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Resveratrol can Reduce the Aggressiveness of Hypoxic Colon Cancer Cells

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Abstract: Colorectal cancer is a significant global health problem characterized by the development of metastasis due to fast cell growth, tolerance to low oxygen levels, and the formation of new blood vessels. Notable advancements have been seen in the management of these instances; however, uncertainties persist about drug resistance and its accompanying adverse effects. Resveratrol, a natural polyphenol derived from several plants, has diverse pharmacological characteristics. The anticancer impact of this has piqued the curiosity of several researchers. The objective of our research was to investigate the impact of resveratrol on the proliferation, migration, and expression of angiogenic factors in hypoxic colorectal cancer cells by the use of resveratrol. Hypoxia was chemically induced using cobalt chloride. Serially diluted concentrations of resveratrol (200, 100, 50, 25, 12.5, and 6.25 μg/ml) were employed to assess the cytotoxic effect through the MTT assay. Smaller concentrations (below IC50) were utilized to investigate the impact of resveratrol on the migration of SW480 cells using the wound healing assay (50 μg/ml). The impact of resveratrol on the expression of vascular endothelial growth factor (VEGF) was assessed using the enzyme-linked immunosorbent assay (ELISA). The findings shown that resveratrol has the ability to effectively decrease cell proliferation in a dose-dependent way, inhibit cell migration and angiogenesis, via suppression of VEGF and HIF-1α. The significance of this etude lies in the enduring inability to effectively manage metastatic colorectal cancer. Suggesting that resveratrol might function as an adjunctive therapy and a useful supplement for those suffering from very metastatic colorectal cancer. The specific method by which resveratrol works is yet unknown, hence more study is needed to conduct experiments in living organisms and clinical trials.

Key Words: Resveratrol, Hypoxia, Colorectal, SW480, Proliferation, Migration, HIF, VEGF

I. INTRODUCTION

Colorectal cancer ranks as the third most prevalent form of cancer globally. In 2020, the number of newly diagnosed cases of colorectal cancer exceeded 1.9 million [1]. Historically, surgery and chemotherapy have been the primary treatment options for individuals with cancer. Nevertheless, the outlook for colorectal cancer has always been unsatisfactory, particularly for individuals with metastatic tumors [2]. Targeted treatment is an innovative alternative method that has effectively extended the overall lifespan of individuals with colorectal cancer. New medicines that inhibit many key pathways are rapidly appearing at an unprecedented pace, after the successful use of the anti-angiogenesis drug bevacizumab and other targeted treatments [3].

Hypoxia, a chronic physiological characteristic of tumors, significantly influences the microenvironment of colorectal cancer [4]. Tumors experience hypoxia when there is an imbalance between the supply and use of oxygen. Hypoxia is strongly linked to the advancement of tumors, heightened aggressiveness, greater ability to spread to other parts of the body, resistance to radiation or chemotherapy, and worse overall survival rates for different kinds of tumors [5].

Hypoxia primarily promotes cancer advancement via hypoxia-inducible factor-1 (HIF-1), which swiftly accumulates inside cells [6]. HIF-1 is responsible for activating crucial genes that govern the vital activities necessary for tumor survival and growth [7]. Research has provided evidence indicating that HIF-1 plays a key role in several aspects of cancer advancement, including proliferation, angiogenesis, and metastasis [8], [9]. Intervening in the HIF-1 pathway has shown promise in clinical studies as a possible treatment for cancer, either on its own or in conjunction with other standard cancer treatments. Additional research demonstrates that hypoxia not only influences tumor biology but also impacts the tumor microenvironment, leading to the emergence of resistance to cancer therapy [10].

Resveratrol, specifically trans-3,4,5-trihydroxystilbene, is a polyphenol compound that is found in several plants including grapes, red wine, peanuts, blueberries, cranberries, and eucalyptus [11]. Resveratrol has been shown to inhibit processes associated with the initiation, progression, and growth of malignancies [12]. It has the potential to induce apoptosis or cellular senescence in cancer cells. Recent findings suggest that resveratrol has anti-angiogenic properties [13]. Resveratrol achieves its anti-angiogenic effects by inhibiting HIF-1α [14] and reducing the expression of VEGF [15]. Research has shown that resveratrol might potentially increase the effectiveness of p53, with the extent of this effect being influenced by the dose. This has been seen in both carcinogenic and noncancerous cell lines [16]. Resveratrol has shown cellular anticancer properties in breast cancer [17], skin cancer [18], and liver cancer [19]. Furthermore, research has shown that resveratrol may be effectively used in conjunction with chemotherapeutic agents to mitigate drug resistance in some cancer therapies [20].

II. MATERIALS AND METHODS

Resveratrol (99%) and cobalt (II) chloride hexahydrate were purchased from Sigma-Aldrich. The MTT cell proliferation and cytotoxicity test kit was purchased from Solarbio, located in Beijing, China. Additional substances were sourced from local marketplaces and subjected to testing prior to use.

A. INDUCTION OF HYPOXIA

By laboratory experiments, cobalt chloride ($CoCl_2$) was shown to function as a hypoxia inducer. It stimulates the production of HIF-1α in cancer cell lines [21]. We conducted experiments with various doses of $CoCl_2$ on the cell line we had developed. The purpose was to assess the dose-dependent relationship during different incubation periods, in order to limit the toxicity of $CoCl_2$ and identify the appropriate dosage for the assay [22]. The most effective concentration of $CoCl_2$for inducing hypoxia in our experiment was 50μg per ml of RPMI1640 culture medium. A solution containing $CoCl_2$was used to prepare dilutions of resveratrol.

B. CYTOTOXICITY ASSAY

The assessment of the impact of resveratrol on cell viability in vitro was performed using the MTT assay, which measures cytotoxicity or stimulatory effects. A volume of 200 μl containing 1×10^5 cells/ml was introduced onto a flat bottom plate (96 well) and incubated at a temperature of 37°C. Once the cells had firmly attached to the walls, the level of confluence reached 70% to 80%, indicating that the wells were prepared for treatment. A $CoCl_2$-containing medium was used to induce chemical hypoxia. A serial dilution of resveratrol was generated using a concentration of 50 μg/ml $CoCl_2$, following the method described in reference [23]. The medium used for exposure was not replaced throughout the specified time period. For the assays, a total of eight sets of quadruplicates were used. The first set of quadruplicates was treated with RPMI-1640 media alone, which served as the normoxic group. The second set of quadruplicates was treated with 50 μg/ml $CoCl_2$in RPMI-1640 media, serving as the hypoxic control group. The remaining sets of quadruplicates were treated with specified concentrations of resveratrol (200, 100, 50, 25, 12.5, and 6.25 μg/ml) and then incubated for 48 hours at 37°C. The medium was withdrawn from the wells after the prescribed duration of 48 hours. The wells were then rinsed with 100 μL of PBS. Subsequently, 100 μL of MTT solution with a concentration of 1 mg/mL was applied to each well, the plate was subjected to a 4-hour incubation period. Following this, the solution was extracted and 100μL of Formazan Dissolving Solution was introduced into each well. By gently agitating in a gyratory shaker for a duration of 10 minutes, ensure that the crystal is fully dissolved. Utilizing a microplate reader at a wavelength of 490 nm. The percentage of inhibition was graphed versus the measured concentration using Microsoft Excel, and the IC_50 value was determined. The calculation of percent proliferation was performed using the equation shown below [24],

$$\%\text{wound closure} = 1 - \left(\frac{\text{width at the indicated time (h)}}{\text{width at zero time}}\right) \times 100\%.$$

C. CELL MIGRATION ASSAY

We conducted a migration test with the SW480 colon cancer cell line. Cells were cultivated and 400 μl of a cell suspension containing 100,000 cells was added to each well of a 24-well plate. In order to produce a wound, we let the cells to grow until they formed a monolayer that covered 95% of the well's surface. The wound was then made by creating a straight line from one edge of the well to the opposite, passing through the center. To manually produce a wound, the simplest and most cost-effective method is to draw a straight line using 200 μl micropipette tips, with the use of a sterile ruler. The cells were rinsed twice with PBS in a gentle manner. The inhibitory impact of resveratrol on the migration of hypoxic colorectal cancer cells may be assessed in vitro using a wound-healing test [25]. Cells in 24 wells were subjected to different treatments. The normoxic group was treated with RPMI-1640 media alone. The hypoxic control group was treated with media containing $CoCl_2$. The combination group was treated with media containing both resveratrol and $CoCl_2$. The cells exhibited migratory behavior thereafter. The wound width was assessed at 12-hour intervals, from the beginning of the experiment (zero time) to 48 hours. Images of the migrating cells along the margins of the wound were acquired using an inverted microscope camera. The experiment was conducted in triplicate, and photos were obtained for each well from three distinct zones of the scratch. The doses used for resveratrol were (50 μg/ml $CoCl_2$-containing medium). A total of five to six images were acquired using an inverted microscope for each well's wound at 0, 6, 12, 18, and 24 hours. The wound's breadth was quantified using Image-J software version-1. The findings were computed to ascertain the proportion of migration inhibition using the following equation,

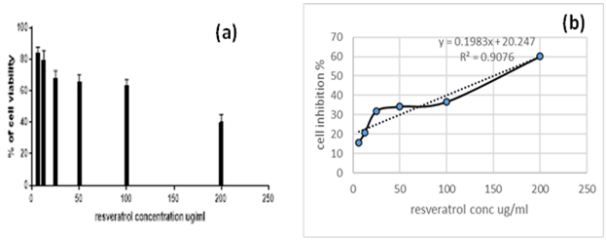

FIGURE 1: Cytotoxicity of resveratrol on hypoxic SW480 cells. (a) The cell viability and (b)inhibition percentages of hypoxic SW480 cells grown with resveratrol concentrations (6.25, 12.5,25,50,100, and 200μg/ml) over 48 hours were determined by MTT test. Values shown are means ± SD from quadruplicates

$$\%\text{wound closure} = 1 - \left(\frac{\text{width at the indicated time (h)}}{\text{width at zero time}}\right) \times 100\%.$$

D. EXPRESSION OF HIF-1 AND VEGF

SW480 cells were seeded in flat bottom 48 well plate (400 μl of 1×105 cells/ml) and incubated at 37°C. When confluence reached 70%-80%, set of triplicates were used for the assays, the first triplicate was treated with RPMI-1640 media only used as normoxic group, the second triplicate treated 50 μg/ml $CoCl_2$ in RPMI-1640 media (hypoxic control group), the rest triplicates were treated with the previously specified concentrations of resveratrol to be incubated for 48 hours at 37°C.

The conditioned medium from cell cultures were collected, and an ELISA assay was conducted following the instructions provided by the manufacturer (Solarbio).

The optical density was measured at a wavelength of 450 nm using a conventional ELISA plate-reader, and the presence of HIF-1, and VEGF in the supernatants was determined [26].

E. STATISTICAL ANALYSES

GraphPad Prism 7.0. was used for statistical analysis. Data for the same group were denoted as the mean ± standard deviation. Tests were evaluated by unpaired Student's t test for two different groups. While one-way analysis of variance was used for more than two groups. Images were analyzed by Image J software analysis. A value of $P < 0.05$ was considered as significant, statistically. 1. Results The impact of resveratrol on the survival of hypoxic SW480 cells (induced by $CoCl_2$) was evaluated using the MTT test, as seen in Figure 1(a). It was observed that greater concentrations of resveratrol led to a considerable reduction in cell viability after 48 hours of incubation, following a concentration-dependent pattern. The IC_50 values of resveratrol, measured after 48 hours of incubation, were around 150 μg/ml (see Figure 1(b)).

Colorectal cancer cells that are deprived of oxygen have a greater ability to spread to other parts of the body compared to cells that have normal oxygen levels. The process of metastasis in cancer cells is contingent upon their capacity for movement. Figure 2 demonstrates that the hypoxic control group exhibited a much higher migration rate, with complete healing of the scratched lesion after 36 hours. In contrast, the normoxic cells (which were not treated with either $CoCl_2$ or resveratrol) showed similar outcomes after 48 hours. Cells experiencing hypoxia exhibited greater aggressiveness compared to cells in a normoxic state. Resveratrol significantly reduced the migratory capacity of SW 480 cells at sublethal concentrations below the IC_50. A significant impact was seen in cells treated with 50 μg/ml resveratrol when compared to the hypoxic control group, as shown in Figure 2.

The data presented in Table 1 clearly demonstrate that hypoxia induced a substantial elevation in HIF-1α levels. We demonstrated that the presence of resveratrol at a concentration of 50 μg/ml in medium containing $CoCl_2$ for a duration of 48 hours dramatically reduced HIF-1α levels in SW480 cells. Furthermore, it has been firmly shown that the levels of VEGF in cells exposed to normal oxygen conditions, low oxygen conditions, and resveratrol treatment are highly associated with the levels of HIF-1α protein expression.

III. DISCUSSION

Hypoxia arises as a result of the fast proliferation of malignant cells in the human body, particularly in the context of solid tumors. In such instances, the local blood vessels are incapable of providing sufficient oxygen and nutrients [26]. Research conducted in recent decades has firmly shown that hypoxia has a significant role in promoting tumor development by stimulating tumor angiogenesis and metastasis [27], [28]. Metastasis may occur as a result of a coordinated and sequential process including cell migration, invasion, and adhesion. The study of active nutraceuticals, which combat endothelial to mesenchymal transmission and angiogenesis, has emerged as an intriguing approach for managing metastatic malignancies [29].

Resveratrol, which is naturally found in several plants and natural foods, has been shown to have potential therapeutic applications in the treatment of numerous disorders, particularly in relation to its impact on a wide range of malignancies. Resveratrol gained popularity in 1997 for its anticancer qualities. Subsequently, researchers and scientists began to take notice of resveratrol due to its extensive array of biological impacts [30], [31]. Higher dosages of resveratrol have the ability to increase the lifespan of animals [32]. Recent research has shown that resveratrol effectively inhibits several types of malignancies, both in laboratory settings and in living organisms, with few to negligible hazardous side effects [33]. Findings indicate that resveratrol might potentially serve as an adjunctive treatment to impede the characteristic features, growth, spread, and infiltration of cancer. These findings align with our discovery that resveratrol has a dose-dependent influence on the growth of SW480 cancer cells. The inhibitory impact of resveratrol was very pronounced ($p<0.05$), as seen in Figure 1, particularly with

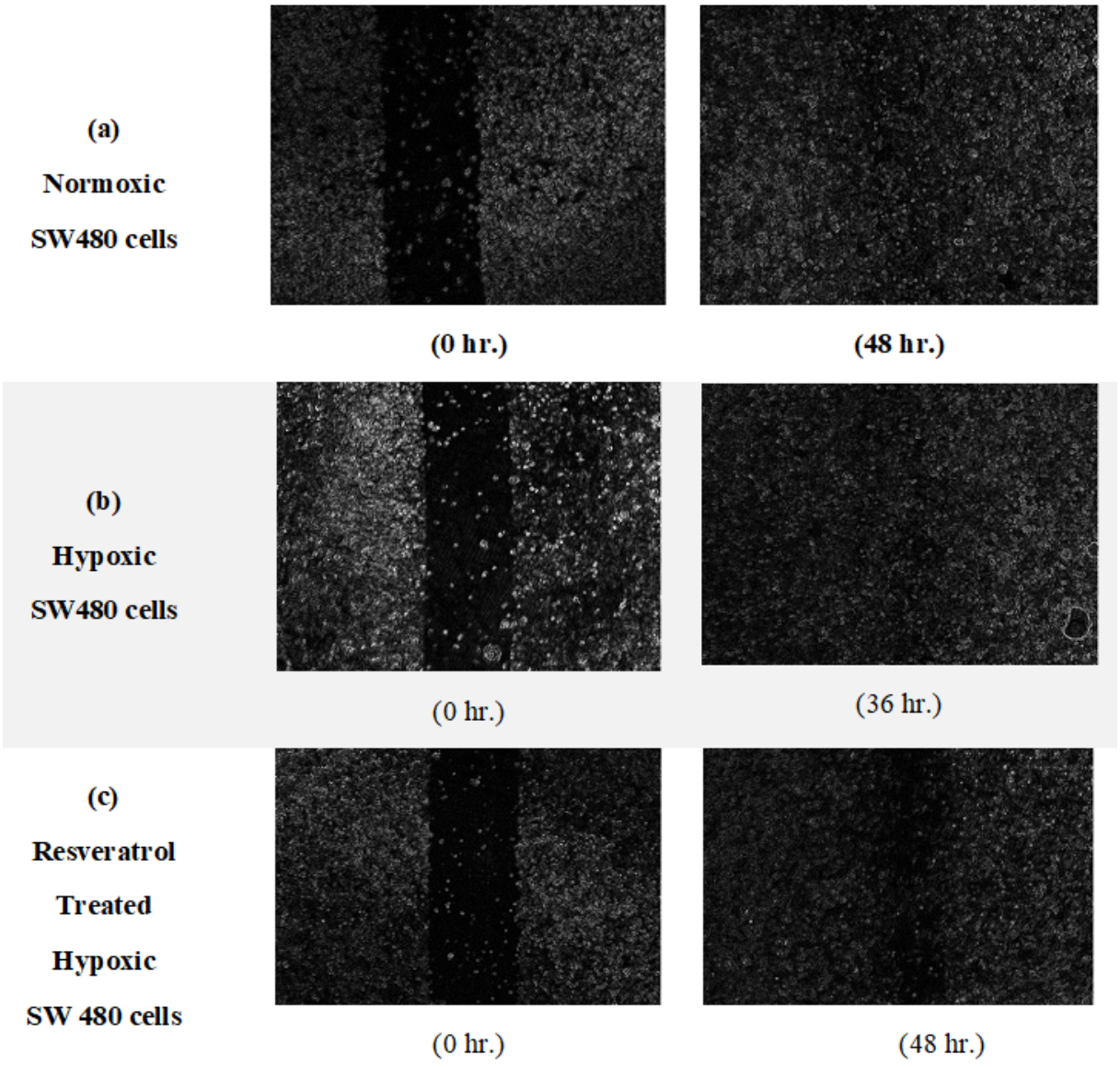

FIGURE 2: Migratory ability of SW480 cells. a) normoxic cells, b) hypoxic cells c) resveratrol treated hypoxic cells. Cell migration was evaluated by wound-healing assay. Images were taken at 0 and 48 hr. for normoxic and resveratrol treated cells and at 0 and 36 hr. for hypoxic cells when a complete healing of scratch obtained.

SW480	Normoxic group	Hypoxic control group	Resveratrol treated group
HIF1α	21.1±0.92	55.2± 3.99	30.08±4.13
VEGF	66.91±5.11	83.14±6.02	51.51±5.12

TABLE 1: Expression of HIF1 α and VEGF in normoxic, hypoxic and resveratrol treated hypoxic SW480 colorectal cancer cells in pg/ml

higher dosages. In order to eliminate the harmful impact of resveratrol on the spread of cells, a lower dose of 50 μg/ml (below the IC_50) was used to investigate the influence of resveratrol on the movement capability of cancer cells under hypoxic conditions. This investigation was conducted using the widely-used wounded healing assay. The findings of our study demonstrated that resveratrol effectively impaired the migratory capabilities of SW480 cells, particularly at the specified dosage used in the experiment.

Researchers have identified that the tumor microenvironment plays a crucial role in facilitating the processes of cell growth, spread to other parts of the body, and the formation of new blood vessels. Huang and his colleagues said that resveratrol caused programmed cell death in SW480 cells and reduced their resistance. However, they did not consider the influence of hypoxia and the connection between the HIF1 signaling pathway and its subsequent consequences.

Prior studies have shown that resveratrol decreases the expression of both HIF-1α [34] and VEGF [35]. The study conducted by Trapp et al. [36] demonstrated the impact of resveratrol under both normal and low oxygen environments, aiming to imitate conditions seen in living organisms more accurately. The researchers discovered that after 48 hours, resveratrol drastically reduced the levels of HIF-1α in three distinct types of cells. They also observed a close correlation between the levels of VEGF in cells treated with resveratrol and HIF-1a protein expression levels in cells exposed to hypoxia or resveratrol treatment [36], [37], these findings aligned with ours.

Our findings indicate that resveratrol inhibits the proliferation and migration of SW480 cells. It disrupts the integration of the HIF-1α-VEGF signaling pathway in colorectal cancer cells, leading to a reduction in the angiogenic response of hypoxia cells. Regrettably, there is a scarcity of evidence about the efficacy of resveratrol in inhibiting the metastatic and angiogenic properties of colorectal cancer cells at specific doses, when subjected to chemical induction of HIF1-α. In summary, this study holds great importance as it demonstrates that resveratrol effectively reduces the aggressiveness of SW480 cells in a laboratory setting. This suggests that resveratrol has the potential to serve as an adjunct therapy and a beneficial supplement for individuals suffering from advanced metastatic colorectal cancer. The precise mechanism of action of resveratrol remains undetermined, necessitating more research for in vivo experimentation and clinical trials.

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CONFLICTS OF INTEREST

No conflicts of interest have been declared by the authors.

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