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IJMCM

International Journal of Molecular and Cellular Medicine

p-ISSN: 2251-9637 o-ISSN: 2251-9645

Cellular and Molecular Biology Research Center

Isoproterenol Alters Metabolism, Promotes Survival and Migration in 5-Fluorouracil-Treated SW480 Cells with and without Beta-hydroxybutyrate

Azam Shakery, Katayoun Pourvali, Ghazaleh Shimi, Hamid Zand*

Department of Cellular and Molecular Nutrition, Faculty of Nutrition Science and Food Technology, National Nutrition and Food Technology Research Institute, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Article type:
Original Article

Received:
2022.02.05
Revised:
2023.10.31
Accepted:
2023.11.12

ABSTRACT

People with cancer often experience long-term physical and psychological stress, which can have a significant impact on tumor metabolism and treatment. The effects of adrenergic signaling on metabolic pathways are well known, but only a few studies have looked into the connection between this signaling and tumor metabolism. This study examined the effects of treatment with isoproterenol (Iso) alone and in combination with β-hydroxybutyrate (βHB), a mitochondrial fuel, on the metabolism, survival, and migration of SW480 colon cancer cells treated with 5-fluorouracil (5FU). The researchers measured the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) to determine the metabolic profile of these cells. They also analyzed the gene expression of PGC-1α, c-MYC, and NANOG to investigate the relationship between metabolic phenotype and stemness status. Scratch assays were used to assess cell migration. The results showed that Iso treatment increased cell viability in both SW480 and 5FU-treated SW480 cells. There was a significant decrease in ECAR and an increase in OCR after Iso treatment in both cell types. The expression of c-MYC and NANOG, genes associated with stemness, increased, while the expression of PGC-1α, a gene related to oxidative phosphorylation, decreased following Iso treatment. Iso treatment also increased the migration potential of both SW480 and 5FU-treated SW480 cells. These findings suggest that under stressful conditions, 5FU-treated colon cancer cells can utilize the oxidative phosphorylation pathway for growth and migration.

Keywords: Beta-adrenergic receptor agonist, isoproterenol, metabolic phenotype, 5FU-treated cells, colon cancer

Cite this article: Shakery A, *et al*. Isoproterenol Alters Metabolism, Promotes Survival and Migration in 5-Fluorouracil-Treated SW480 Cells with and Without Beta-hydroxybutyrate. *International Journal of Molecular and Cellular Medicine. 2023; 12(2):144-158.* **DOI:** 10.22088/IJMCM.BUMS.12.2.144

∗Corresponding: Hamid Zand
Address: National Nutrition and Food Technology Research Institute, Faculty of Nutrition Science and Food Technology, Shahid Beheshti University of Medical Sciences,
E-mail: hamid_zand@sbmu.ac.ir

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Introduction

Epidemiological observations have demonstrated the role of stress and sympathetic nervous system activation in tumorigenesis, cancer progression, and metastasis (1, 2). Further studies have shown that the long-term use of beta-blockers decreases tumor progression in several types of cancers (3, 4). The presence of large numbers of beta-adrenergic receptors on tumor and stromal cells' surfaces including colon cancer cells, (5, 6) shows that they can be influenced by both systemic epinephrine and local sympathetic nerves. For example, histological examinations of breast and ovarian carcinomas have shown localized nerve expansion of catecholaminergic fibers in parenchymal tumor tissues due to chronic stress. Moreover, repeated stimulation increases the density of these fibers (7, 8). Similarly, mouse xenograft models confirm that stress is enhancing the effects on the growth and dissemination of tumor cells (9).

Activation of β-adrenergic receptors results in increased intracellular cyclic AMP following the activation of G-protein mediated adenylyl cyclase. Cyclic AMP, as the main intracellular mediator of the β-adrenergic receptor, induces two opposite effects in cancer cells. The majority of published studies have explored the effect of cyclic AMP on the regulation of apoptosis in tumor cells. In some types of cancers, cyclic AMP has a pro-apoptotic effect. This has been demonstrated in lung carcinoma, (10) melanoma, (11) and ovarian cancer, (12) conversely, in other cancers, augmentation of cyclic AMP promotes tumor growth and dissemination. These cancers include colorectal cancer (13) and lymphoma cells treated with DNA damaging agents (14). β-adrenergic associated protein kinase A (PKA) activation and Ras-Raf pathway are involved in controlling a range of cellular processes, such as inflammation, angiogenesis, apoptosis, cellular immune responses, DNA-damage repair, differentiation, movement, invasion, secretion, and proliferation by regulating the various expression of genes (15).

Despite the well-known effects of adrenergic signaling on cancer progression, and considering that tumor cells are metabolically diverse and flexible to maintain survival and growth, few studies have explored the precise role of catecholamines in cancer progression and the potential impact of metabolic alterations. Thus, the pro-carcinogenic effects of β-adrenergic signaling, specifically the role of β-adrenergic receptors in the switch between oxidative metabolism and glycolysis, require further investigation.

We have recently suggested that a sub-population of grade IV colorectal adenocarcinoma, treated with 5FU for 3 days, shows a preference for recruiting βHB as a fuel in their mitochondrial oxidative pathways. βHB also promoted the expression of stemness genes, increased migration, and enhanced the invasiveness of the cancer cells (16).

In this study, we focused on the effect of β-adrenergic receptor stimulation using Iso on the shift of metabolism from glycolysis to oxidative phosphorylation or vice versa in SW480 cells and 5FU-treated cells, both in the absence and the presence of βHB. We also investigated the effects of Iso and βHB on cell viability and migration in these cells.

Materials and methods

Colon Cancer Cell Line and Culture

SW480 cells from Grade III-IV human colon cancer cell line (obtained from the National Cell Bank of Iran, Pasteur Institute, Iran) that are undifferentiated, invasive, and tumorigenic were compared with

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chemotherapy-treated SW480 cells. These treated cells, according to the protocol described in our previous study, (16) survived and grew 72 hours after treatment with 20 µM of 5FU (equivalent to the calculated IC_{50} dose) (Ebewe PHARMA, AUSTRIA). They were cultured in DMEM medium with 10% fetal bovine serum (GIBCO, Life Technologies), 1% L-glutamine, 1% penicillin/streptomycin (Sigma, St. Louis, USA) under standard conditions for single layer culture of eukaryote cells at 37 °C temperature, 95% humidity, and 5% CO. Confluent cells were harvested by EDTA-trypsin (Sigma, St. Louis, USA) and their old media was replaced twice a week.

Survival of Cells after Iso Treatment with MTT Test

We investigated the viability of SW480 and 5FU-treated SW480 cells after treatment with Iso at 20, 100, and 150 µM concentrations (Isoprenalina Cloridrato, Monico, Italy), as well as, Iso in combination with 250 µm βHB that was calculated and described in our previous study as the EC_{50} dose of it. The EC_{50} dose of Iso was calculated by plotting the concentration curves against the percentage of live cells. After determining the EC_{50} dose of Iso, 3000 cells/well were seeded in a 96-well plate. After 24 hours, they were treated with 100 µM Iso alone or its combination with 250 µM βHB for 72 hours. Following this, the old media was removed, and 200 µL of a 5 mg/ml solution of MTT salts (Sigma, St. Louis, USA) dissolved in phosphate-buffered saline (PBS) was added to each well. The plate was then placed in the incubator for 4 hours. Then, the supernatant of the cells was replaced with 200 µL DMSO to dissolve the formazan crystals. The optical absorption at 570 nm was measured by an Elisa reader (ELx808, BioTek, USA) to assess cell viability.

Determination of Metabolic Phenotype Based on Measurements of Oxygen Consumption and Extracellular Acidity

OCR in living cells in real-time can be used as an indicator of oxidative phosphorylation. The OCR of SW480 and 5FU-treated SW480 cells was investigated by analyzing the changes in fluorescence over time using the MitoXpress® Xtra kit (Luxcel Bioscience, Cork, Ireland # MX-200) and calculating ECAR as a measure of glycolysis using a pH-Xtra kit (Luxcel Bioscience, Cork, Ireland # PH-200). Approximately 70000 cells for each well of 96-well plate were cultured overnight. The cells were treated with Iso (100 µM) and a combination of Iso and βHB (250 µM) for 24 hours. As per the MitoXpress® Xtra kit protocol, the fluorescence signal intensity was measured using a plate reader (Cytation 3, Bio-Tek) at 37 °C every one and a half minutes for 150 minutes. This measurement was taken immediately after adding the drug treatments, the prepared fluorescent probe, and mineral oil (to prevent oxygen exchange). The excitation wavelength was 365 nm, and the emission wavelength was 650 nm. The device was set in the mode of time-resolved fluorescence (TRF) mode, with delay times of 30 µs and 70 µs. According to the protocol of the kit, the presence of CO residue can lead to acidity and affect ECAR measurements. Therefore, it was crucial to make an effort to eliminate all CO. To do so, the plate containing the cultured cells was incubated in a carbon-free incubator for three hours before adding the drug treatments. Once added, in conjunction with the respiratory buffer and pH-Xtra reagents, which were prepared according to the kit protocol, the plate was immediately read on the fluorescent plate reader at 37°C every one and a half minutes for at least 120 minutes. The excitation wavelength used was 380 nm, and the emission wavelength was 615 nm. The device was set to time-resolved fluorescence (TRF) mode.

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Finally, the fluorescence values measured for both tests were plotted against time after being corrected based on the values of blank samples that lacked reaction materials. The linear section of the signal profile was selected and the oxygen and extracellular acidity were determined by calculating the regression line slope. The mean values of two sets of triple repetitions for each sample were statistically analyzed.

cDNA Synthesis and RT-PCR Assay

The RNX-Plus solution was used to extract total RNA from cells that were treated for 72 hours, following the manufacturer's protocol (Cinagene, Iran). Then 1 µg of RNA was subjected to cDNA synthesis using the cDNA synthesis kit from Vivantis Technologies in Malaysia. Finally the PCR reaction for PGC-1α, c-MYC, and NANOG with GAPDH as an internal control was performed using the designed primers (Table 1), the MasterMix, and the appropriate temperatures as recommended by the manufacturer.

Table 1. Details of the primer pairs used in this study.

Gene	Forward primer/ reverse primer	Length (nt)
NANOG	F:5'AACTCTCCAACATCCTGAACCTC3' R: 5'CGTCACACCATTGCTATTCTTCG3'	119
c-MYC	F: 5'GCATACATCCTGTCCGTCCAAG3' R: 5'TTCCTTACGCACAAGAGTTCCG3'	124
PGC-1α	F: 5'GTCACAACACTTACAAGCCAAACC3' R: 5'GCAGTTCCAGAGAGTTCCACAC3'	135
GAPDH	F: 5'CATCAAGAAGGTGGTGAAGCAG3' R: 5'GCGTCAAAGGTGGAGGAGTG3'	130

Evaluation of Cell Migration Ability by Scratch Assay

To investigate the effect of Iso versus the combination of Iso with βHB on cells' ability to migrate, we created a scratch using a crystalline sampler tip in a 90% confluent single-layer cell culture. The cells were incubated with mitomycin C (Sigma, St. Louis, USA) for 2 hours to prevent cell division. After scratching, the cells were washed twice with PBS to remove any remaining mitomycin C and cell debris. After that, the cells were incubated in a culture media containing 0.5% serum (to inhibit cell proliferation) and treated with Iso and its combination with βHB. We analyzed photos taken at 0, 36, and 72 hours after scratching by using a camera attached to the microscope, and subsequently, the migration rate of the cells was calculated based on the percentage of scratch closure.

Statistical Analysis

Statistical tests were performed using SPSS 21 software and were determined by Student's t-test and one-way ANOVA (with Tukey post-hoc test). Data were presented as mean ± standard deviation (S.D.) and $P < 0.05$ was considered statistically significant. The scratch area and its changes were measured at different times with Image J software. Figures and the EC_{50} calculations were performed using GraphPad Prism 6 (La Jolla, CA 92037 USA) software.

Results

Cell Viability

Based on our observations, treatment of cells with Iso in different concentrations (0, 20, 100, 150 µM) for 72 hours showed increasing survival rates in both SW480 and 5FU-treated SW480 cells (Figure 1A).

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Thus, using the dose-response curve ability of GraphPad Prism software, the EC_{50} values at 100.3 and 103.1 µM for Iso in 5FU-treated SW480 and SW480 cells were calculated respectively and shown in figure 1B. Therefore, in the next experiments, we used 100 µM of Iso for cell treatment. The treatment of cells with Iso , along with 250 µM βHB as an aerobic metabolic fuel, significantly increased cell viability compared to the control group ($p<0.05$) in both cell types, as shown in Figure 1C.

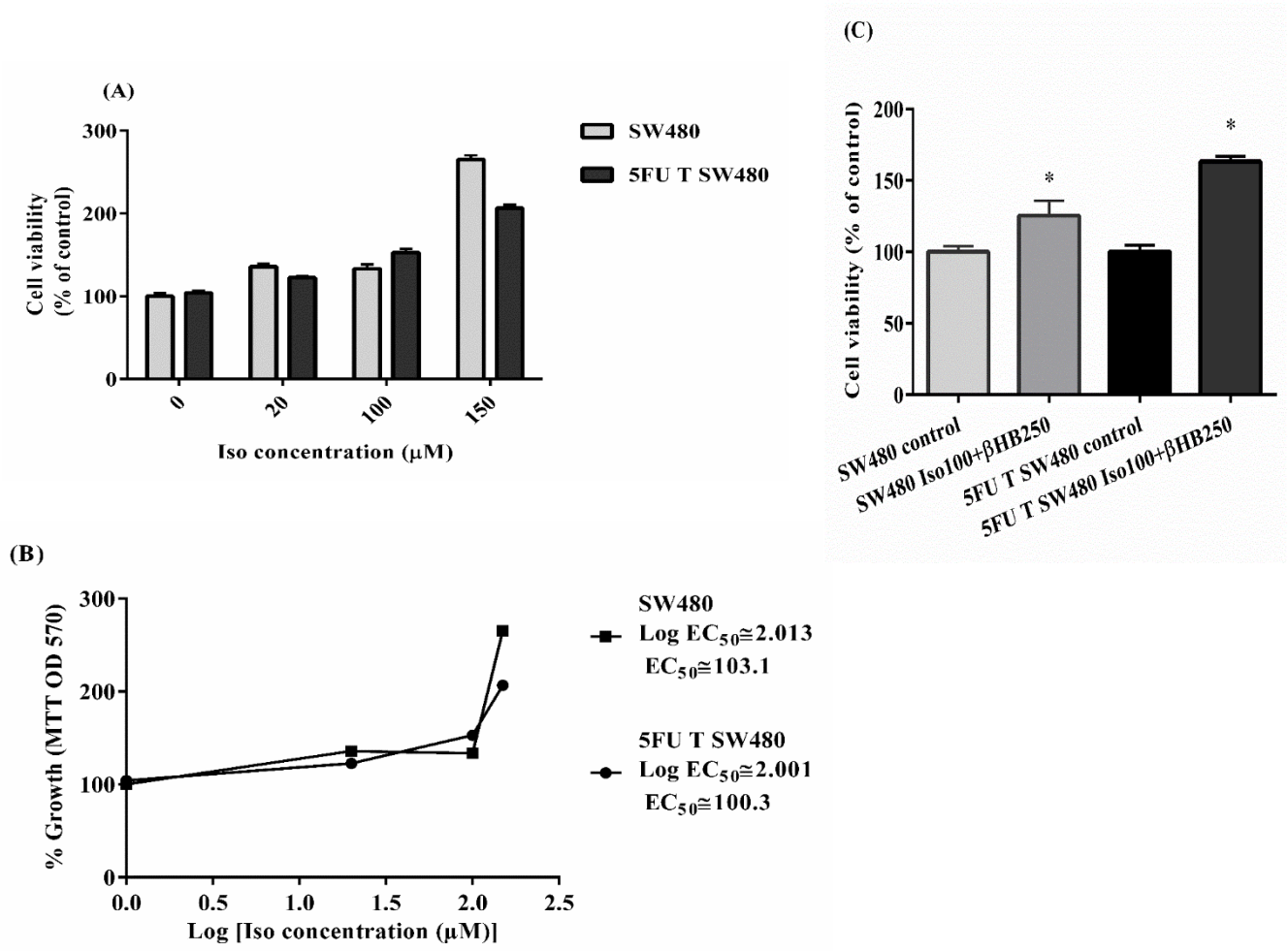


Fig.1. Increased cell viability after Iso treatment in SW480 and 5FU-treated SW480 cells. (A) Cell viability was measured with the MTT method after 72 h treatment of SW480 and 5FU-treated SW480 cells with different concentrations of Iso (0, 20, 100, 150µM) for 72 hours (B) EC_{50} was calculated at about 100µM using dose-response curve for both cell types (C) Significant increase in cell viability after Iso and its combination with βHB treatment at their EC_{50} dose. The results are provided as mean values with standard error from at least three independent experiments and normalized to 100% of the control group. *$p < 0.05$ *vs.* control.

Oxygen Consumption Rates

The OCR, as a measure of oxidative phosphorylation, examined the impact of treatment with Iso alone as a stress inducer, or Iso combined with βHB as an energy source for mitochondrial metabolism, on the metabolic phenotype in both SW480 and 5FU-treated cells. Results showed that treatment with Iso and Iso in combination with βHB caused a significant increase in OCR compared to the control group ($p<0.0001$). However, there was no significant difference between the two treatment groups. No significant difference in OCR was observed between the two cell types, as shown in Figure 2A-B.

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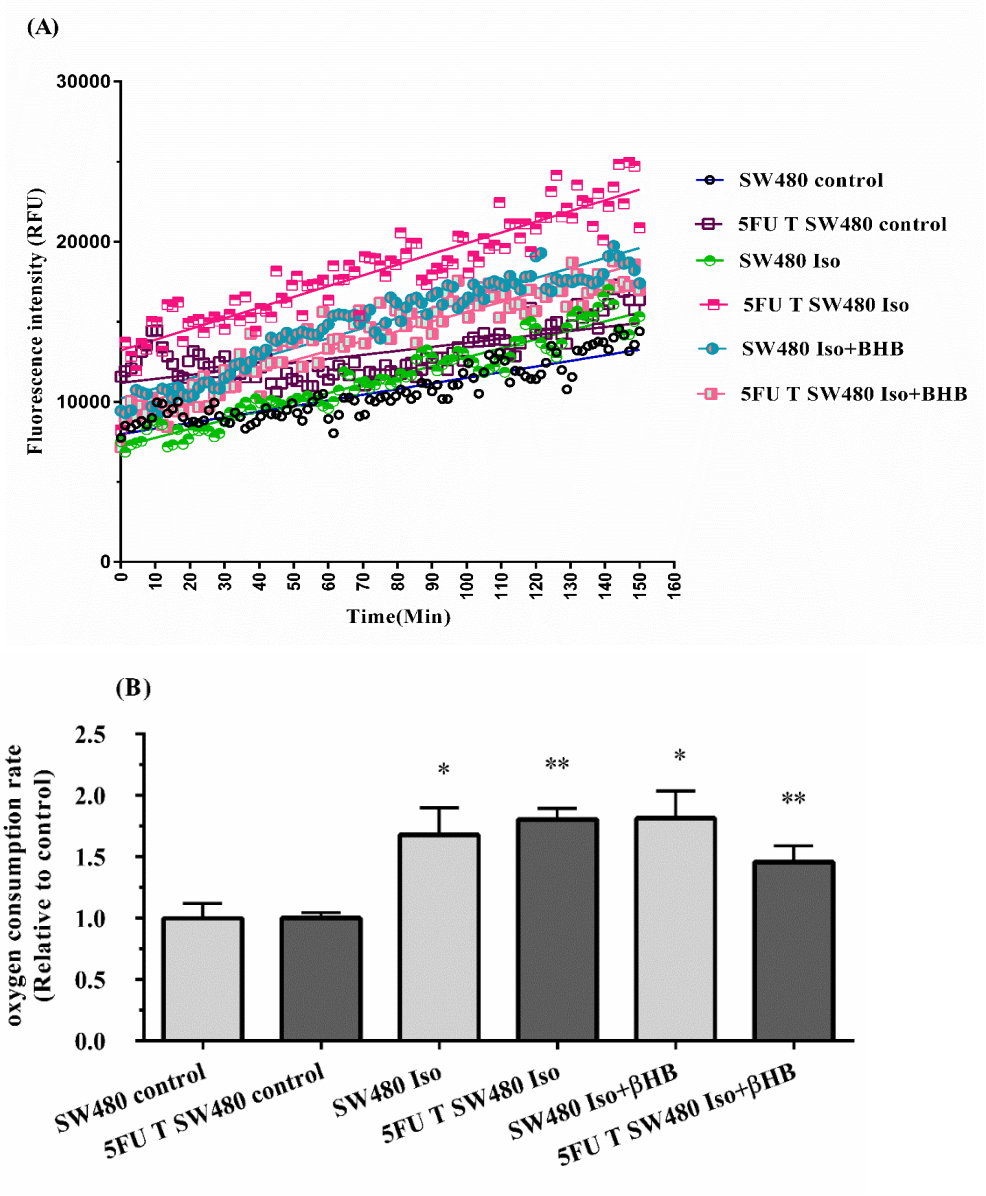


Fig.2. An increase in oxygen consumption rate after Iso and Iso + βHB treatment in SW480 and 5FU-treated SW480 cells. (A) Real-time responses as the average fluorescent emission value over 150 minutes of fluorophores in the MitoXpress-Xtra kit as a measure of OCR (mean ± SEM, n = 6) in SW480 and 5FU-treated SW480 cells relative to the control (B) OCR calculated based on the linear portion of the regression line of real time-responses of average fluorescence versus time which is normalized to control in SW480 and 5FU-treated SW480 cells. *$p < 0.0001$ *vs.* control in SW480 cells. **$p < 0.0001$ *vs.* control in 5FU-treated SW480 cells.

Extracellular Acidification Rates

Alterations in the ECAR were used as indicators of glycolysis and considered as a method for examining the metabolic phenotype after treatment with Iso and Iso combined with βHB in 5FU-treated SW480 and SW480 cells. Treatment with Iso and Iso in combination with βHB significantly reduced ECAR compared to the control group in SW480 cells treated with 5FU ($p<0.0001$). However, in SW480 cells, Iso significantly decreased ECAR ($p<0.0001$). However, no significant difference in ECAR was observed after Iso plus βHB treatment compared to the control, as shown in Figure 3A-B.

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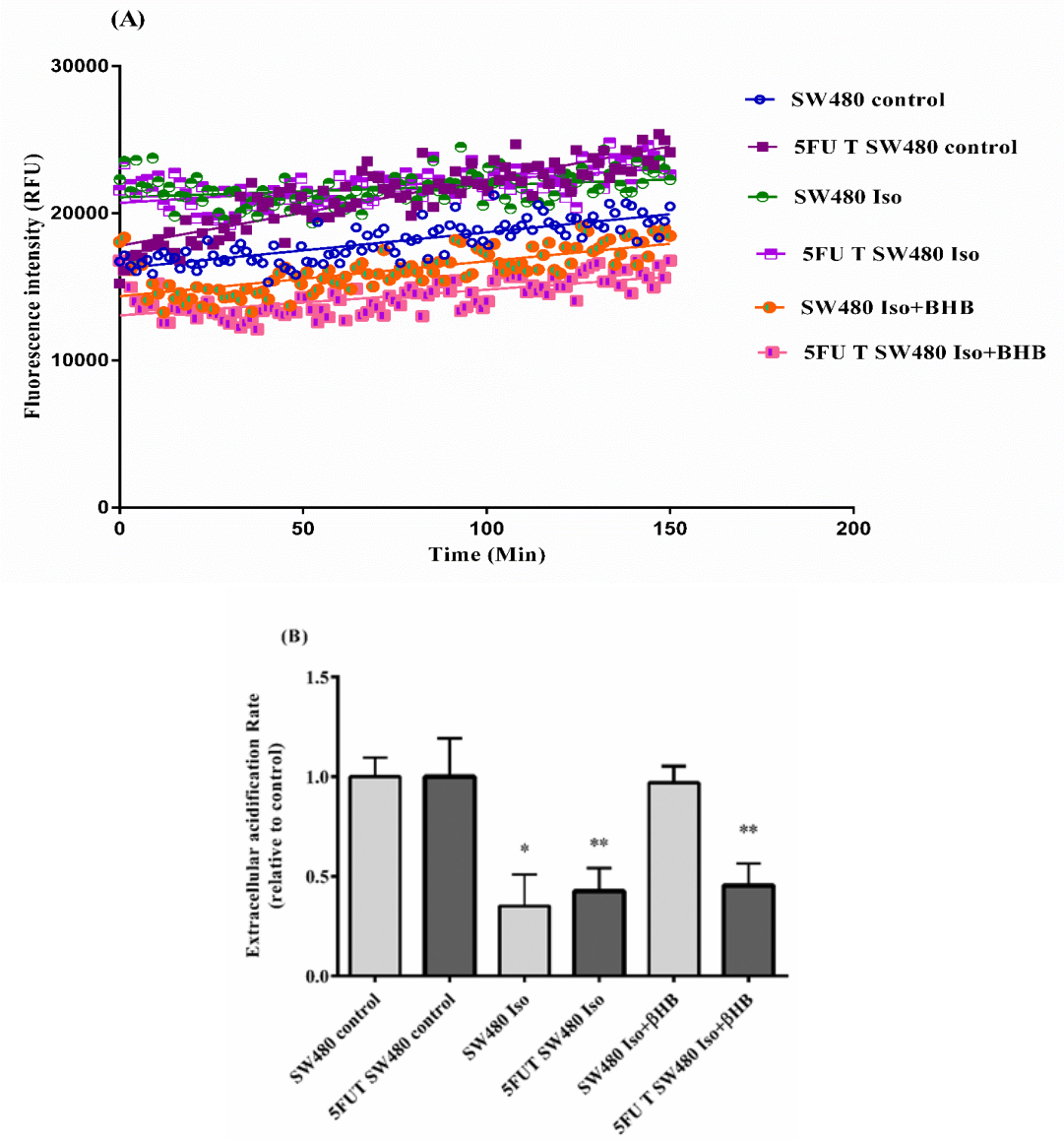


Fig.3. Decrease in extracellular acidification rate after Iso and Iso + βHB treatment in SW480 and 5FU-treated SW480 cells. (A) Real-time responses as the average fluorescent emission value over 120 minutes of fluorophores in the pH-Xtra kit assessed as an indication of ECAR (mean ± SEM, n = 6) in SW480 and 5FU-treated SW480 cells relative to the control (B) ECAR calculated by using the slope of the linear portion of real time-responses of average fluorescence versus time which is normalized to control in SW480 and 5FU-treated SW480 cells. *$p < 0.0001$ *vs.* control in SW480 cells. **$p < 0.0001$ *vs.* control in 5FU-treated SW480 cells.

Analysis of the Expression of Genes

To investigate the correlation between gene expression and the favored metabolic pathway in SW480 cells treated with 5FU and those not treated with 5FU, we assessed the expression of specific gene markers associated with mitochondrial biogenesis, glycolysis, and stemness. These markers included PGC-1α, c-MYC, and NANOG. The analysis was conducted after 72 hours of treatment with Iso and its combination with βHB.

In 5FU-treated SW480 cells, as shown in Figure 4A, the expression of PGC-1α, a master regulator of mitochondrial biogenesis and increased oxidative phosphorylation metabolism, significantly decreased

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($p<0.0001$) after Iso and Iso in combination with βHB treatments. The expression of oncogenic c-MYC which is assumed to be associated with the glycolytic program showed a significant increase compared to the control after Iso and Iso in combination with βHB treatments ($p<0.05$). A significant increase was also observed in the expression of the stemness marker gene NANOG after treatment with Iso and its combination with βHB, compared to the control ($p < 0.05$).

In non-5FU-treated SW480 cells, as shown in Figure 4B, the expression of PGC-1α significantly decreased compared to the control in both Iso and Iso plus βHB treatments. The highest decrease was observed after treatment with only Iso ($p<0.0001$). Iso treatment did not make any significant difference in the expression of c-MYC compared to the control. However, treatment with Iso plus βHB significantly reduced c-MYC expression ($p<0.001$). No significant difference was observed in NANOG expression among these cells after Iso and Iso plus βHB treatments.

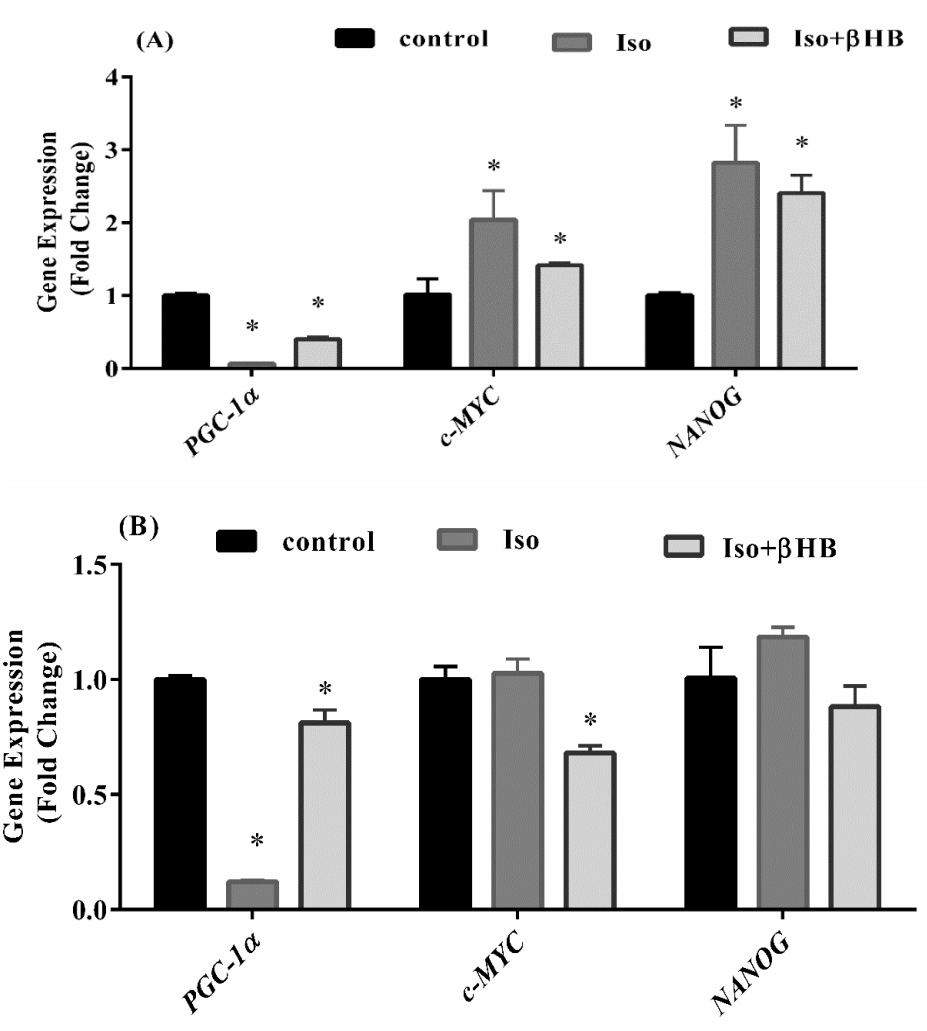


Fig.4. Relative gene expression. (A) PGC-1α, c-MYC, and NANOG relative mRNA expression levels determined by real-time PCR after 72 h treatment with Iso and Iso + βHB in 5FU-treated SW480 cells (B) Relative expression of mRNA of above-mentioned genes after 72 h treatment with Iso and Iso + βHB in SW480 cells. Each column represents the mean ± SEM of data from two independent experiments in duplicate reactions. *$p < 0.05$ *vs.* control.

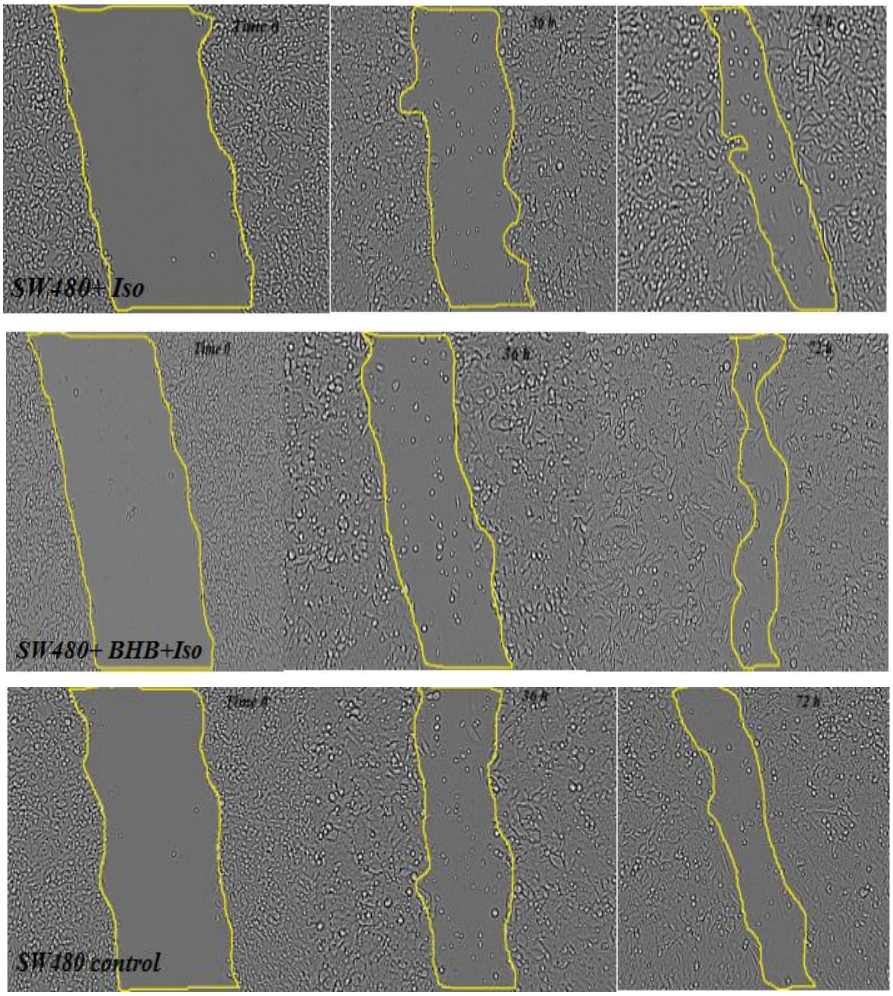
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Cell Migration

The effects of treatment with Iso and Iso plus βHB on cell migration were evaluated using a scratch assay. The cells from both groups migrated to the scratched area, and the percentage of scratch closure was calculated at 0, 36, and 72 hours (Figure 5A and 5B). In SW480 cells, treatment with Iso and Iso plus βHB did not significantly alter the migration compared to the control (Figure 5C). However, as seen in Figure 5D, in 5FU-treated SW480 cells, treatment with Iso plus βHB significantly increased migration compared to the control after 36 hours ($p < 0.05$). However, Iso alone did not result in a significant difference in scratch closure percentage. Interestingly, after 72 hours, the migration rate of the cells in both groups was significantly higher than that of the control group ($p < 0.05$).

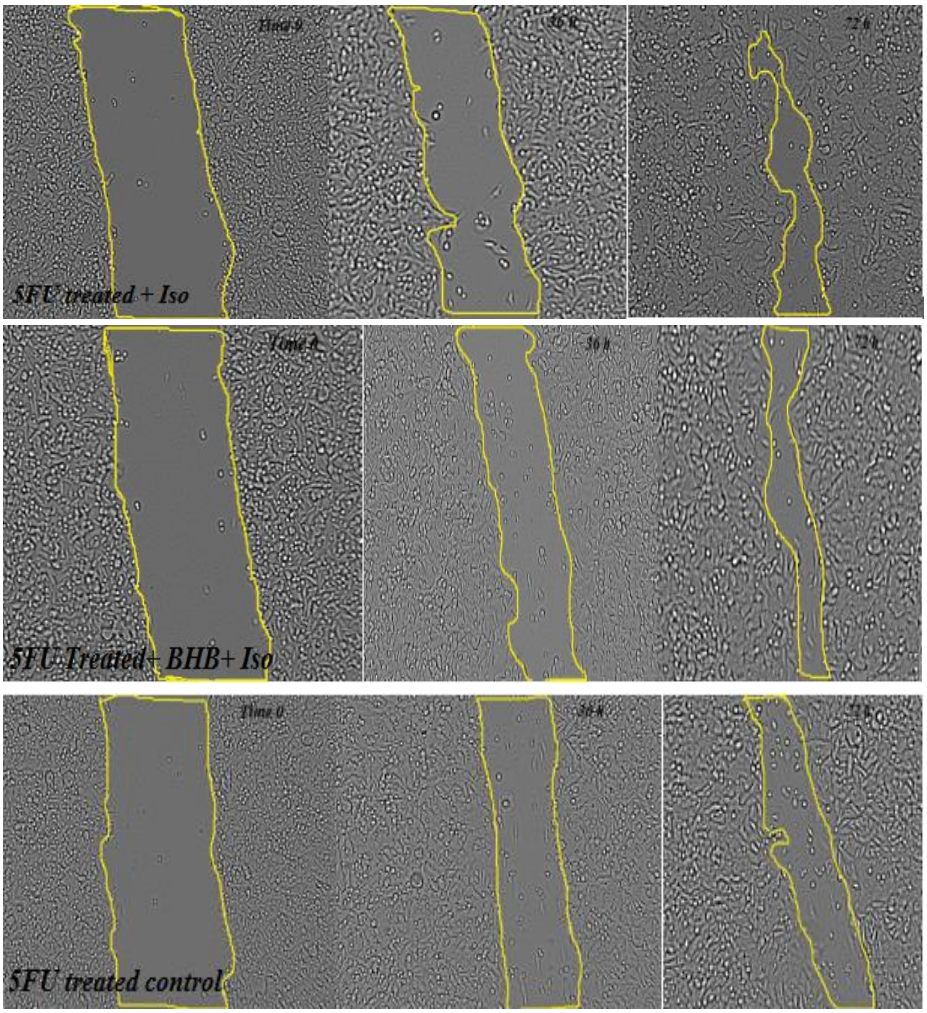
(A)



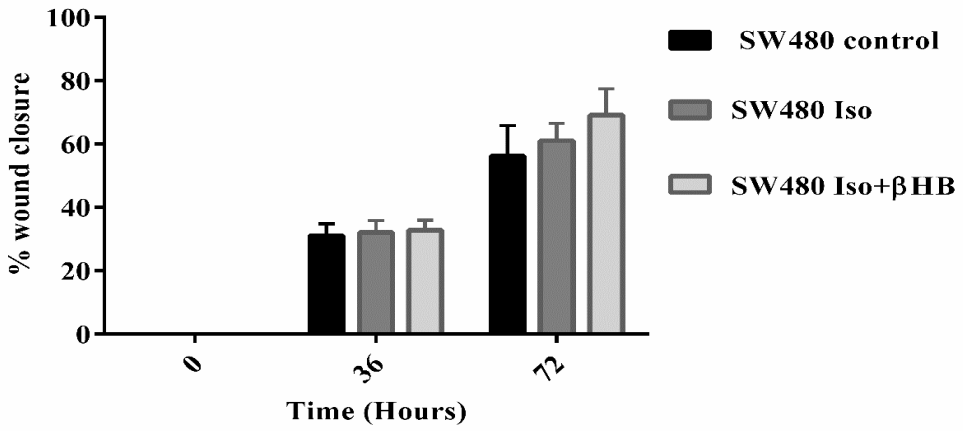
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(B)



(C)



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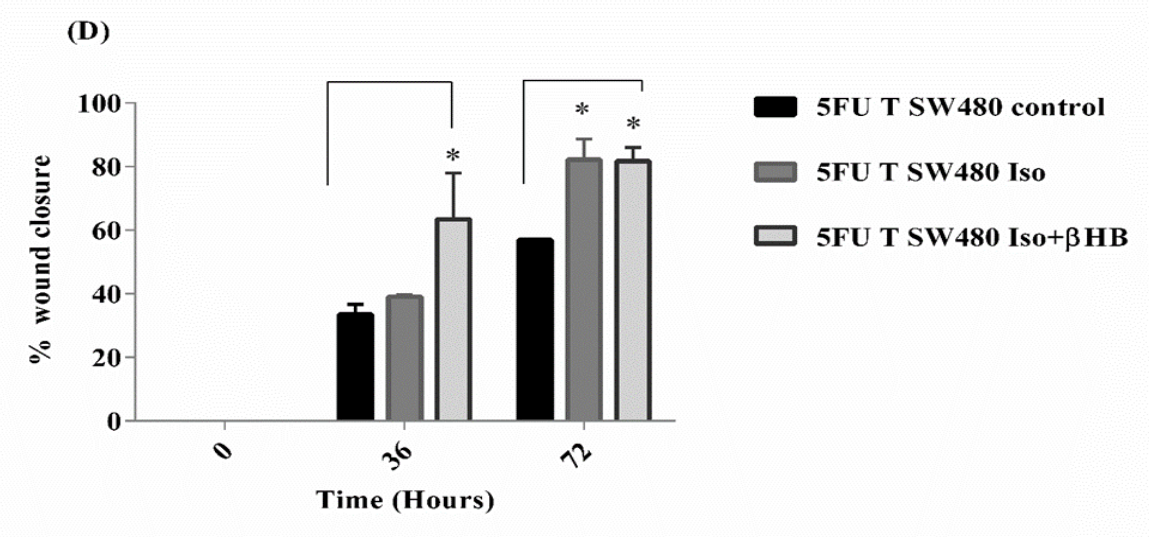


Fig.5. Migration of SW480 and 5FU-treated SW480 cells. (A) Photographs of wounded SW480 cells. The photos were captured immediately (Time = 0) after scratching and after 36 and 72 h following Iso treatment using an inverted microscope equipped with a digital camera. The area restricted to lines indicates the boundary of migrated cells at the desired time point (B) Photographs of wounded 5FU-treated SW480 cells (C) The effect of βHB treatment in migration was plotted as a percentage of wound closure in SW480 cells (D) Migration as a percentage of wound closure in 5FU-treated SW480 cells. The results are provided as mean values with standard deviations from at least three independent experiments. *$p < 0.05$ *vs.* control after 36 and 72 h.

Discussion

In this study, we discovered that Iso can shift metabolism towards oxidative phosphorylation to provide suitable survival and migration conditions under the presence or absence of βHB in 5FU-treated SW480 colon cancer cells.

Our results agree with the vast majority of studies conducted in this area of medicine suggesting that beta-adrenergic receptor agonists may cause an increase in SW480 cell survival. (15) In addition, we also observed that the presence of βHB in combination with Iso caused an increase in cell survival. Several downstream mechanisms are suggested for the pro-cancerous effects of beta-adrenergic stimulation in tumor cells. These mechanisms include the β-arrestin-dependent activation of STAT3 and pro-inflammatory signaling, cyclic AMP/PKA-dependent phosphorylation, activation of CREB/ATF, GATA transcription factors, and the cyclic AMP/EPAC-associated ERK1/2 pathway (5). It has been shown that the activation of β-adrenergic receptors promotes the acceleration of metastasis and invasiveness in many types of tumor cells, including those found in breast, pancreatic, and prostate cancer (17-19). We have previously shown that βHB accelerates the proliferation and invasiveness of 5FU-treated SW480 cells. (16) In this study, we found that beta-adrenergic receptor agonists enhance the effect of βHB on the growth and migration capacity of cells treated with 5FU. Furthermore, previous studies have shown that beta-adrenergic receptor antagonists can suppress the growth, movement, and infiltration of cancer cells in the breast, colon, and liver (20). Moreover, it seems that the beta-adrenergic system enhances the migratory capacity of these cells by reprogramming their metabolism and suppressing the immune system to facilitate metastasis (21).

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Our study results indicated that Iso and the combination of Iso with βHB treatment significantly increased oxidative phosphorylation and reduced glycolysis in 5FU-treated SW480 cells compared to the untreated group. It remains unclear whether glycolysis or oxidative phosphorylation is the dominant metabolic pathway amongst cancer stem cells, but the results of studies in this area remain contradictory. While cancers such as lung and colon tumors, which are similar to somatic cells, are dependent on oxidative phosphorylation, embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and certain cancers like breast and liver rely primarily on glycolysis (22-28). In our previous study, we observed that treating 5FU-treated SW480 cells with βHB resulted in an approximately 5-fold increase in oxidative phosphorylation. However, in this study, despite increasing oxidative phosphorylation and reducing glycolysis, Iso did not exacerbate the effect of βHB on oxidative phosphorylation. It seems that tumor cells do not rely solely on glycolysis and, it is clear that at least a subgroup of them is able to shift their metabolism toward oxidative phosphorylation based on their microenvironment.

The study of gene expression patterns associated with metabolism showed a decrease in the expression of PGC-1α and an increase in the expression of c-MYC, after treatment with Iso. Sancho *et al*. demonstrated that the decrease in c-MYC expression was linked to a reduction in glycolysis, achieved by downregulating glycolytic enzymes. This, in turn, led to an upregulation of PGC-1α expression and oxidative phosphorylation (27). On the other hand, c-MYC has been recognized as a transcription factor that promotes growth and metastasis in tumors. Increased lactate production may be one of the mechanisms causing these effects, although the role of lactate metabolism is not well understood. In principle, this factor induces oxidative phosphorylation by promoting mitochondrial biogenesis and increasing glucose utilization as a metabolic substrate (29). In general, depending on the existing conditions, it seems that Iso, can alter the metabolic adaptation of cancer cells to survive and grow. Nevertheless, further molecular research is needed to clarify the mechanism and effect of this interaction on cancer cell metabolism.

Our results indicated that treatment with Iso and the combination of Iso with βHB in 5FU-treated SW480 cells caused an increase in the expression of the NANOG transcription factor. NANOG is a well-known stem cell marker that plays a role in tumor progression. However, the mechanisms of cancer pathogenesis are still not fully understood. A study by Chen et al. linked NANOG with the reprogramming of mitochondrial metabolism and the inhibition of mitochondrial ROS production. These processes contribute to preserving the ability of self-renewal and metabolic programming, which induce resistance to chemotherapy in liver cell carcinoma (30). It appears that the increase in stemness following upregulation of NANOG, along with c-MYC after Iso treatment, is consistent with other studies in this field.

Fluorouracil is commonly used as the initial treatment for most types of cancer. Studies have shown that it can stimulate mitochondrial biogenesis and induce shifts in metabolism towards oxidative phosphorylation by activating AMPK and PGC1-α. It has been found that the SIRT1/PGC1-α axis plays a role in resistance to chemotherapy and increased oxidative phosphorylation in metastatic colon cancer cells. Therefore, it may be possible to attribute some of the differences in the gene expression and other outcomes between the two cell groups to the effects of 5FU (31-35). It should be noted that our study does not provide information on the mechanism of these phenomena. Stress and sympathetic nervous system activation play a role in tumorigenesis, cancer progression, and metastasis. Long-term use of beta-blockers can decrease

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tumor progression in several types of cancers. Due to limited research, further investigations, particularly regarding protein expression, are necessary to elucidate the specifics.

The findings of this research indicate that administering Isoprotrenol to SW480 colon cancer cells treated with 5FU, which serve as a representation of stressed cells, leads to an increase in the utilization of oxidative phosphorylation as a metabolic pathway. Additionally, it enhances the capacity of cells to survive and migrate. Therefore, based on the findings of our study, which are consistent with numerous other studies in this field, stress and the use of beta-adrenergic receptor agonists may contribute to the development of cancer. On the contrary, it seems that beta-blockers have been found to have a beneficial effect on tumor progression in various types of cancers when used over a long period of time. Further research is needed to understand the mechanisms and effects of these interactions on cancer cell metabolism.

Acknowledgments

The study was funded by Shahid Beheshti University of Medical Sciences, Tehran, Iran (grant number 10242-8-1). The present authors would like to express their gratitude to their colleagues of the Laboratory of Cellular and Molecular Nutrition Research, National Nutrition and Food Technology Research Institute, Faculty of Nutrition Science and Food Technology, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

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