Screening of Chemotherapeutic Drugs Against Colorectal Cancer Based on Different Oxygen Environments in Vitro

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Abstract. With the deepening of tumor research, the concept of the tumor microenvironment has gradually come into people's view. A significant characteristic of the microenvironment is hypoxic. A large number of vitro experiments have confirmed that hypoxic environments can induce angiogenesis, anti-apoptosis, changes in drug sensitivity, and tumor metastasis. Now, chemosensitivity tests are all carried out under normoxic conditions. Therefore, some scholars have pointed out that if it is possible to simulate the hypoxic state of the tumor microenvironment during the chemosensitivity tests in vitro, it may be able to match better the effect of chemotherapeutic drugs on killing tumor cells in vivo. This study clarified the differences in the chemosensitivity for colorectal cancer cells in normoxic and hypoxic environments. The result of CCK-8 detections has shown that the toxicity of drugs to cells is much higher under normoxic conditions. To further figure out the different mechanisms of the chemotherapy drugs under normoxic and hypoxic conditions, we conducted cellular uptake analysis and western blot, which demonstrated that the uptake of Doxorubicin in normoxic cells was significantly higher than that in hypoxic cells. While the result of the western blot has shown that the expression of VDAC (Voltage-dependent anion channel) and cleaved caspase-3 is higher under normoxic conditions. This study provides a specific basis for future research on the chemosensitivity of chemotherapy drugs in hypoxic environments.

Keywords: Colorectal cancer; Hypoxia; Chemosensitivity; Doxorubicin.

1. Literature Review

1.1 Introduction of cancer

Cancer is a major disease plaguing human health. With the aging of the global society and the deterioration of the ecological environment, the incidence and mortality of malignant tumors show an increasing trend year by year. In 2019, annual cancer cases grew by 23.6 million people and 10 million deaths worldwide, representing increases of 26% and 21% over the previous decade, respectively. By 2040, the total number of cancer patients will reach 27.5 million and cause 16.3 million death worldwide, according to the AACR Cancer Progress Report 2020. The most common cancers are breast, lung cancer, colon, and prostate cancer. Among them, Colorectal cancer (CRC) is the third most common cause of cancer-related death worldwide.

Malignant tumors are difficult to cure because of their special microenvironment, including hypoxia, high osmotic pressure, and dense extracellular matrix. Patients with CRC usually die of metastatic lesions. And researchers have found that hypoxic colorectal cancer cells can enhance the invasion and migration ability of normoxic colorectal cancer cells in vitro and lung metastasis potential in vivo through the paracrine pathway. Traditional treatment methods include surgery, chemotherapy, radiotherapy, and immunotherapy. Among these methods, chemotherapy is the most widely used and researched treatment. However, traditional chemotherapeutic drugs have low tumor selectivity, resulting in highly toxic and other side effects, which seriously limits their clinical application. In addition, hypoxic areas in solid tumors have been found to induce tumor resistance to chemotherapeutic drugs, further reducing the effectiveness of chemotherapy.

1.2 The concept and the causes of hypoxia

Sixty years ago, Thomlinson R.H. and Gray L.H. et al. hypothesized the existence of the hypoxic environment in tumors based on the observation of the distribution of necrosis and vessels, and they confirmed that chronic hypoxia and necrosis had been demonstrated at approximately 100-200 μm from the blood vessels in patients with lung cancer. In the following decades, the study of hypoxia in tumor tissue has been one of the hot spots in tumor research.

The cause of intratumoral hypoxia is that solid tumors (such as lung cancer, liver cancer, etc.) often grow out of control, and the rate of angiogenesis is relatively lagged, resulting in some tumor cells far away from blood vessels,

beyond the range of oxygen diffusion. Studies have shown that hypoxic cells are present in almost all solid tumors.

The normal cellular vascular system is well organized, and only the blood vessels that are sufficiently close to the cells can ensure an adequate supply of all nutrients and oxygen. Vessels in tumors are chaotic, dilated, and tortuous, usually far apart from each other and with slow blood flow, while hypoxic and necrotic areas are often far away from the vessels. In addition to these chronic hypoxic areas, acute hypoxic areas may occur in tumors due to temporary closure or reduced flow in certain blood vessels. Therefore, blood vessels provide oxygen and nutrients unevenly, leading to the phenomenon of hypoxia.

1.3 The result of hypoxia

The hypoxic environment is a double-edged sword: on the one hand, it leads to apoptosis or necrosis directly; on the other hand, it increases anaerobic glycolysis, genetic instability, angiogenesis, and other adaptive changes by activating the transcription of genes related to metabolism, protein synthesis, etc. Hypoxic tumor cells are significantly less sensitive to radiotherapy and are also resistant to chemotherapy due to changes in the tumor microenvironment caused by hypoxia, drug delivery, etc.

In recent years, a number of studies have shown that tumor hypoxic microenvironment can increase the ability of malignant transformation and metastasis of tumor cells and may be the main factor inducing the increase of drug resistance of tumor cells.

At the same time, these studies suggest that the adaptation of tumor cells to hypoxic environments is related to the expression of specific genes and proteins in hypoxic regions. For instance, the high expression of HIF-1α (Hypoxia-inducible factor-1α) will lead to the expression of downstream signaling factors such as angiogenic factors, thereby inducing angiogenesis, leading to increased tolerance to external treatment and thus reducing the effect of traditional treatment.

1.4 The necessity of screening chemotherapy drugs under hypoxia

To improve the prognosis of patients, academia put forward the individualized treatment of tumors. At present, individualized treatment for tumors mainly relies on in vitro chemosensitivity tests. Although the chemosensitivity test has certain effects, its consistency with the actual clinical situation still needs to be improved. Some scholars have pointed out that the inconformity between the in vitro test and the clinical situation may relate to the failure to simulate the tumor microenvironment. The tumor microenvironment is the internal environment in which tumor cells grow. Its physical and chemical properties, cell components, etc. can regulate the chemosensitivity of tumor cells. Hypoxia is one of the major physical and chemical properties of the tumor microenvironment. Many in vitro experiments have confirmed that hypoxic environments induce tumor angiogenesis, anti-apoptosis, drug resistance, and tumor metastasis. Kumar et al. in 2022 further confirmed that the regulation pathways and the response to chemotherapy drugs of tumor cells showed an obvious difference in the hypoxic and normoxic environment. And characteristics such as the metabolic state of cells under hypoxic treatment are more similar to tumor cells in the body. Thus, screening drugs under hypoxic conditions may be more effective.

2. Materials and Methods

2.1 Materials

2.1.1 Doxorubicin (Dox)

Doxorubicin is a type of chemotherapy drug called anthracycline and is a treatment for many different types of cancer. Doxorubicin is also known as Adriamycin.

Doxorubicin slows or stops the growth of cancer cells by blocking an enzyme called topo isomerase 2, which cancer cells need to divide and grow.

2.1.2 Fluorouracil (5-Fu)

Fluorouracil, also known as FU or 5FU, is one of the most commonly used drugs to treat cancer. It is most often used in combination with other cancer drugs to treat many types of cancer, including breast, head and neck cancer, anal cancer, stomach cancer, colon cancer, and some skin cancers.

Fluorouracil is part of a group of chemotherapy drugs known as anti-metabolites. Anti-metabolites are similar to normal body molecules, but they have slightly different structures. These differences mean that anti metabolites stop cancer cells from working properly. They stop the cells from making and repairing DNA. Cancer cells need to make and repair DNA to grow and multiply.

2.1.3 Cisplatin (Pt)

Cisplatin （in the platinum-based antineoplastic family of medications） is a chemotherapy medication used to treat a number of cancers. It works in part by binding to DNA and inhibiting its replication.

Cisplatin crosslinks DNA in several different ways, interfering with cell division by mitosis. The damaged DNA elicits DNA repair mechanisms, which in turn activate apoptosis when repair proves impossible. In 2008, researchers showed that the apoptosis induced by cisplatin on human colon cancer cells depends on the mitochondrial serine-protease Omi/Htra2.

2.1.4 MC38 Cell

MC38 is a murine colon adenocarcinoma cell line from a C57BL/6 mouse. The cells are adherent and have a fibroblast morphology.

2.1.5 4T1 Cell

4T1 is a murine mammary carcinoma cell line from a BALB/cfC3H mouse. The cells are adherent and have an epithelial morphology.

2.2 Methods

2.2.1 Thawing of Cells

Quickly thaw the cryovial in a 37 ºC water bath until the last sliver of ice melts. Pipette the solution into a sterile tube (15 ml) and add more than 10 times cell culture media (DMEM medium + 1% PS + 10% FBS). Centrifugation (1000 rpm, 3 min). Discard the supernatant, add culture media to resuspend the cells, and count and adjust the cell density. Inoculate cell suspension in a petri dish and place it in the 37 ºC incubator. Change the growth medium the day after seeding.

2.2.2 Subculture

Subculture the cells when they are 60%-80% confluent. First, aspirate culture medium and wash cells 2 × with 4 ml PBS. Then, Cover the cells with 1 ml of Trypsin/EDTA solution. Examine the cell layer microscopically. Allow the trypsinization to continue until approximately 90% of the cells are rounded up. Add 6 ml of complete medium and aspirate cells by gently pipetting. Collect the solution in a 15 ml centrifuge tube. Centrifugation (1000 rpm, 3 min). Resuspend the cells in a complete culture medium and add them to a new flask.

2.2.3 Cell Counting Kit-8

WST-8[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] produces a water-soluble formazan dye upon reduction in the presence of an electron mediator. CCK-8, being nonradioactive, allows sensitive colorimetric assays for the determination of the number of viable cells in cell proliferation and cytotoxicity assays. WST-8 is reduced by dehydrogenases in cells to give an orange-colored product(formazan), which is soluble in the tissue culture medium. The amount of the formazan dye generated by dehydrogenases in cells is directly proportional to the number of living cells.
Inoculate cell suspension (100 μl/well, 6000 cells/well) in a 96-well plate. Pre-incubate the plate in a humidified incubator (at 37 ºC, 5% CO2) overnight. Treated cells with Doxorubicin (Dox, at 21% and 0.1% O2), Fluorouracil (5-Fu, at 21% O2), and Cisplatin (Pt, at 21% O2) in different concentrations, which was shown in the following table.

Table 1. The drug concentration (μM)

Dox	12.5	6.25	3.125	1.5625	0.78	0.39	0.19	0.08
5-Fu	1920	960	480	240	120	60	30	15
Pt	62.5	31.25	15.625	7.8125	3.90625	1.95	0.97	0.48

After 24 hours, add 10 μl of the CCK-8 solution to each well of the plate. Incubating the plate for 1-4 hours in the incubator. An ELISA microplate reader was used to read the absorbance of the CCK-8 solution at 450 nm (excitation)/600 nm (emission).

2.2.4 Cellular uptake analysis——Confocal

The MC38 cells (1 ml/plate, 80000 cells/ml) were cultured in DMEM medium supplemented with 10% (v/v) FBS and maintained at 37 °C in a humidified environment with 5% CO2. Cells were incubated with Doxorubicin (0.08 μM) for 2 h and 6 h, respectively. Then the cells were washed and labeled with Hoechst 33342 and observed by a single photon laser confocal imaging system (Zeiss 710) and pictures were acquired by using ZEN 2.6 blue edition software.

2.2.5 Western Blot

2.2.5.1 Extract protein and measure its concentration

Incubate MC38 cells in six-well plate（80000 cells/well）for 24 hours in different oxygen concentrations(21% and 0.1%). Treat the cells with Doxorubicin (0.08 μM) for 24 hours. Wash each well with PBS three times and add RIPA Buffer (100 μl/well). Then scrape the plate and collect the solution in a centrifuge tube. Centrifugation (10000 g, 10 min). Collect the supernatant.
Prepare the BCA working reagent by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A: B). Pipette 10 μl supernatant and 120 μl working reagent into a 96-well plate. Incubate for 15 min. Measure the absorbance at 562 nm using a microplate reader. RIPA Buffer was added to adjust the concentration of all samples to the same. Then add 6×loading buffer (95 °C, 10 min).

2.2.5.2 SDS-polyacrylamide gel electrophoresis

Makeup separation glue and concentrated glue. The formula is shown in the following table.

Table 2. The formula of the glue

	Separation glue (12%)	Concentrated glue (5%)
ddH2O	1.6 ml	1.4 ml
30% Acrylamide(4℃ keep in dark)	2.0 ml	0.33 ml
1.5MTris-HCl(pH8.8)	1.3 ml	-------
0.5MTris-HCl(pH6.8)	-------	0.25 ml
10% SDS	50 μl	20 μl
10% APS	50 μl	20 μl
TEMED	2 μl	2 μl

Pull out the sample comb carefully after polymerization. Samples are loaded into wells in the gel. One lane is usually reserved for a marker, which is a commercially available mixture of proteins of known molecular weights, typically stained so as to form visible, colored bands. Applied a voltage at 80 V，30 min, then 120 V，45 min.

2.2.5.3 Electroblotting

Electroblotting uses an electric current to pull the negatively charged proteins from the gel towards the positively charged anode and into the PVDF.
Pretreat PVDF membrane with methanol for 1 min. Trim off the concentrated glue. The placement order is anode core(black), sponge pad, filter paper, membrane, gel, filter paper, sponge pad, and cathode core(white). Remove any bubbles with the Blotting Roller. Start transfer at 250 mA for 1.5 h.
Use TBST buffer to prepare a 5% skim milk powder blocking solution （10 ml）, blocking under room temperature for 1-1.5 h. Washed the membrane with TBST for 15min+5min+5min. Diluted primary antibody in TBST wash buffer, incubated with the membrane under gentle agitation overnight at 4 °C. Following incubation, the membrane is washed several times (15 min+5 min+5 min+5 min) in wash buffer to remove unbound primary antibody and thereby minimize background. After rinsing the membrane, expose it to the secondary antibody (1:3000), and incubate it under gentle agitation for 1 hour. Wash the membrane with TBST for 15 min+5 min+5 min+5 min.

2.2.5.4 Detection

Chemiluminescent detection methods depend on the incubation of the western blot with a substrate that will luminesce when exposed to the reporter on the secondary antibody. The light is then detected by CCD cameras which capture a digital image of the western blot or photographic film.

3. Results

MC38 cells were incubated overnight under normoxic conditions and treated with three different chemotherapeutic agents for 24 hours. Add CCK-8 to detect the survival rate. Survival rate = （OD experimental group/OD control group）×100% use GraphPad Prism 9.4.0 to create the graph.

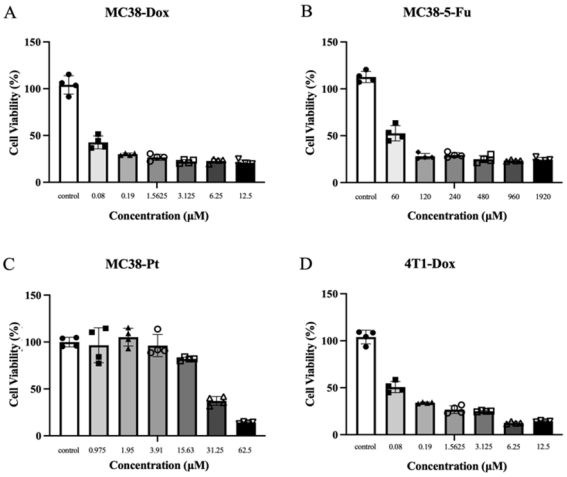

Fig. 1. The cell viability of cancer cells after different chemotherapeutic under 21% O_2 for 24 h **a.**MC38 cells were treated with Doxorubicin. **b.**MC38 cells were treated with Fluorouracil. **c.**MC38 cells were treated with Cisplatin. **d.** 4T1 cells were treated with Doxorubicin

Under the normoxic condition, the semi-inhibitory concentration (IC50) of the three different chemotherapeutics: Dox, 5-Fu, and Pt on MC38 cells was about 0.0069 μM, 60 μM, and 26.73 μM, respectively (Fig. 1a, b, c). This suggests that MC38 cells are most sensitive to Doxorubicin. Replacing MC38 cells with 4T1 cells (whose IC50 is 0.057 μM), it was found that they were also very sensitive to Doxorubicin (Fig.1 d).

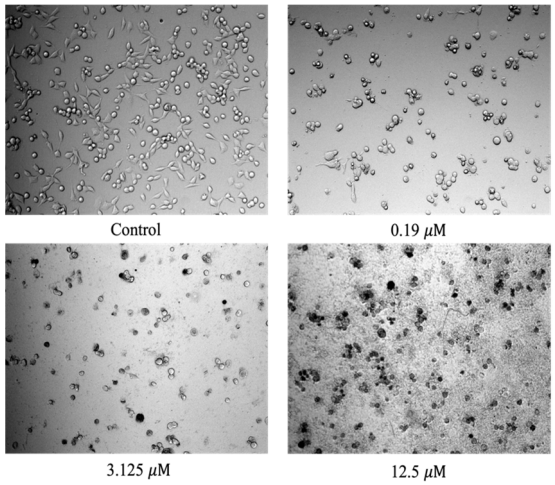

Fig. 2. MC38 cells treated with different concentrations of Doxorubicin（24 h）

MC38 cells treated with Doxorubicin were observed under an inverted fluorescence microscope. At a lower concentration of Doxorubicin （0.19 μM） , the cell density decreased, and the cell morphology changed from spindle to round. At a higher concentration（3.125 μM and 12.5 μM） , only a few cells remained adherent, and most of them were ruptured.

To understand how the drug acts on and kills cells, MC38 cells were cultured overnight in Confocal dishes, treated with Dox for 2 and 6 hours, respectively, and observed under a confocal microscope.

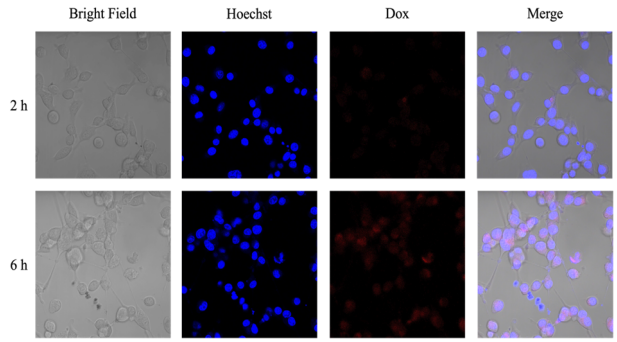

Fig. 3. MC38 cells treated with Dox for 2 h and 6 h under 21% O_2

Under the confocal microscope, most of the cells were still spindle-shaped after two hours of drug treatment. The red fluorescence of Dox was very weak, and the drug was mainly concentrated in the cytoplasm. After six hours of treatment, most of the cells were round, and the red fluorescence was significantly stronger. The coincidence of red fluorescence (Dox) and blue fluorescence (nucleus) indicates that the drug enters the nucleus and functions.

Under normoxic conditions, cancer cells are very sensitive to the drug. However, tumor cells are often in a hypoxic environment in the human body. Therefore, in order to better simulate the sensitivity of tumor cells to Doxorubicin in the in vivo environment, MC38 cells were cultured in a hypoxic device, and the same concentrations of Doxorubicin were applied.

The hypoxic environment was simulated with 0.1% oxygen concentration, and Doxorubicin was applied after one day of cell culture in the hypoxic apparatus. CCK-8 was used to detect cell viability after 24 hours of drug treatment.

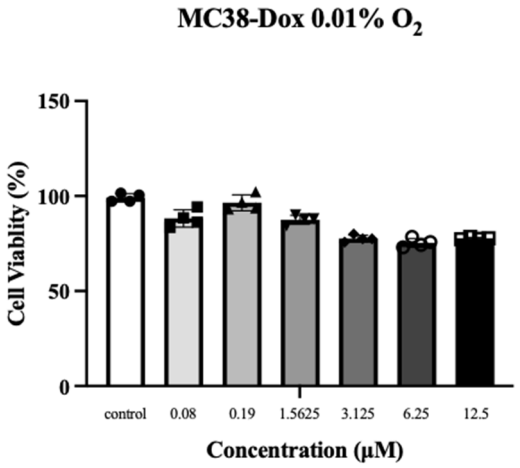

Fig. 4. the cell viability of MC38 cells after Dox under 0.1% O_2 for 24 h

Compared to the normoxic group, even under the highest concentration (12.5 μM) MC38 cells still have a survival rate close to 80%. IC50 of MC38 cells treated with Dox under hypoxic condition (1727 μM) indicated that MC38 was no longer sensitive to Dox under the hypoxic environment in comparison to the IC50 of it with the same treatment under normoxic condition (0.0069 μM).

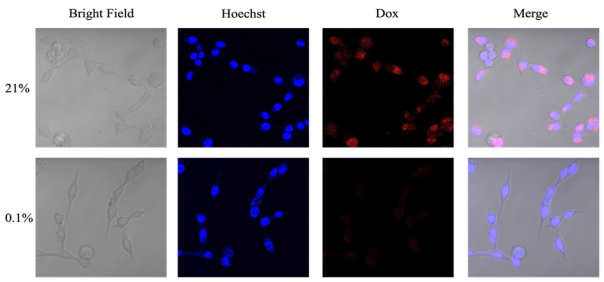

Fig. 5. MC38 cells treated with Dox under 21% and 0.1% O_2 for 6 h

To better understand why MC38 cells are no longer sensitive to the drug, we compared MC38 cells' intake of Dox under normoxic and hypoxic conditions. After incubating the cells for 6 hours, the red fluorescent of the normoxic group was significantly stronger than the hypoxic group, which suggested that the intake of Dox was greatly reduced in MC38 cells in the hypoxic environment.

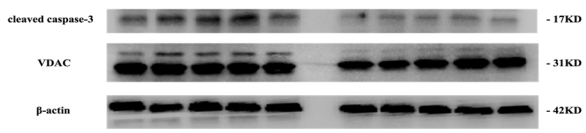

Fig. 6. Western blots of MC38 cells treated with DOX in 21% and 0.1% O_2

By using western blot, the detection of the expression of apoptosis-related proteins was used to further compare the killing effect of Dox on MC38 cells under normoxic and hypoxic conditions. We chose cleaved caspase-3 and VDAC as the biomarker.

3.1 Caspase-3

Caspases are a family of proteases that play essential roles in programmed cell death. The precursor form of all caspases comprises a prodomain and a large (17–20 kDa) and small (10–12 kDa) catalytic subunits. Activation of caspases is generated by several stimuli, including ligand-receptor interactions, growth factor deprivation, and inhibitors of cell function. Cleaved caspase-3 is an activated form of caspase-3. When apoptosis initiates, caspase-3 is cleaved and activated to produce cleaved caspase-3.

3.2 VDAC

VDAC (Voltage-dependent anion channel) is a kind of porin ion channel located on the outer mitochondrial membrane. It is involved in transporting metabolic and energetic flux across the outer mitochondrial membrane and plays a key role in regulating the communication between mitochondria and the rest of the cell. Recent studies have found that VDAC is also a functional binding site for mitochondrial interaction with other proteins and can interact with a variety of apoptotic regulatory proteins (such as HK-I/II, Bcl-2 protein family, tubulin, MAP2/4, etc.) and non-protein regulatory factors. Therefore, VDAC is a key target protein in the mitochondrial apoptosis pathway.

The result has shown that under normoxic conditions, the expression of cleaved caspase-3 has increased with the increased concentration of Dox. This proved that apoptosis of MC38 cells increased. While under hypoxic conditions, cleaved caspase-3 was also expressed but was obviously weaker than the normoxic group. The result of VDAC appears to have the same rules, which is the same as the result of CCK-8 and confocal shown previously. All the results suggest that the effects of chemotherapeutic drugs under normoxic and hypoxic conditions are different.

4. Discussion

4.1 Simulation of hypoxic environments

In recent years, hypoxia as a vital part of the tumor microenvironment has attracted more and more attention. Currently, it can be roughly divided into two classical modeling forms: chemical and physical. The commonly used inducer for chemical methods is cobalt chloride, which can inhibit the degradation of HIF-α protein, enhance the hypoxic-related pathway mediated by HIFs protein, and finally simulate the cellular response under the hypoxic environment. In the literature, different cells have different doses of cobalt chloride to simulate hypoxia. After applying this method, HIF protein expression should be detected to confirm whether the induction is successful.

The physical hypoxia method mainly simulates the hypoxic environment by reducing the oxygen content in the culture environment. Current experiments show that in vitro physical hypoxia model can better maintain the characteristics of tumor cells under hypoxic conditions in vivo, while cobalt chloride cannot better induce the related metabolic pathways involved in hypoxic regulation. Therefore, in this research, we choose the physical method to simulate the hypoxic environment.

4.2 Mechanism of drug resistance in tumor cells under hypoxic conditions

The result of the experiment has shown that MC38 cells are much less sensitive to Doxorubicin under hypoxic conditions. Also, the intake of the drug is greatly reduced too. (Fig.4 Fig.5) There are several possible reasons for the high survival rate of MC38cells under hypoxic conditions.

4.2.1 Transmembrane proteins pump the drug out of the cell

P-gp is a transmembrane protein associated with tumor resistance to chemotherapy drugs. The pump of P-GP reduces the concentration of the drug in the tumor cells by transporting the drug out of the cell, thereby reducing the chemosensitivity of the drug.

4.2.2 Alteration of the cell cycle

Some chemotherapeutic drugs function primarily through direct DNA damage during synthesis and promoting apoptosis. Doxorubicin, which was used in this experiment, works in part by interfering with the function of DNA. Cyclin-dependent kinase KIPI (p27) can be activated by the hypoxic microenvironment, blocking the tumor cells in the G1 phase, thus prolonging the cell cycle. And the killing efficiency against these tumors with prolonged cell cycle of the above chemotherapeutic drugs has decreased.

4.2.3 Alteration of certain genes or proteins within the cell

The lack of oxygen in the tumor microenvironment can alter the expression of several genes and proteins in tumor cells. Among the factors that are closely associated with chemosensitivity are p53 and MT. Alterations in gene and protein expression may cause a decreased apoptotic potential of tumor cells or the inability of drugs to kill tumors due to protein-drug affinity.

5. Conclusions and Future Study

The sensitivity of tumor cells to chemotherapeutic drugs varies greatly under different oxygen environments. Under hypoxic conditions, the killing effect of drugs on tumor cells was significantly reduced. When conducting in vitro drug screening, the effect of the hypoxic environment needs to be considered to improve the effectiveness of the screening.

Due to the particularity of the tumor microenvironment, hypoxia can be targeted to develop therapeutic strategies in the future. HIF-1α is a major inducer of hypoxia. It is ubiquitous in mammalian cells. HIF-1α can regulate many genes to adapt to hypoxic environments in the information

pathway of hypoxic gene expression. The increase of angiogenesis and glycolysis in response to HIF-1α are two essential features of tumors. These characteristics enable the tumor to adapt rapidly to hypoxic environments during development and metastasis. Since the transcriptional regulation of HIF-1α is at the center of the compensatory hypoxia process, it may be an effective therapeutic approach to treating tumors by regulating the activity of HIF-1α.

Acknowledgments

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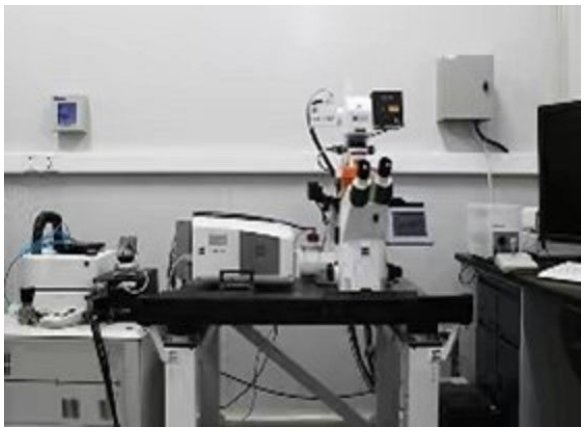
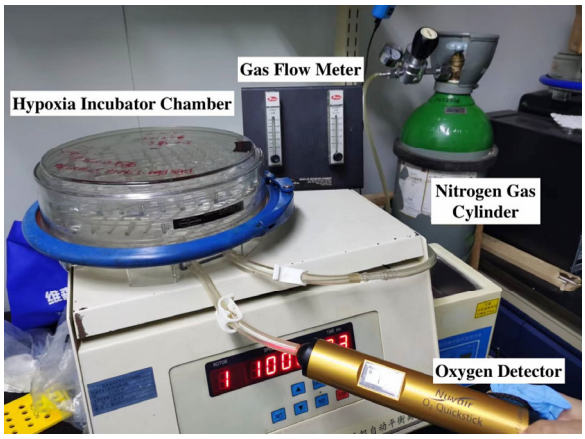
Appendix

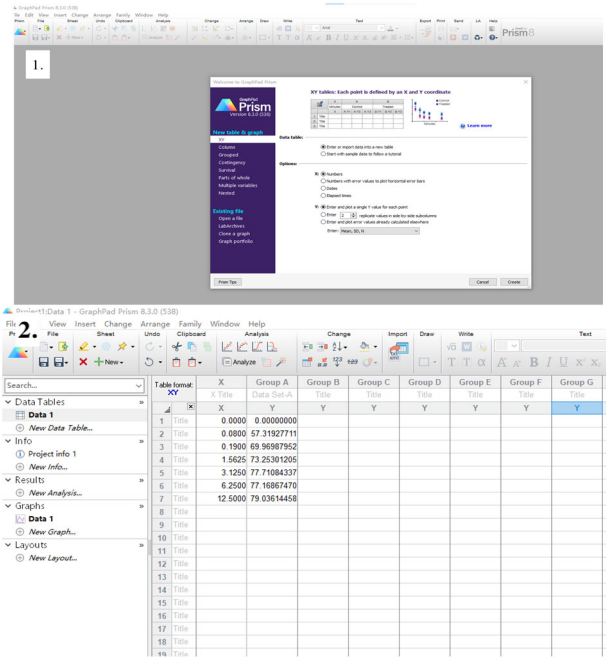


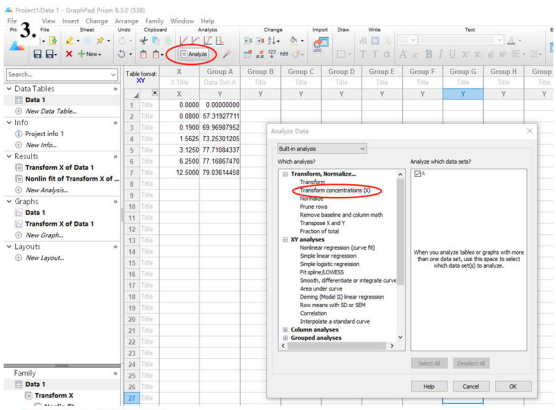
Appendix. Fig.1. Single Photon Laser Confocal Imaging System (Zeiss 710)



Appendix. Fig. 2. The Hypoxia System

The Calculation Process of IC50:

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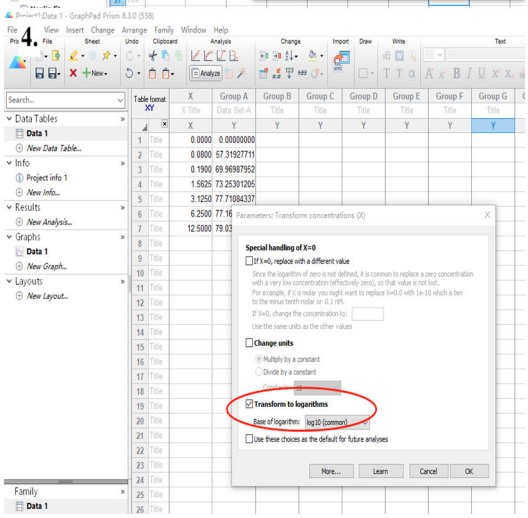
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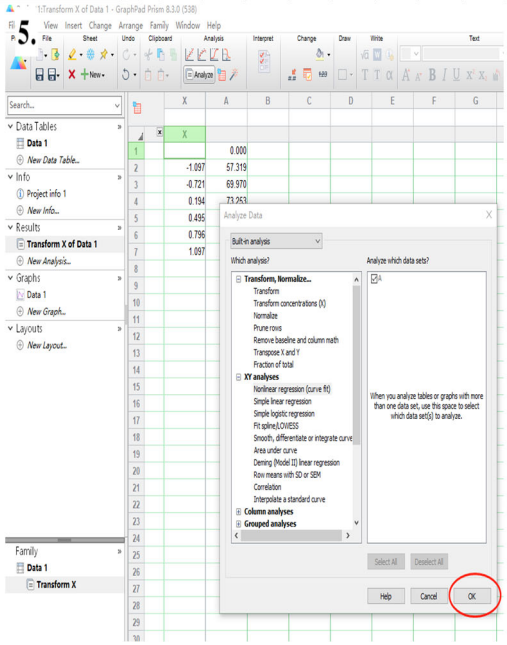
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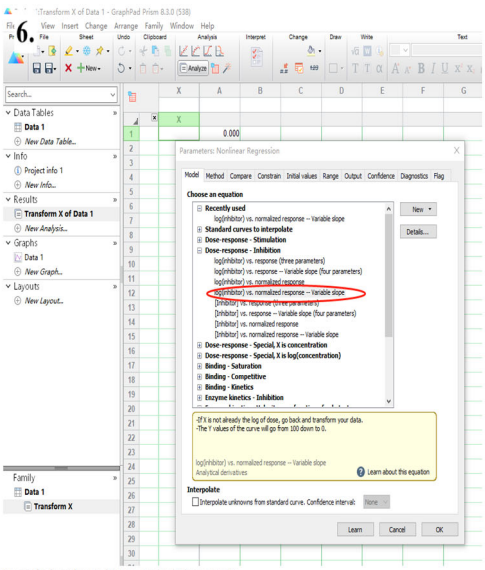
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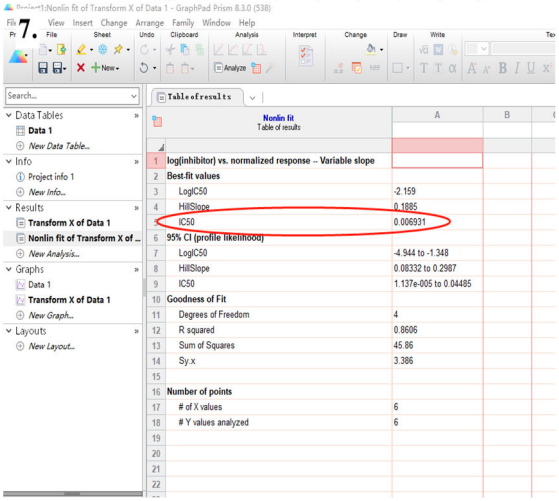
7.