in proliferating cells. This alteration in mevalonate cycle further enhances the protein production. This elevated production of proteins facilitates cholesterol synthesis and its cellular uptake mechanisms. Therefore, cholesteroluptake was increased in proliferating cells due to the alteration in mevalonate derivatives towardthe post-translational modification of protein targets. This modification plays an essential role in the regulation of cellular proliferation.

Cholesterol synthesis pathway is a sub-branch of the mevalonate pathway. Mevalonate pathway is required for the production of several key metabolic end-products. Cholesterol, isoprenoids, dolichol, ubiquinone, and isopentenyladenine are the metabolic-end products of mevalonate pathwya **(Figure 1.1)**. Several research groups have associated cancer cell progression and transformation with the mevalonate pathway [8, 9]. Enzyme 3-hydroxy-3-methylglutaryl-CoAreductase (HMGCR) is the initial step in this pathway which is used in the synthesis of mevalonate.

HMGCR is inhibited in normal cells which triggers a robust homeostatic feedback response that ensures the cells up-regulate and restore the mevalonate pathway [10]. However, a lacking feedback control of HMGCR is reported in number of tumors [11]. Different end-products of the mevalonate pathway have been previously associated with cancer. In transformed cells de novo synthesis is shown to be elevated [8] and cholesterol is reported to be required for the growth of cancer cells [8, 9].

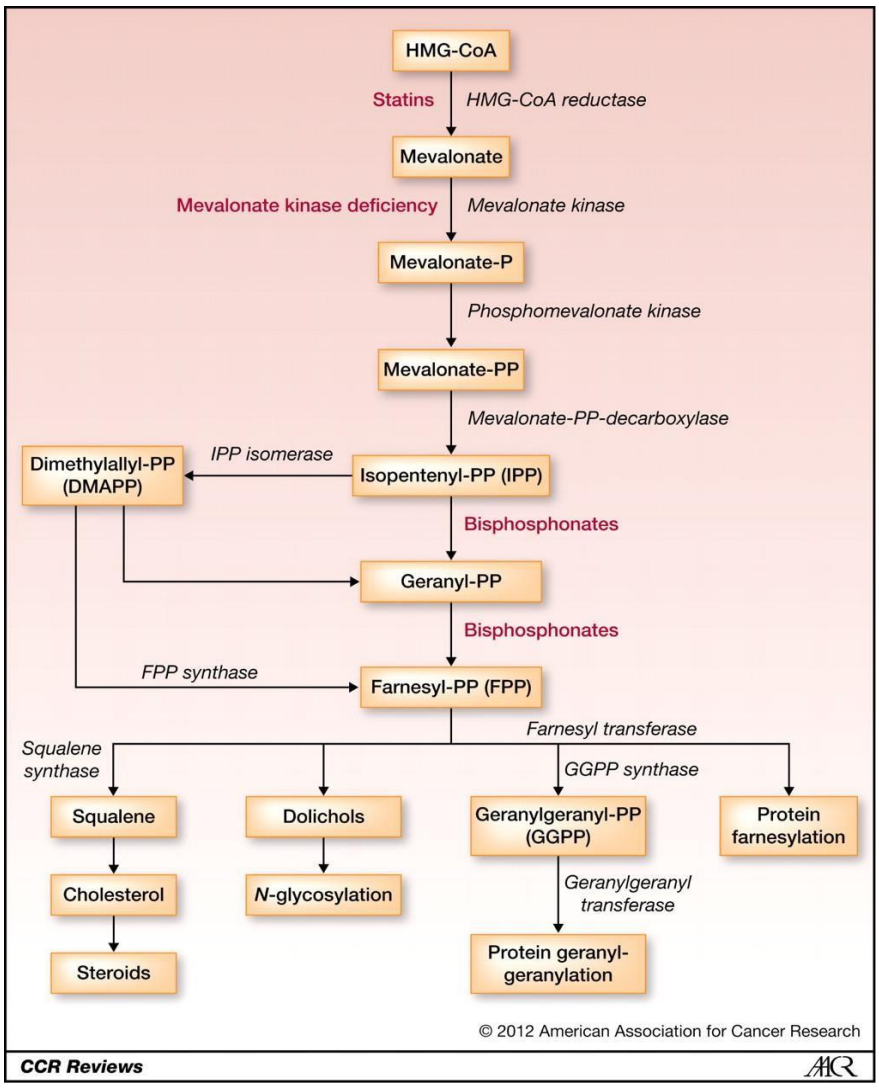

Figure 1: Schematic representation of the Mevalonate pathway. (Courtesy: Martin Thurnher *et al.* DOI: 10.1158/1078-0432.CCR-12-0489)

Hypoxic cancer cells are known to accumulate large quantities of lipids, particularly of triglycerides (TGs) [12]. Formation of lipid droplets –that contain triglycerides and cholesterol esters– is a hallmark for hypoxic cancer cells and is co-related to the capability of cells to persist under oxidative stress [13]. A recent study suggests that inhibition of adipose triglycerides lipase (ATGL)-mediated lipolysis occurs due to hypoxia. Proteomics and functional analysis revealed hypoxia-Inducible Gene 2 (HIG2) is responsible of mediating lipolytic inhibition in hypoxia which act as an inhibitor of ATGL Breakdown of LDs and fatty acid oxidation enhanced by the removalof HIF-2 leads to increased apoptosis and ROS production in hypoxic cancer cells. Meanwhile HIG-2 acts downstream of HIF-1 and promotes the LD accumulation in hypoxic cancer cells.

The effect of hypoxia on cholesterol accumulation has not been investigated in detail. A recent study has shown that hypoxia in combination with serum-deprivation induces overall decrease in cholesterol ester levels in colon and lung cancer cells [14]. However, hypoxia (48hrs) alone was not able to induce any changes. It has been speculated that increasing the duration of hypoxia may have some impact on cholesterol accumulation in cancer cells. Further studies are required to investigate the effect of hypoxia on cellular cholesterol content in cancer cells.

2. MATERIALS & METHODS

2.1 Cell culture

The HCT-116 (Colon Adenocarcinoma cell line) (Appendix-I) was purchased from American Type Culture Collection (ATCC). Cell line was cultures in RPMI 1640 (ATCC, 30-2001) supplemented

with 10% FBS (ATCC, 30-2021) and penicillin-streptomycin solution (ATCC, 30-2300). Cell culture was maintained in the atmosphere of 5% CO_2 and $37C^0$ temperature. This cell line was allowed to grow in $25cm^2$ cell culture flask until it became 85-90% confluent. For cells detachment,tumor cell monolayer was washed with phosphate buffered saline (PBS), appropriate concentration of trypsin enzyme was added to flask and incubated the cells at $37C^0$ for 2-5 min. cells detachment were checked under microscope and the trypsin enzymes were neutralized with complete medium. Cell suspensions centrifuged at 1000 rpm for 5 min. supernatant were removed, tapped the tube andre-suspended cell pellet in 1ml of complete growth medium. Cell count was done by using trypan blue assay. Cell suspension was diluted to desired concentration.

2.2 Induction of hypoxia

2.2.1 Parafilm seal method

Hypoxic conditions were provided to HCT-116 cell plates by using parafilm. All hypoxic plateswere sealed with parafilm. Normoxic plates were not sealed with parafilm. Plates were incubated under appropriate conditions.

2.2.2 Trypan blue assay

Trypan blue dye exclusion assay were used to analyze the growth and viability of HCT-116. Cells were trypsinised and combined with the suspended cells in the culture medium at the specific time-intervals. Centrifuge the culture medium to form cell pellets at 1000 rpm for 5 minutes and resuspended in PBS (500ul). Cell suspension was mixed with equal volume of trypan blue solution. In hemocytometer, stained cell suspension was loaded and counted under microscope. Total number of viable cells per ml of culture was calculated by following formula:

*Number of viable cells per 1ml culture= average number of cells *104*volume of cell suspension*

2.2.3 MTT assay

HCT-116 cells were seeded in 100ul of each dilution in 96-well microtiter plate in triplicate. Medium control was added in triplicate. HCT-116 cells were incubated under appropriate conditions. 10ul MTT reagent was added to each well, including controls. Plates were wrapped with aluminum foil to avoid exposure to light. Plates were placed in an incubator for 2-4 hours. Cells were observed at periodic intervals under an inverted microscope for the presence of intracellular needle-shaped, dark purple colored precipitates. When the purple precipitates are clearly visible under microscope, 100ul of solubilization solution was added to the wells. Plateswere stirred gently on a shaker to enhance dissolution of the crystals. Absorbance was measured at 570nm with the reference wavelength higher than 650nm on a spectrophotometer. Average values were determined from triplicate readings at 570nm and subtract from this value the average value for blank (i.e. medium control) and average value at the reference wavelength.

Specific value= absorbance $_{(570nm)}$ (test)- absorbance$_{(570nm)}$ (blank)- absorbance $_{(>650nm)}$ (test)

Final value was determined by using the following formula:

% viable cells= absorbance$_{(sample)}$ - absorbance$_{(blank)}$ / absorbance$_{(control)}$ -absorbance $_{(blank)}$*100

Absorbance was plot against cell density.

2.2.4 Oil red O staining (ORO)

Lipid accumulation of cell line were determined by Oil red O staining. For ORO staining cellswere grown in both hypoxic and normoxic conditions. Cells were fixed in 10% formalin at room temperature for 15 minutes. Then, formalin was discarded, and fresh formalin was addedfor overnight at room temperature. Next day, formalin was removed, and plates were washed with 60% isopropanol and dried completely. ORO reagent (Himendia Ref TC 256-25G) was added for 10 minutes at room temperature. After removing the ORO plates were washed with distilled water for 4 times. Cells were stained with Mayer's hematoxylin (Himendia Ref TC259-25G) for counterstaining. Then they were washed with the distilled water to get a clearbackground. Lipid droplets appeared as light red spots

in cytoplasm. Cells were imaged by using an inverted microscope fitted with digital camera. Different fields per plate were imagedand analyzed.

2.2.5 Lipid extraction assay

Intracellular lipids of cancer cells were extracted by using a modified Bligh Dyer method (Appendix-V). To a 2ml of Eppendorf tube containing the sample, 700ul magnesium chloride ($MgCl_2$) and 800ul of chloroform: methanol (2:1) were added. Samples were resuspended by vigorous vertexing. The tubes were centrifuged for 2 minutes. At 10000 rpm. Upper layer was removed. Then 500ul magnesium chloride ($MgCl_2$) and 700ul of chloroform: methanol (2:1) were added, vortexed and the tubes was centrifuged. Upper layer was removed and 200ul magnesium chloride ($MgCl_2$) and 400ul of chloroform: methanol (2:1) were added to tubes, vortexed and centrifuged. Nitrogen was passed to evaporate the organic layer completely.

2.2.6 Determination of cellular cholesterol-load

Cellular cholesterol-load in lipid extracts was determined spectrophotometrically by using a commercially available kit (Analyticon Biotechnology AG, 4046). For cellular extraction,200ul of cholesterol reagent was added and incubated for 1 hour at 37°C. At 540nm absorbance was measured. Cellular cholesterol concentration was calculated by comparing the standard curve. Standard cholesterol solutions were made by using HPLC (High-performance liquid chromatography) grade cholesterol standard (Sigma-Aldrich Company) to form standard curve.
Standard curve of different dilutions was prepared by plotting a graph between standarddilutions concentrations on X-axis and OD on Y-axis

2.2.7 Expression analysis

Total RNA was extracted from HCT-116 cells grown under hypoxic and normoxic conditions for 48 hours, using TRIZOL reagent (One step RNA reagent, BIO BASIC INK: BS409A and BS410A, Canada). mRNA levels were determined by reverse transcriptase polymerase chain reaction (RT-PCR) using appropriate primer sets according to the manufacturer's instructions. Total RNA was reverse transcribed into cDNA by using USB® first strand cDNA Synthesis Kit (Product number 75780). Amplification of genes involved in tumor metabolism by PCR in the presence of specific primers was done by using Kit: Taq™ PCR Master Mix, 2X (Product No. 71162). The PCR was performed to analyses the expression of fatty acid synthase (FASN) in normoxic and hypoxic HCT-116 cells. PCR product was analyzed using 1.5% agarose gel electrophoresis using ImageJ software.

2.2.8 Statistical analysis

For statistical analysis *Graph Pad Prism Software (version 6)* was used. Significant relation between two variables was assessed by *unpaired t-test* with 95% confidence intervals. Results were expressed as mean±SD). All the experiments were performed at least three times and showed consistent reproducibility. A p-value of ≤ 0.05 was considered as statistically significant.

3. RESULTS

3.1 Study Strategy

For the presented study HCT116 cells were selected as these cells have been previously reported to store large quantities of lipids[15]. **Figure 3.1** displays study-plan for the presented work. HCT116 cells were seeded for initial 24 hours under normoxic conditions (see materials and methods for details). After that hypoxia was induced. After 48 hours of hypoxia induction yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed to assess cell proliferation rates. From separate wells of the culture plate cells were trypsinized and pelleted *via* centrifugation. Three cell pellets were used to extract protein and three were used for lipid extraction. The protein extracts were subjected to protein estimation while lipid extracts were further processed and total cholesterol content in these samples was assessed using standardprotocols (see materials and methods for details). The same protocol was repeated at 72hours of hypoxia induction.

The data was analysed and results were obtained (see below). Each experiment was repeated at least 3 times.

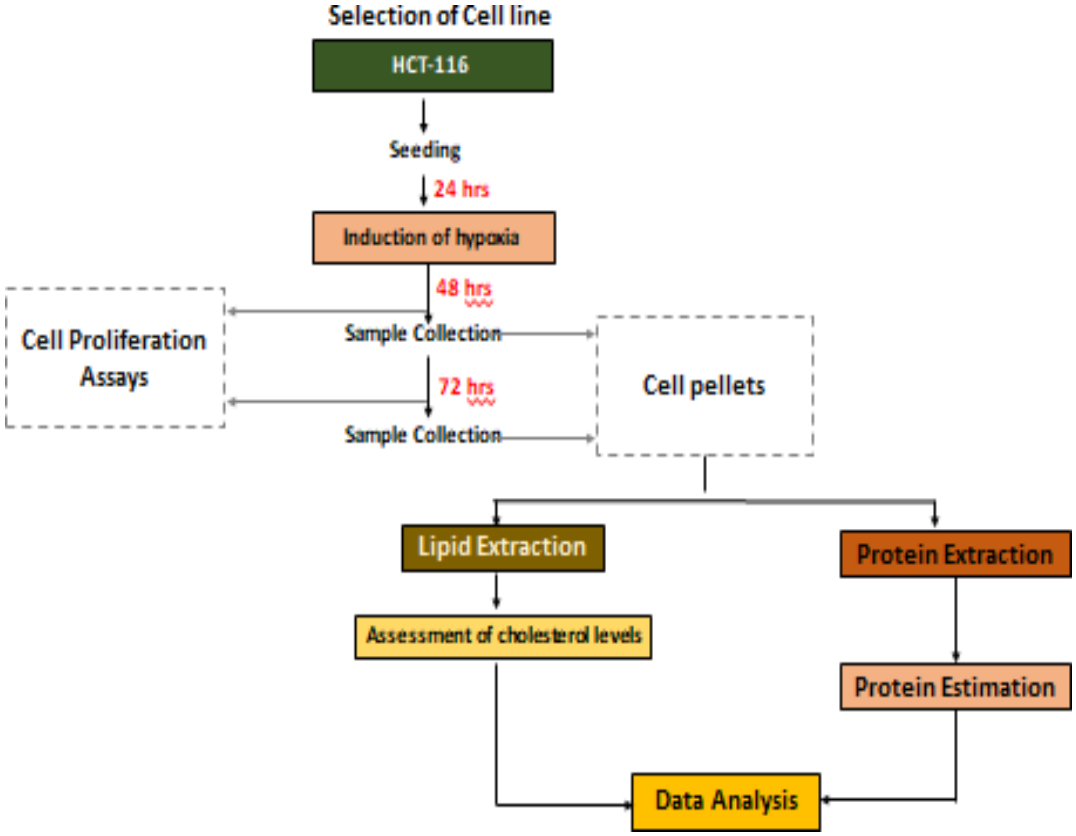

Figure 3.1: Study-Design.

HCT-116 cells were seeded and cultivated in normoxic conditions. After 24 hours, cell culture plates were sealed with paraffin film for the induction of hypoxia. Cell proliferation assays were performed at 48 and 72hr time points. From the separate wells cells were trypsinized and pelleted for further processing. Lipids wereextracted from cell pellets using a modified Bligh and Dyer method after 48 and 72 hours incubation. Total cholesterol content in the lipid extracts was spectrophotometrically determined using commercially available kit. For total protein quantification Bradford assay was used. Graph Pad Prism Software was used for statistical analysis.

3.2 Effect of different durations of hypoxia on cell proliferation rates in HCT-116 cells

MTT assay was performed to assess cell viability under normoxic and hypoxic conditions. As shown in **figure 3.2(a)** the cell proliferation rates were decreased under hypoxic condition in comparison to normoxic conditions. MTT assay is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored (dark purple) formazan product. The cells are then solubilized withan organic solvent (eg. Isopropanol) and the released, solubilised Formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells. For the presented work cancer cells were under the hypoxic stress that affects metabolic activity of cancer cells. Therefore, MTT assay mightnot be the most appropriate method for determining cell proliferation rates.

Therefore cell viability was also assessed via trypan blue assay **(Figure 3.2b)**. Again, it was observed that cell proliferation rates were significantly reduced under hypoxia (48 hrs) in comparison to normoxia. However, the number of viable cells after 72 hours of hypoxia was higherthan that of 48 hours of hypoxia. It shows that cells were still proliferating under hypoxic conditions but proliferation rates were significantly reduced in comparison to normoxic condition.

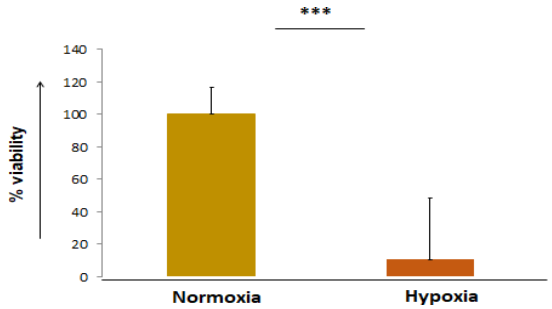

Figure 3.2(a): Comparison of cell proliferation in normal oxygen verses hypoxic conditions using MTT assay. Significance was determined by unpaired t test. * Significance difference (* $p < 0.05$; **$p < 0.01$; ***$p \leq 0{,}001$).

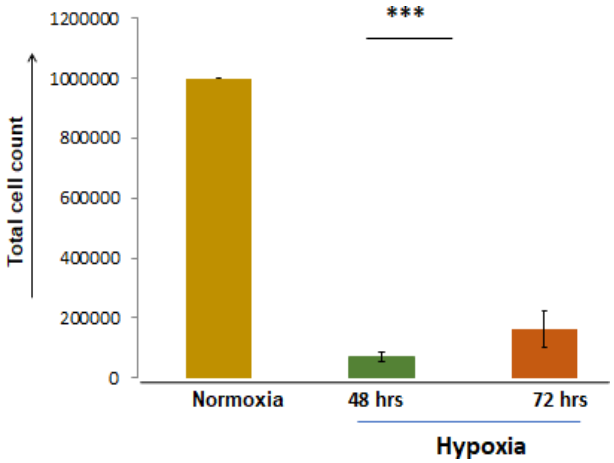

Figure 3.2(b): Comparison of cell proliferation in normoxic verses hypoxic conditions using trypan blue assay. Significance was determined by one-way ANOVA. * Significant difference (* $p < 0.05$; **$p < 0.01$; ***$p \leq 0{,}001$)

3.3 Effect of different durations of hypoxia on cholesterol-load in HCT-116 cells

Next, the impact of hypoxia on cholesterol-deposits was determined. As shown in **figure 3.3** the cholesterol-load was slightly decreased after 48hours of hypoxia however this difference did not reach statistical significance. After 72 hour of hypoxia the cholesterol-load was same as under normoxic conditions. The cellular lipid-load was also assessed by Oil Red-O (ORO) staining. **Figure 3.4 (a)** displays micrographs of ORO-stained HCT-116 cells under normoxic and hypoxic cancer cells. No visible differences were noted in these cells. After that lipids were extracted from these cells and optical density (OD, 490nm) was measured to assess ORO content in the lipid extracts. Once again no significant differences were noted between hypoxic and normoxic cells **Figure 3.4 (b)**.

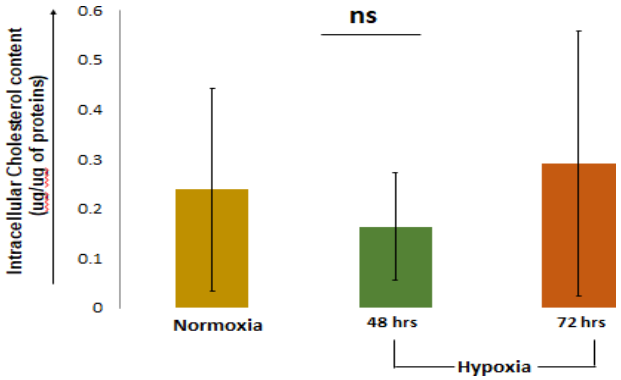

Figure 3.3: Comparison of total cholesterol content of cancer cells in normal oxygen vs hypoxic conditions at 48 and 72 hour time points. Significance was determined by one-way ANOVA. * Significant difference (* $p < 0.05$; **$p < 0.01$; ***$p \leq 0.001$, ns not significant ($p > 0.05$).

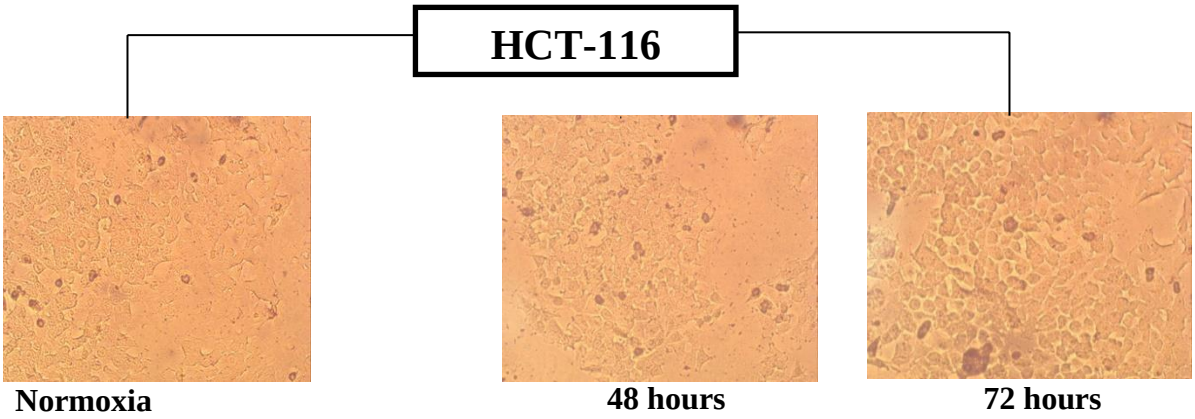

Fig 3.4(a): Oil Red O staining for lipid accumulation in HCT-116 cell line in normal and hypoxic conditions. Cells were examined by using inverted microscope and images were taken by inverted-phase microscope at 20X magnifications with the help of digital camera.

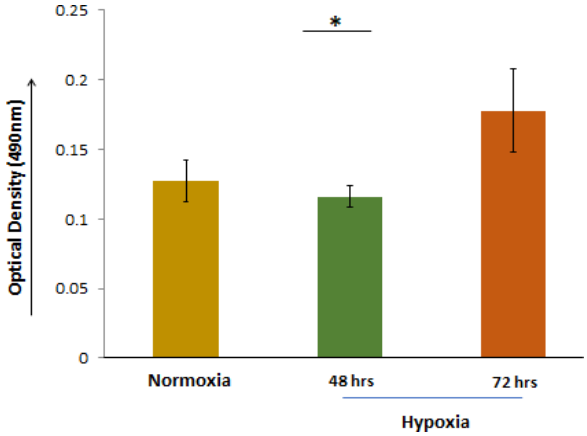

Figure 3.4(b): Comparison of lipid accumulation in normal oxygen verses hypoxic condition using Oil Red-Ostaining. Significance was determined by unpaired t test. * Significant difference (* $p < 0.05$; ** $p < 0.01$; *** $p \leq 0{,}001$).

3.4 Expression of genes involved in lipid metabolism under hypoxia

As described above the cholesterol-load was slightly decreased after 48hours of hypoxia, while after 72 hourof hypoxia the cholesterol-load was same as under normoxic conditions. To assess the underlying molecular mechanism the expression of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) –the rate- limiting enzyme of mevalonate pathway– was assessed. No significant difference was observed in HMGCR expression between normoxic and hypoxic cells (**Figure 3.5**).

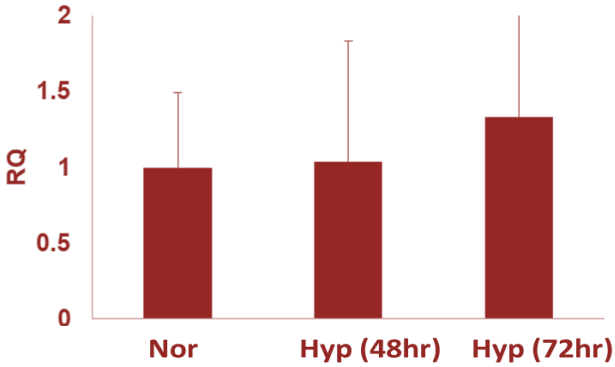

Figure 3.5. Bar-graphs representing the HMGCR gene expression by quantitative RT-PCR in Normoxic and Hypoxic cancer cells. The results are calculated as RQ values. Bars represent mean ± SD. The results were statistically analyzed by Kruskal-Wallis test ($p < 0.0001$).

4 DISCUSSION

Proliferation and physiology of cancer cells is largely affected by changes in supply of oxygen and nutrients [2]. Cancer cells within in vivo tumours are often exposed to fluctuations in these two factors. Hypoxia –oxygen deprivation– affects a number of tumours associated factors e.g.cell growth rate, metastasis, neovascularization, and response to treatment. In addition to that hypoxia is also known to affect lipid metabolism pathways in cancer cells. Hypoxic cancer cellsare also known to accumulate large quantities of lipids, particularly of triglycerides (TGs) [12]. The effect of hypoxia on cholesterol accumulation has not been investigated in detail. A recent study has shown that hypoxia in combination with serum-deprivation induces overall decrease in cholesterol ester levels in colon and lung cancer cells [14]. However, hypoxia (48hrs) alone was not able to induce any changes It has been speculated that increasing the duration of hypoxia may have some impact on cholesterol accumulation in cancer cells. The presentedwork aimed to study the impact of varying durations of hypoxia on cell proliferation rates cholesterol content in cancer cells.

For the presented study HCT116 cells were selected as these cells have been previouslyreported to store large quantities of lipids[15]. Hypoxia was applied for two different time- durations –48 hours and 72 hours. It was observed that cell proliferation rates were significantly reduced under hypoxia (48 hrs) in comparison to normoxia. However, the number of viable cells after 72 hours of hypoxia was higher than that of 48 hours of hypoxia. It shows that cells were still proliferating under hypoxic conditions but proliferation rates were significantly reduced in comparison to normoxic condition. Next, the impact of hypoxia on cholesterol-deposits was determined. It was observed that cholesterol-load was slightly decreased after 48hours of hypoxia however this difference did not reach statistical significance. After 72 hour of hypoxia the cholesterol-load was same as under normoxicconditions. A recently published work has also shown that total cholesterol ester load issignificantly reduced when hypoxia is induced in combination with serum-derivation [14]. However, hypoxia under normal growth conditions (full serum) did have any impact on cholesterol load in cancer cells. . Hence, it is possible that under low-serum conditions cancer cells depend on previously stored LD content for the release of free fatty acids. That causes decrease in cholesterol ester levels. It has been previously reported that under low-cholesterol media cancer cells mobilize their stored cholesterol ester [16]. Cancer cells are shown to consume fatty acid (FA) through fatty acid β-oxidation (FAO) that is considered as the dominant bioenergetic pathway in non-glycolytic tumors. It has been shown that the dependence of cancer cells on FAO is further heightened in nutrient-deprived conditions [17].

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