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Fucoxanthin induces apoptosis in acute lymphoblastic leukaemia cell line by damaging the endoplasmic reticulum

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Abstract

PURPOSE: The purpose of this study was to examine the impact of fucoxanthin on acute lymphoblastic leukemia (ALL) cell line Nalm6 and the roles played by mitochondria and the endoplasmic reticulum in this process.

METHODS: *In vitro*experiments were conducted using the acute lymphoblastic leukemia cell line Nalm6 to evaluate the cytotoxic effects of fucoxanthin and dexamethasone, both as individual agents and in combination. The mechanism of action of fucoxanthin on ALL cell lines was investigated using MTT assays, flow cytometry, Seahorse assays, western blot and transmission electron microscopy.

RESULTS: Compared to the control group, fucoxanthin significantly suppressed the proliferation of Nalm6, triggered apoptosis by regulating the expression of apoptosis-related protein, and arrested the cell cycle at the G0/G1 phase in the in vitro experiments. When combined with the clinical drug dexamethasone, fucoxanthin exhibited synergistic effects in inducing apoptosis. Transcriptomics sequencing revealed that the oxidative phosphorylation and protein processing in endoplasmic reticulum pathway was notably affected. Following treatment with fucoxanthin, the mitochondrial membrane potential became abnormal, the mitochondrial ATP consumption rate decreased, and the mitochondrial ATP production rate were reduced. Mitochondrial damage, including the disappearance of mitochondrial cristae and matrix swelling, was observed via transmission electron microscopy, along with signs of endoplasmic reticulum stress. The Western blot results indicate that the GRP78-PERK-CHOP signalling axis is activated following treatment with fucoxanthin. The co-application of the calcium chelator BAPTA-AM with fucoxanthin mitigated the mitochondrial damage caused by fucoxanthin.

CONCLUSION: Our findings suggest that fucoxanthin induces apoptosis by initiating endoplasmic reticulum stress, which subsequently leads to mitochondrial damage. Fucoxanthin holds potential for further investigation as a treatment for acute lymphoblastic leukemia.

Introduction

Acute lymphoblastic leukemia is a highly heterogeneous group of hematological malignancies that originate from hematopoietic stem cells. Treatment strategies, guided by cytogenetic and molecular biological features and risk stratification, have significantly improved the prognosis of patients with ALL in recent years. However, the prognosis for adult patients with ALL remains poor[1]. This poor prognosis is often associated with the emergence of resistance to the current first-line chemotherapy regimen, leading to relapse of the patient's ALL condition that is refractory to treatment. Additionally, chemotherapy-related adverse effects are also closely related to patient prognosis, and in both pediatric and adult ALL patients, some have developed serious and even life-threatening adverse drug reactions after chemotherapy[2]. Therefore, there is a need to explore a novel anticancer active substance that has few side effects on patients and can act in combination with current chemotherapeutic agents.

Fucoxanthin is a naturally occurring marine carotenoid primarily found in brown algae and some microalgae. The chemical structure of fucoxanthin features a unique allylic bond and an epoxy group, which bestow upon it potent anti-inflammatory, antioxidant, and anticancer properties[3]. Consequently, it has garnered significant interest in the food and medical fields. Despite demonstrating robust biological activity and pharmacological effects in vitro, fucoxanthin exhibits poor stability in vivo, similar to most carotenoids. It is metabolized in the gut to the alcohol derivative fucoxanthinol (Fxol), which is then absorbed by the body and further metabolized to amarouciaxanthin A in the liver, with very little pure fucoxanthin being directly absorbed[4]. To enhance the bioavailability of fucoxanthin, researchers have developed several methods to improve drug absorption, such as mixtures of fish oil and mesoacyltriolglycerol[5], combinations of oleic acid and bovine serum albumin[6], and natural emulsion-based delivery systems with various types of emulsifiers[7]. These methods can promote the stabilization of fucoxanthin. Consequently, numerous animal and clinical trials involving fucoxanthin have been conducted in recent years. These trials have shown that fucoxanthin does not induce any significant toxic side effects in experimental subjects, whether in human clinical trials or in vivo experiments with mice[8, 9].

The anticancer activity of fucoxanthin has been demonstrated in several tumor models, including breast cancer[10], osteosarcoma[11], melanoma[12], and others. In these studies, fucoxanthin was able to induce apoptosis by affecting several signaling pathways. In MT4, a common adult T-cell leukemia (ATL) cell line, it induced apoptosis by decreasing the expression of the BCL2 anti-apoptotic protein, blocked the cell cycle by decreasing the expression of CDK2, CDK4, and cell cycle protein D1, and inhibited the NF-κB signaling pathway[13]. However, there are fewer research reports on the effects of fucoxanthin in acute lymphoblastic leukemia, and its killing effect and mechanism on this type of leukemia remain unclear, meriting further study.

Before conducting the present study, our technical method for extracting fucoxanthin from Laminaria japonica had been thoroughly verified[14]. Building upon this, we further investigated the mechanism of action of fucoxanthin. In this paper, we used the B-ALL cell line Nalm6 as cell model to investigate the anticancer mechanisms of fucoxanthin on the B-ALL cells *in vitro*. We also explored the synergistic effects and mechanisms between fucoxanthin and dexamethasone, a widely used clinical drug in ALL. Based on the findings, we believe that fucoxanthin is a natural compound with potential clinical applications.

Results

Inhibition of cell viability of acute lymphoblastic leukaemia cell lines by fucoxanthin

To confirm the cytotoxic effect of fucoxanthin on the B-ALL cell line Nalm6, we assessed the impact of fucoxanthin on the viability of Nalm6 cells after 48 hours of treatment using MTT assays. As depicted in Figure 1A, various concentrations of fucoxanthin were incubated with the B-ALL cell line for 48 hours.

The IC_{50} for the Nalm6 cell line was calculated by GraphPad Prism and determined to be 8.383±0.213 μM. Considering this result, the concentrations of fucoxanthin investigated in this study were 2.5 μM, 5 μM, and 10 μM. Building upon this, we proceeded to evaluate the influence of fucoxanthin on the proliferation rates of Nalm6 at 24, 48, and 72 hours post-treatment. As illustrated in Figures 1B, two-way ANOVA indicated that both the duration of treatment and the concentration of the drug significantly inhibited the proliferation rates of the cells.To further investigate the inhibitory effect of fucoxanthin on cell growth, we analyzed cell cycle changes using flow cytometry. The group treated with 10μM fucoxanthin exhibited an excessively high proportion of cell death, making it impossible to plot the cell cycle data; therefore, the result was not included in the discussion. As shown in Figure 1C and 1D, the proportion of cells in the G0/G1 phase significantly increased, while the proportion in the S phase correspondingly decreased, indicating that fucoxanthin inhibits proliferation by blocking the cell cycle at the G0/G1 phase.

Fucoxanthin induces apoptosis by regulating the expression levels of proteins in the BCL2 apoptosis protein family.

To further investigate the effects of fucoxanthin on the biological behavior of ALL cells, we employed flow cytometry to assess the apoptosis alterations in ALL cells influenced by fucoxanthin. As shown in the figure 2A and 2B, flow cytometry analysis revealed significant apoptosis in cells treated with fucoxanthin, exhibiting concentration-dependent characteristics. The BCL-2 apoptosis protein family serves as the most classical indicator of apoptosis[15]. Therefore, we employed Western blotting to detect changes in the expression levels of the apoptosis-related proteins BCL2, BAX, and caspase-3 in both the control group and the fucoxanthin-treated group. The results, presented in Figures 2C and 2D, demonstrate that fucoxanthin-induced activation of caspase-3 was increased, while the expression of the anti-apoptotic protein BCL-2 was reduced and that of the pro-apoptotic protein BAX was elevated, indicating active apoptosis in the cells under fucoxanthin treatment.

The combination of fucoxanthin and dexamethasone has synergistic killing effect on ALL cells.

In the previous study, we demonstrated through flow cytometry and Western blot that fucoxanthin can induce apoptosis by regulating the expression of the BCL2 apoptosis protein family. Currently, dexamethasone, the most commonly used glucocorticoid in clinical practice, plays an indispensable role in chemotherapy regimens due to its ability to induce apoptosis in ALL cells [16]. Based on this, as a drug capable of inducing apoptosis, we hypothesize that fucoxanthin may exhibit a synergistic effect with dexamethasone in jointly inducing apoptosis. To validate this hypothesis, we initially determined the IC_{50} of dexamethasone on ALL cells and subsequently chose appropriate concentrations of both dexamethasone and fucoxanthin based on these results. Additionally, we assessed the combined cytotoxicity of dexamethasone and fucoxanthin on Nalm6 cells and calculated the synergy score using the ZIP model. As illustrated in Figure 3A and 3B, the combination of dexamethasone and fucoxanthin exhibited a potent synergistic effect in killing cells. To delve deeper into whether the two drugs synergize in inducing apoptosis, we examined the apoptosis rate of cells treated with each drug individually and in

combination. The findings presented in Figure 3C indicate that the co-application of both drugs significantly enhances the apoptosis rate, surpassing the effects of either drug administered alone ($P<0.05$).

Transcriptomic sequencing results indicate mitochondrial and endoplasmic reticulum damage following fucoxanthin treatment

Nalm6 cells underwent transcriptomics sequencing after being treated with 5 μM fucoxanthin for 24 hours. As illustrated in Figure 4A, the Nalm6 group yielded a total of 5780 differentially expressed genes (DEGs), with 2180 up-regulated and 3600 down-regulated, compared to the control group. KEGG enrichment analysis of the aforementioned differentially expressed genes, as shown in Figure 4B, revealed enrichment of apoptosis signaling pathways and cell cycle signaling pathways within the Cellular Processes category, consistent with previous experimental findings[17]. To further investigate the mechanism of fucoxanthin's cellular effects, we examined potential mechanisms in Genetic Information Processing and Metabolism categories. Specifically, protein processing in the endoplasmic reticulum and oxidative phosphorylation showed higher enrichment scores across more differentially expressed genes. GESA enrichment curve analysis demonstrated that fucoxanthin induces positive enrichment in protein processing within the endoplasmic reticulum and negative enrichment in oxidative phosphorylation, suggesting potential mitochondrial dysfunction and endoplasmic reticulum stress under fucoxanthin exposure—a finding requiring further experimental validation.

Fucoxanthin affects mitochondrial function in ALL cells

To verify the effect of fucoxanthin on mitochondrial function, we investigated the impact of fucoxanthin on the mitochondrial membrane potential of ALL cells using flow cytometry. The results, depicted in Figure 5A and 5B, revealed that the proportion of cells with abnormal mitochondrial membrane potential increased with the concentration of fucoxanthin, demonstrating a concentration-dependent effect($P<0.05$). Furthermore, we plan to conduct the Seahorse assay to examine, on one hand, the changes in mitochondrial function under fucoidan treatment, and on the other hand, the alterations in intracellular ATP generation rates under fucoxanthin stress. As shown in Figures 5C and 5D, under fucoxanthin treatment, the basal respiration, ATP production, and maximal respiration levels of mitochondria were significantly reduced; the mitochondrial ATP generation rate decreased, while the cytoplasmic glycolytic ATP generation rate increased, resulting in a overall reduction in the total intracellular ATP generation rate.

Fucoxanthin affects endoplasmic reticulum function in ALL cells and induces intracellular calcium overload to damage mitochondria

To further examine the impact of fucoxanthin on mitochondria, we observed morphological changes in mitochondria using transmission electron microscopy. As illustrated in Figure 6A, mitochondrial morphology in Nalm6 cells was notably altered after fucoxanthin treatment (indicated by red arrows), with changes encompassing the rupture of mitochondrial membranes, a reduction and disappearance of

mitochondrial cristae. Furthermore, we noted the presence of numerous vesicular vacuoles(indicated by black arrows) in cells post-fucoxanthin treatment, indicating that endoplasmic reticulum stress occurs, leading to fragmentation of the endoplasmic reticulum. Next, we employed Western blot to analyze changes in the expression levels of the classic endoplasmic reticulum stress signaling pathway—GRP78-PERK-EIF2A-ATF4-CHOP—as shown in Figures 6B–D. Under 10μM fucoxanthin treatment, the expression levels of all proteins in this signaling pathway were significantly elevated, demonstrating that fucoxanthin induces endoplasmic reticulum stress in cells. Moreover, we monitored the changes in intracellular calcium ion levels under the influence of fucoxanthin. The findings indicated that the intracellular calcium ion concentration increased after 1H of fucoxanthin treatment($P<0.05$), and the level of intracellular reactive oxygen species (ROS) also rose ($P<0.05$). To ascertain the causal relationship between mitochondrial and endoplasmic reticulum damage, we employed the calcium chelator BAPTA-AM to sequester calcium ions, aiming to mitigate their detrimental effects on mitochondria. If the cellular ROS levels were not significantly reduced by the concurrent use of BAPTA-AM and fucoxanthin, it would suggest that mitochondrial damage is not linked to endoplasmic reticulum damage. Instead, mitochondrial damage appears to be closely related to elevated calcium ion levels. The results, as depicted in Figure 6G, showed that the intracellular ROS content was diminished following the combined application of BAPTA-AM and fucoxanthin ($P<0.05$).

Discussion

- Acute lymphoblastic leukaemia (ALL) is a neoplastic disease arising from immature lymphocytes of the B-cell or T-cell lineage. Patients with this condition primarily present with symptoms of haematopoietic dysfunction, including anaemia, bleeding, infection and extramedullary infiltration. In recent years, research into ALL has made significant progress in the fields of genomics, transcriptomics and epigenomics[18]. The main drugs currently used in clinical practice include glucocorticoids, cytarabine, anthracyclines, cyclophosphamide and targeted therapies. Consequently, whilst treatment for ALL aims to eradicate tumour cells, it also imposes a certain burden on the body [19]. Therefore, the development of a drug that can produce synergistic effects with existing chemotherapeutic agents in eliminating tumour cells, whilst causing no significant adverse reactions to the body, holds significant clinical importance.
- Fucoxanthin is essentially a carotenoid synthesised primarily from β-carotene through a series of complex chemical reactions; consequently, its main structural features include an epoxide ring, multiple hydroxyl groups and unique isoprene functional groups, amongst others. These complex chemical groups confer upon fucoxanthin a strong ability to scavenge oxygen free radicals, endowing it with antioxidant activity[20], which has attracted widespread attention within the scientific community in recent years. In normal cells, the presence of ROS is a key component of the intracellular redox balance and plays a role in important functions such as cell signalling [21]. ROS is also closely associated with the biological behaviour of tumour cells, including proliferation and differentiation. A wealth of research data indicates that ROS levels are elevated in tumour cells; however, this does not lead to cell death but rather promotes tumour cell proliferation by activating

growth-related signalling pathways[22]. At the same time, ROS provides the intracellular environment that facilitates drug resistance in tumour cells. Research suggests that moderate levels of ROS can influence the expression of drug efflux pumps and anti-apoptotic proteins by regulating signalling pathways, thereby causing cells to develop drug resistance[23]. In summary, the differences in ROS levels and their physiological significance in normal and tumour tissues provide a theoretical basis for the anticancer application of antioxidant drugs.

- The redox balance within the endoplasmic reticulum provides an optimal environment for protein folding. The endoplasmic reticulum exhibits a more oxidising environment than the cytoplasm, owing to the formation of disulphide bonds, which generates the by-product H_2O_2 [24]. Extensive research indicates that disruption of this balance affects protein folding and protein activity within the endoplasmic reticulum, thereby triggering the unfolded protein response (UPR) and leading to endoplasmic reticulum stress[25]. In this study, we confirmed that fucoxanthin induces endoplasmic reticulum stress within cells. Given the antioxidant activity of fucoxanthin, we propose that upon entering the cell, it disrupts the redox balance within the endoplasmic reticulum, thereby triggering endoplasmic reticulum stress. Endoplasmic reticulum stress is closely associated with mitochondrial damage and apoptosis. Once endoplasmic reticulum stress occurs, it can induce cell apoptosis through multiple mechanisms. Firstly, following the upregulation of the endoplasmic reticulum stress marker protein GRP78, downstream CHOP is upregulated via various signalling pathways, such as the PERK/ATF4 axis and the IRE1/MAPK axis, thereby mediating changes in the expression levels of the BCL2 family of apoptotic proteins and leading to cell apoptosis[26]. Secondly, massive calcium efflux from the endoplasmic reticulum leads to intracellular calcium overload, which disrupts the mitochondrial membrane potential, causing mitochondrial damage and the release of substances such as cytochrome C into the cytoplasm, thereby inducing cell apoptosis[27]. Therefore, we believe that the antioxidant activity of fucoxanthin, by influencing endoplasmic reticulum function, may offer new insights and approaches for cancer treatment.
- Combination therapy is the most commonly used treatment strategy in the clinical management of cancer. Compared with monotherapy, the combined use of anticancer drugs with different mechanisms of action can achieve a better tumour-killing effect at the same concentration; or, by reducing the drug concentration, it can minimise adverse reactions whilst achieving the same or even better anticancer efficacy[28]. This study aims to further validate the synergistic cytotoxic effects of fucoxanthin and conventional chemotherapeutic agents; therefore, dexamethasone was selected as the partner drug for the combination therapy. This drug was chosen because dexamethasone is the most commonly used chemotherapeutic agent for acute lymphoblastic leukaemia (ALL); it induces apoptosis in ALL cells whilst having minimal impact on normal cells—a characteristic that is widely utilised in ALL treatment regimens[29]. The mechanism by which dexamethasone eradicates acute lymphoblastic leukaemia (ALL) cells has been extensively studied: it binds to the glucocorticoid receptor (GR) to form a complex; upon entering the cell nucleus, this complex functions as a transcription factor, thereby influencing protein expression, specifically by downregulating anti-apoptotic proteins and upregulating pro-apoptotic proteins[30]. However, in

glucocorticoid-resistant cell lines, the primary causes of resistance are low expression levels of the glucocorticoid receptor GRγ[31] and elevated expression levels of BCL2[32]. In this study, we experimentally demonstrated that fucoxanthin can act in concert with dexamethasone to induce cell apoptosis. This suggests that inducing cell apoptosis through different pathways offers greater efficacy in the combined killing of tumour cells. This indicates that fucoxanthin holds potential for future clinical applications.

- At present, we believe that, on the one hand, fucoxanthin has not caused any apparent toxic side effects in experimental subjects; on the other hand, in vitro studies suggest that its combination with dexamethasone has potential clinical value, making it a natural compound with promising clinical applications. However, this study has limitations. Although fucoxanthin did not induce any significant toxic side effects in other in vivo experiments, there are differences between the animal models used and the acute lymphoblastic leukaemia model; further research is required to determine whether fucoxanthin can produce the same effects in an acute lymphoblastic leukaemia animal model as observed in in vitro experiments. In summary, fucoxanthin is a drug with clinical potential; its mechanism of action requires further exploration and warrants more in-depth research.

Methods and materials

Cell culture

Nalm6 cells, an acute B-lymphoblastic leukaemia cell line, were purchased from the American Type Culture Collection (ATCC) and preserved at the Institute of Hematology at Fujian Medical University's Union Hospital. The two cells were cultured by Roswell Park Memorial Institute(RPMI) 1640 culture medium mixed with 10% fetal bovine serum (Cegrogen Biotech GmbH, A0500-3010) and 1% 100X penicillin-streptomycin solution (Tagene, ABK0005C, China), and were cultured in the 37°C cell culture incubator, 5% CO_2.

Seperation and purification of fucoxanthin

The powder of Laminaria japonica Aresch (10kg, Xiamen City, Fujian Province, China) was extracted with 80% aqueous methanol (50 L) at room temperature for 10 minutes and the extract was subsequently evaporated under vacuum to produce the crude extract. This crude extract was eluted with a mixture of petroleum ether and ethyl acetate, with the elution ratio gradually changing from 10:1 to 1:1 to obtain various fractions. The active components were further purified using preparative high-performance liquid chromatography (DAC-80). Fucoxanthin powder was produced by vacuum evaporation of the composition containing fucoxanthin[14]. Utilizing this method, the yield of fucoxanthin was 0.22 grams per kilogram of dried Laminaria. The purity of fucoxanthin exceeded 98%, as determined by high-performance liquid chromatography (HPLC). The fucoxanthin powder was dissolved by dimethyl sulfoxide(MP,0219605580) and stored in the refrigerator at -20℃.

Reagents

Dexamethasone was purchased from the Union Hospital of Fujian Medical University and stored in a refrigerator at 4℃. BAPTA-AM was purchased from MedChemExpress(MCE, HY-100545)，dissolved by dimethyl sulfoxide and stored in the refrigerator at -20℃. MTT solution was prepared by dissolving MTT powder (Beyotime, ST1537-5g) in ddH_2O to a concentration of 5 mg/ml and stored in a refrigerator at 4℃.

Cell viability

The killing effect of fucoxanthin and dexamethasone on the cell lines was detected by MTT. The MTT solution was mixed with the cell-drug mixture in the ratio of 1:10. After complete precipitation of crystals, the supernatant was discarded and DMSO was added to dissolve the crystals. Subsequently, the cell viability was assessed on a multifunctional enzyme marker.

Western blot

The cells were seeded in a 6-well plate and treated according to the control group (using only culture medium) and the fucoxanthin treatment groups (5μM and 10μM). After 24 hours of treatment, the cells were collected, washed with PBS, and centrifuged to obtain cell pellets. The cell pellets were lysed using a cell lysis buffer containing phosphatase inhibitors, protease inhibitors, and PMSF, then centrifuged at 13,000 rpm for 5 minutes at 4°C to collect the supernatant. The protein concentration in the supernatant was determined by the BCA assay, and the corresponding loading volume was calculated. The remaining supernatant was mixed with loading buffer in a 4:1 ratio, homogenized, and treated with a metal bath at 100°C for 5 minutes. The samples were stored at -20°C. Proteins of different molecular weights were separated by SDS-PAGE gel electrophoresis at 80 mV for 30 minutes, followed by 120 mV electrophoresis for 60 minutes. The electrophored gel and adhered proteins were transferred to NC membrane via 360 mA current transfer for 60 minutes. After washed with TBST, the NC membrane was blocked with 5% skim milk for 1 hour, then incubated with primary antibody overnight at 4°C. The NC membrane was removed the next day, washed with TBST, and incubated with secondary antibody for 1 hour at room temperature. After incubation, the membrane was washed again with TBST, developed using a developer solution, and the protein exposure was visualized. The images were processed and analyzed using ImageJ software. The primary antibodies used include: Bcl2(3498, CST),BAX(5023,CST),Tubulin(A12289,abclonal),caspase3(A19654,abclonal),cleaved-caspase3(A28349,abclonal),PERK(24390-1-AP,proteintech),P-PERK(AP1501,abclonal),EIF2A(ET7111-34,Huabio),P-EIF2A(AP0692,abclonal),ATF4(ET1612-37,Huabio),GRP78(ET1706-50,Huabio),CHOP(15204-1-AP,proteintech).

Flow cytometry analysis

The cells were mixed with the drug and placed in a 37℃ cell culture incubator, 5% CO_2 for 24 hours. Cells were subsequently harvested and washed with phosphate buffered saline (Servicebio, G4202-100ML). The washed cells were processed according to the instructions in the kit and assayed on flow cytometry(BD Accuri C6 Plus). The kits for flow cytometry assays used in this article include:

mitochondrial membrane potential assay kit (Beyotime, C2003S), AnnexinV-APC/7-AAD apoptosis assay kit (Elabscience, AK19730), cell cycle assay kit (BD Pharmingen, 550825), reactive oxygen detection kit(Beyotime, S0033M) and Fluo-4 calcium ion detection kit(Beyotime, S1061M).

Transcriptome sequencing

Nalm6 cells were seeded in 6-well plates and pre-treated with fucoxanthin for 24 hours. Next, cells were collected and washed with PBS. After the cells were lysed with TRIZOL (abkbio, abk0005m), they were stored in a -80℃ refrigerator. Transcriptome sequencing was done by Metware(China, Wuhan).

Seahorse mitochondrial function test

The cells were centrifuged into cell pellets and stored in the refrigerator at -80℃.The Seahorse analyzer (Agilent Technologies XFe24) was pre-warmed and the probe plate was hydrated one day before the assay.After the samples were defrosted at room temperature, the cells were processed according to the instructions in the kits. The kits used include: Seahorse XF Real-Time ATP Rate Assay Kit (Agilent technologies, 103592-100) and Seahorse XF Mitochondrial Stress Test Kit (Agilent technologies, 103015-100). After the preparation was done, the mitochondrial function of the cells was assayed in the Seahorse analyzer. The Seahorse assay was done by Genelily, Inc(China, Shanghai).

Transmission electron microscope imaging

Nalm6 cells were seeded in 6-well plates and pre-treated with fucoxanthin for 24 hours. Next, cells were collected and washed with PBS.After that, the cells were resuspended with electron microscope fixative under room temperature for 2 hours, and then stored in the refrigerator at 4℃.The samples were mainly processed through the steps of pre-embedding, fixation, dehydration-permeable embedding, polymerisation, sectioning, and staining, and were viewed and imaged by transmission electron microscope (HITACHI HT7700 80kv) after the treatment.TEM imaging was done by Servicebio(China, Wuhan).

Static analysis

All experiments were repeated at least three times.Unless otherwise specified, the data were processed using Graphpad Prism 9.0 statistical software (GraphPad Software, San Diego, USA): for data satisfying normal distribution, the experimental results were expressed as x ± SD. The comparisons between means were performed using the t-test. For data that did not satisfy normal distribution, the rank sum test was used, and two-way ANOVA was used for data involving two independent variables. $p<0.05$ was considered statistically significant.

Declarations

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CRediT authorship contribution statement

Boheng Wu conceived, designed the study and wrote the original graft. Yan Huang and Xiang Li performed data analysis. Xiaomin Xi assisted in the experimental design. Qing Cai and Yanxin Chen reviewed and edited the manuscript. Jianda Hu, Yiping Zhang and Ping Chen reviewed the manuscript. All authors reviewed and approved the manuscript.

Declaration of competing interest

The authors have no relevant financial or non-financial interests to disclose.

Data Sharing Statement

The Transcriptome sequencing datasets generated and/or analysed during the current study are available in the NCBI repository, https://dataview.ncbi.nlm.nih.gov/object/PRJNA1494775?reviewer=uev8vbh7bbf58glrlcn6i1rct1. The data of the vitro experiments can be reasonably obtained from the corresponding author.

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Figures

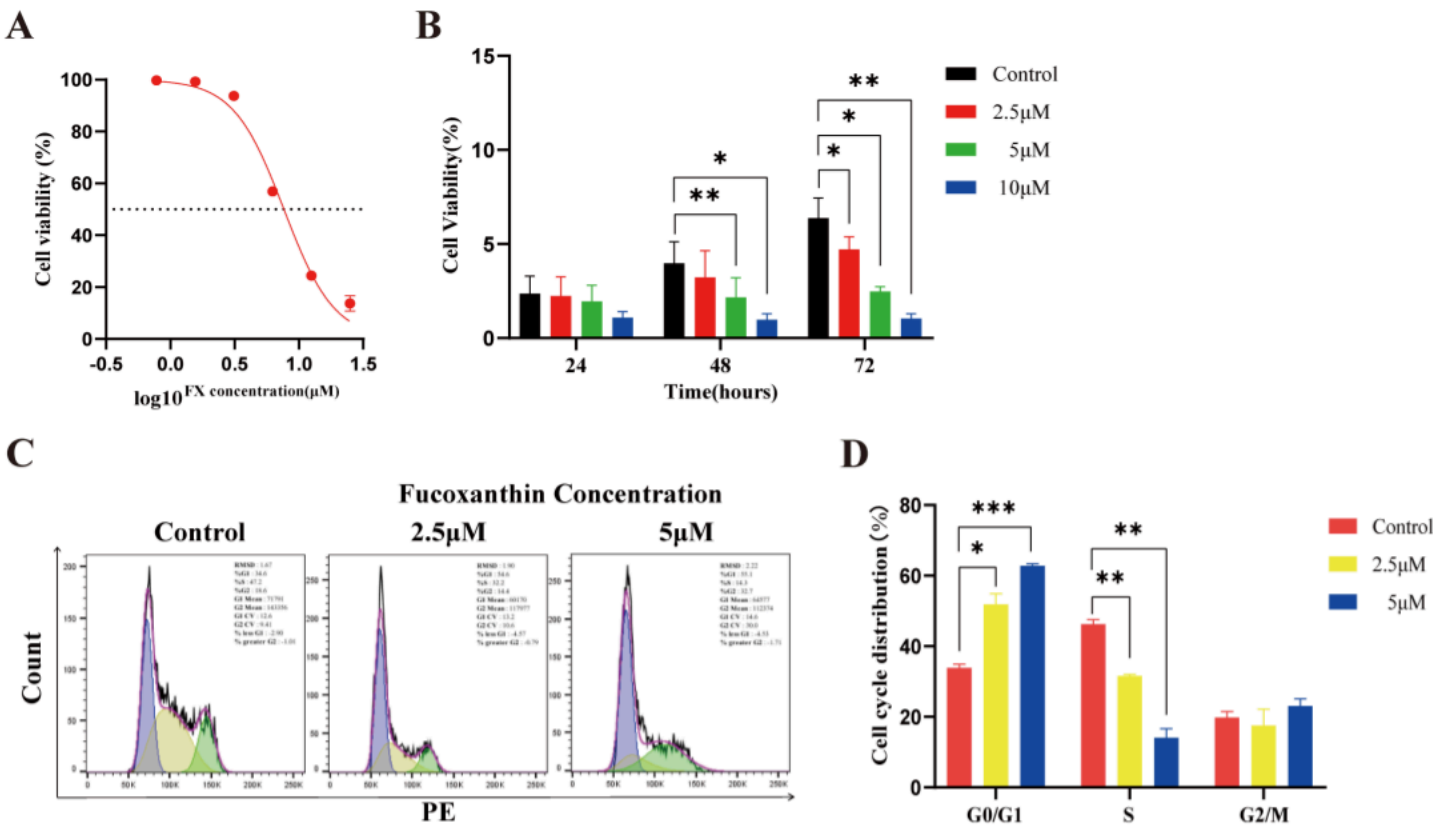


Figure 1

Effect of fucoxanthin on the survival rate of Nalm6 cells. (A) Nonlinear fitting curve of the survival rate of Nalm6 cell lines under fucoxanthin treatment. Nalm6 cells were cultured for 48 hours in media containing either solvent (control group) or 3–50 μM fucoxanthin (experimental groups). Cell viability was assessed using the MTT assay, and the percentage of cells in each experimental group relative to the control group was calculated. The obtained inhibition rates were processed with GraphPad Prism software to generate the nonlinear fitting curve. The dashed line represents the 50% survival rate line, and the x-coordinate of its intersection point with the nonlinear fitting curve corresponds to the IC_{50}. (B) Effects of fucoxanthin treatment for 24,48, and 72 hours on the proliferation rate of Nalm6 cells. (C) Effect of fucoxanthin on the cell cycle of Nalm6 cell lines. Nalm6 cells from both control and experimental groups were cultured with fucoxanthin for 24 hours, fixed with absolute ethanol, stained with PI solution, and analyzed for cell cycle changes using flow cytometry. The results were fitted to the cell cycle using FlowJo software and plotted. (D) Bar chart showing the proportions of cells in the G1/G0, S, and G2/M phases of the Nalm6 cell cycle at different fucoxanthin concentrations. (n=3; *P <0.05, **P <0.01, ***P <0.001).

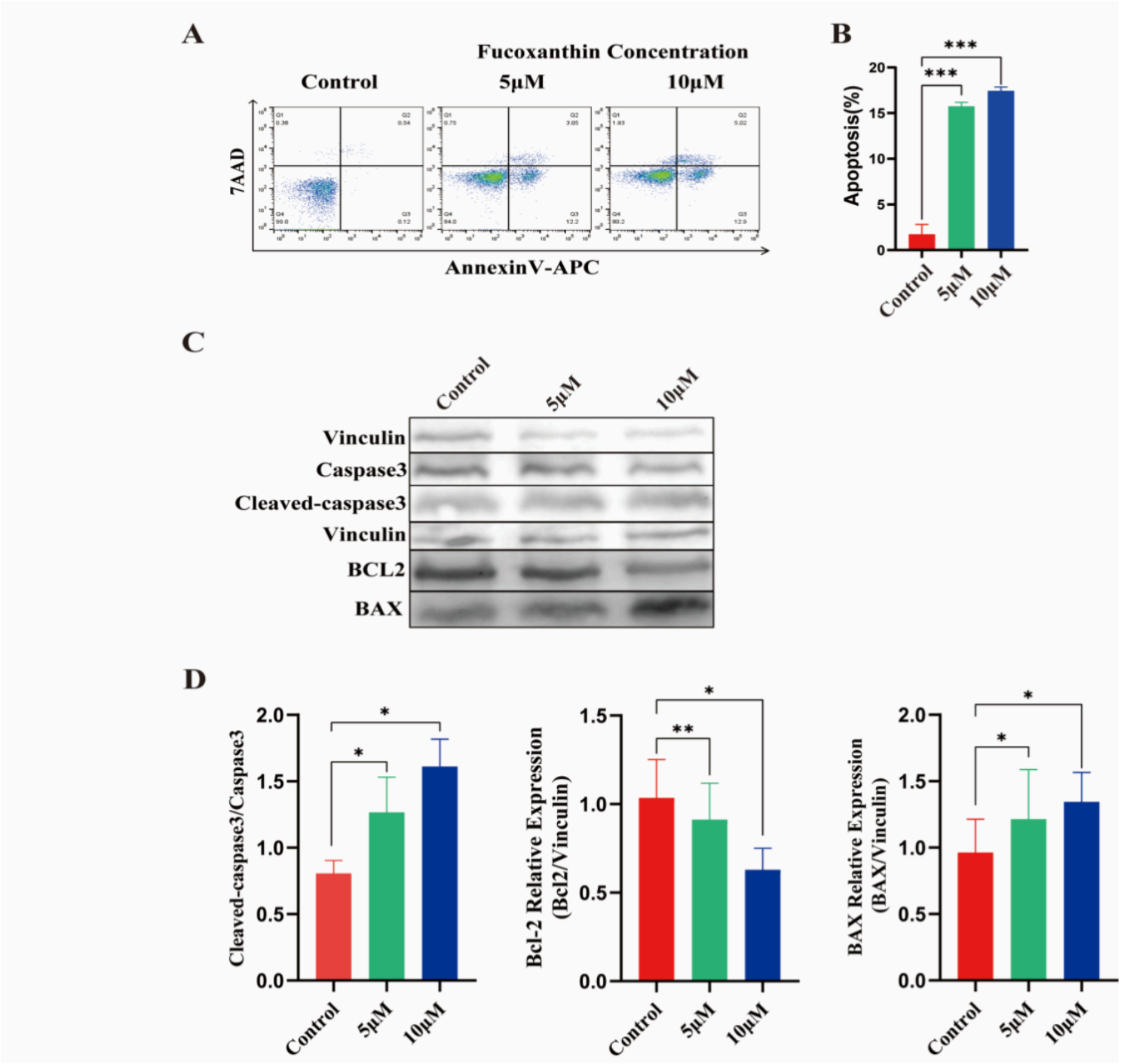


Figure 2

Fucoxanthin regulates the expression levels of the Bcl2 apoptosis protein family to induce apoptosis in Nalm6 cells. (A) Fucoxanthin induces apoptosis in Nalm6 cells. The apoptosis levels were detected using the Annexin V-APC/7AAD double-staining method and flow cytometry. (B)Histogram of apoptosis percentages in Nalm6 cells at different fucoxanthin concentrations. The apoptosis results were analyzed using Flowjo software, and the sum of cell percentages in the Q2 and Q3 quadrants was calculated and presented as a histogram. (C)Western blot experiments confirmed that fucoxanthin regulates the expression levels of apoptosis-related proteins. D. Expression levels of BCL2, BAX, Caspase3, and their activated forms in cells after fucoxanthin treatment (n=3. *P <0.05, **P <0.01).

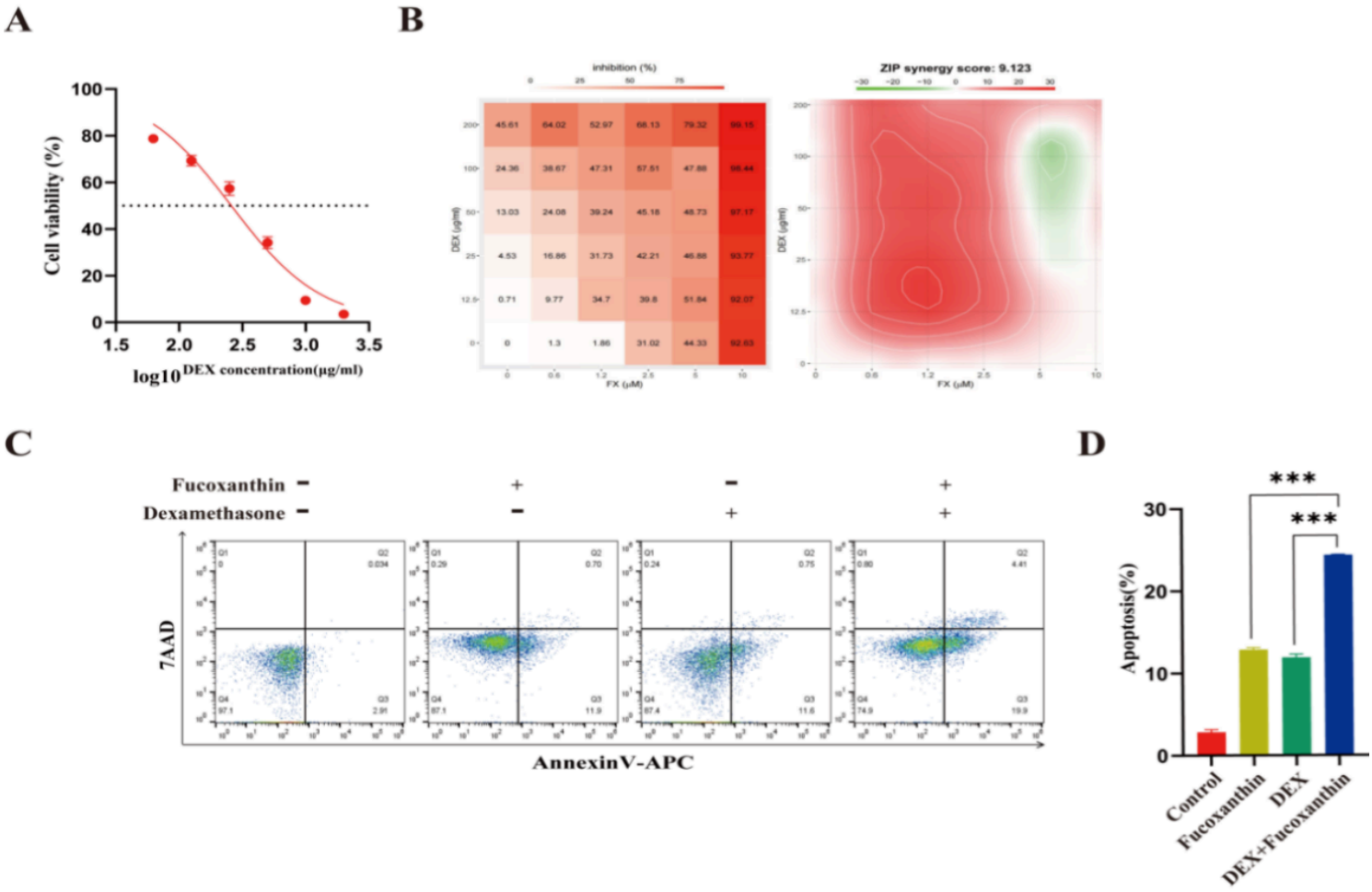


Figure 3

Fucoxanthin and dexamethasone exhibit a synergistic effect in inducing apoptosis in Nalm6 cells. (A)Nonlinear fitting curve of dexamethasone's effect on cell survival rate in the cells (dashed line represents the 50% cell survival rate). (B)Heatmap showing the survival inhibition rates and synergistic index of Nalm6 cells treated with different concentrations of fucoxanthin (0.6–10μM) and dexamethasone (12.5–200μg/mL) in combination. (C)Fucoxanthin and dexamethasone synergistically induce apoptosis in Nalm6 cells. Apoptosis levels were detected using Annexin V-APC/7AAD double staining and flow cytometry in the control group (no fucoxanthin or dexamethasone), experimental groups (treated with 2.5μM fucoxanthin alone, 25 μg/mL dexamethasone alone, or 2.5μM fucoxanthin and 25μg/mL dexamethasone combined). (D)Histograms showing the apoptosis percentages of Nalm6 cells treated with fucoxanthin alone, dexamethasone alone, or the combination of both drugs. Apoptosis results were analyzed using Flowjo software, and the sum of cell percentages in the Q2 and Q3 quadrants was calculated and presented as a histogram. (n=3; *P <0.05, **P <0.01, ***P <0.001).

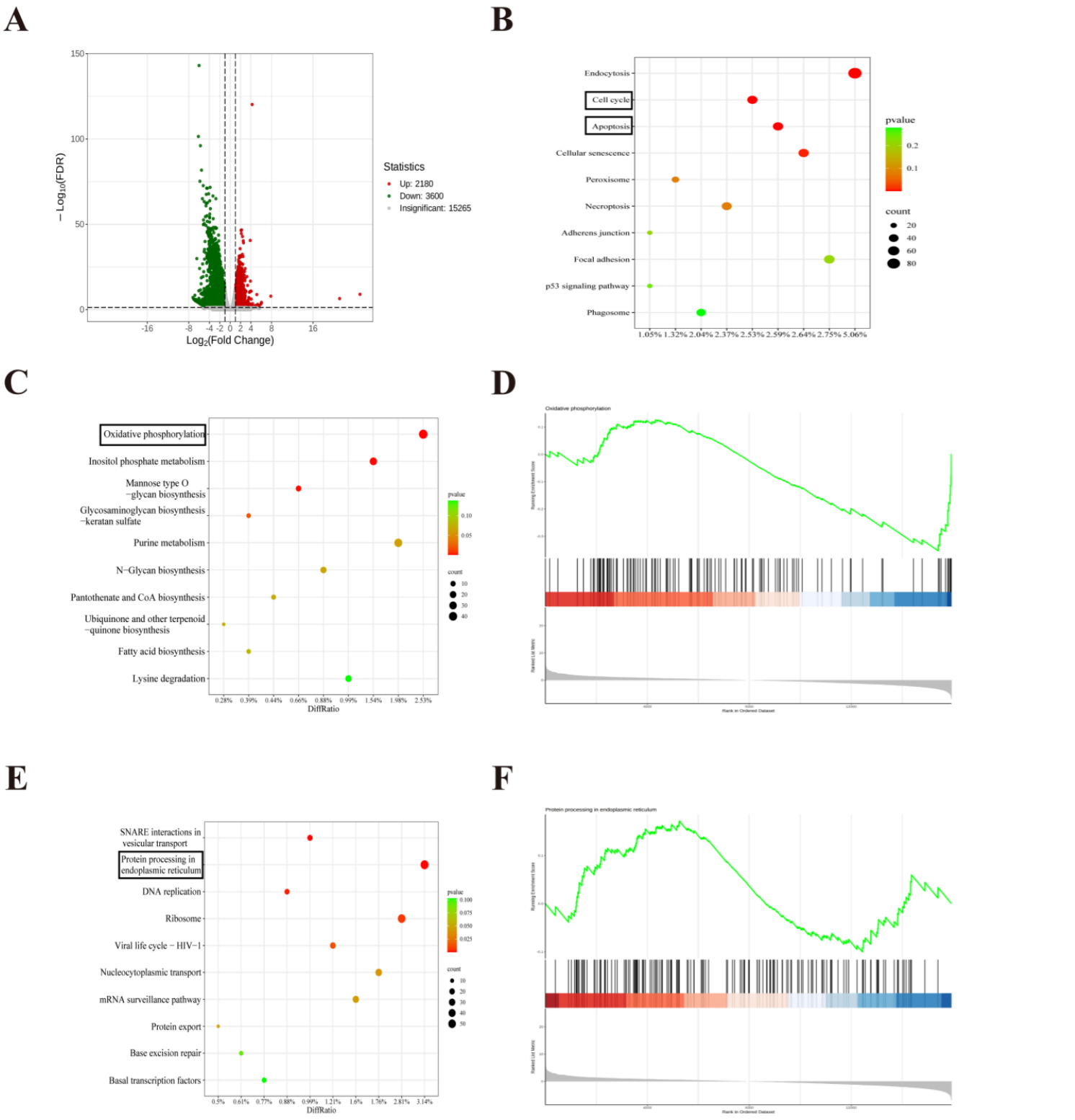


Figure 4

Transcriptomic sequencing results indicate mitochondrial and endoplasmic reticulum damage following fucoxanthin treatment. (A)The volcano plot of differentially expressed genes in Nalm6 cells treated with 5 μM fucoxanthin. (B, C, E)KEGG enrichment analysis results of differentially expressed genes. The identified differentially expressed genes were subjected to KEGG enrichment analysis. Based on KEGG classification, the top 10 signaling pathways were ranked according to P-values under Cell Processes,

Metabolism, and Genetic Information Processing catagories, and plotted as bubble charts. (D, F)GESA enrichment curve analysis results for oxidative phosphorylation and protein processing in the endoplasmic reticulum.

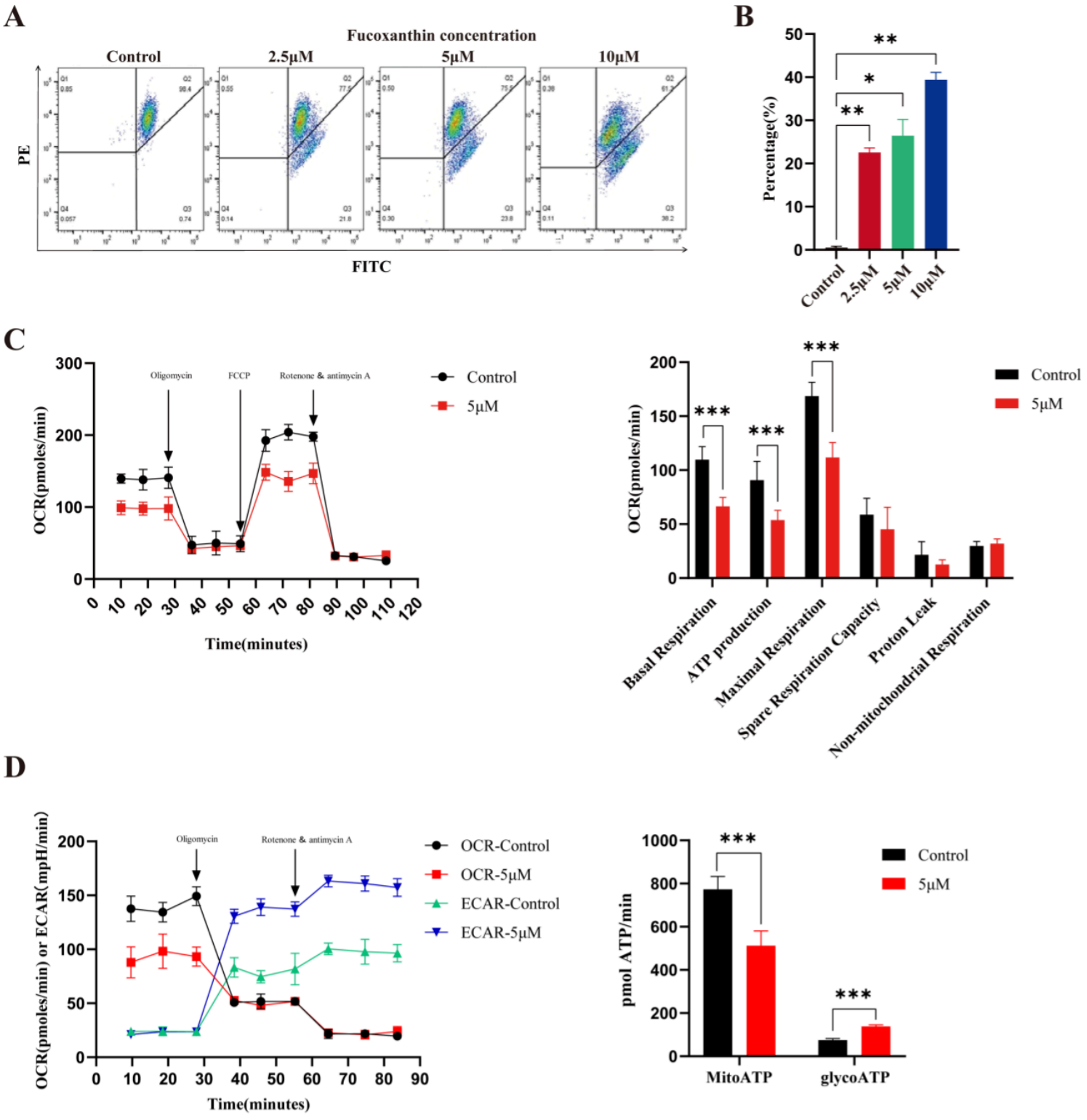


Figure 5

Fucoxanthin induces mitochondrial damage and impaired ATP synthesis in Nalm6 cells. (A)Fucoxanthin causes abnormal mitochondrial membrane potential. The mitochondrial membrane potential levels were

measured using JC-1 staining and flow cytometry. (B)Histogram showing the percentage of Nalm6 cells with reduced mitochondrial membrane potential under different concentrations of phycobilipene. JC-1 assay results were analyzed using Flowjo software to calculate the percentage of cells in the Q3 quadrant and plotted as a histogram. (C)After 24-hour exposure to 5μM fucoxanthin, the oxygen consumption rates(OCR) of Nalm6 cells under stress conditions induced by Oligomycin, FCCP, Rotenone, and Antimycin A were measured. (D) After 24-hour exposure to 5μM fucoxanthin, changes in oxygen consumption rates(OCR) and extracellular acidification rates(ECAR) of Nalm6 cells under stress conditions induced by Oligomycin, Rotenone, and Antimycin A were assessed. The corresponding mitochondrial ATP synthesis rates and glycolytic rates were calculated and plotted. (n=3; *P <0.05, **P <0.01, ***P <0.001).

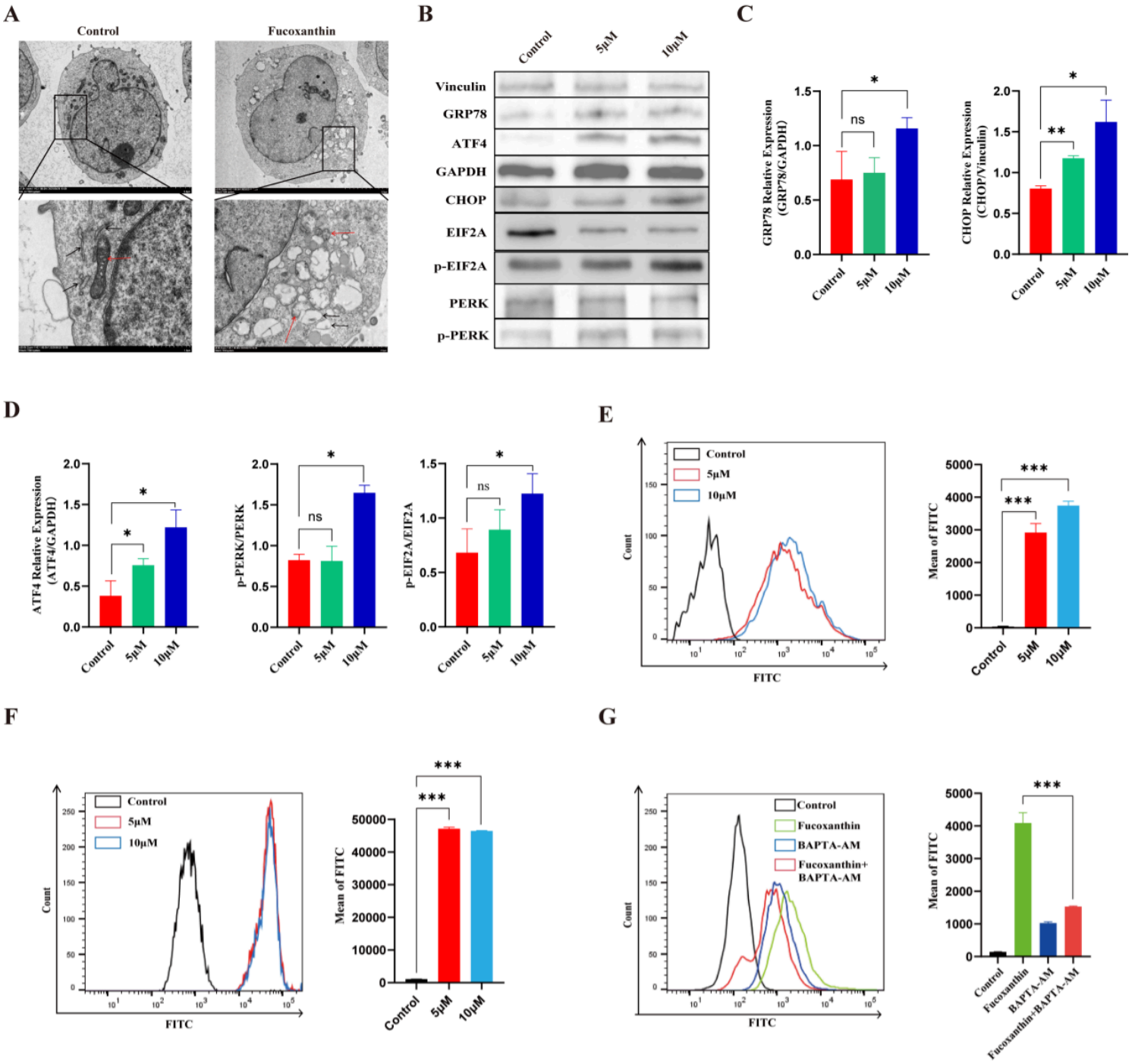


Figure 6

Fucoxanthin induces mitochondrial dysfunction by damaging the endoplasmic reticulum. (A) Mitochondria and ER ultrastructural changes were observed in Nalm6 cells after fucoxanthin treatment. After fixing and preparing slides from Nalm6 cells treated with 5μM fucoxanthin for 24 hours, the intracellular ultrastructure was examined under transmission electron microscopy. Mitochondrial structures are indicated by red arrows, while endoplasmic reticulum structures are indicated by black arrows. (B) Western blot confirmed that fucoxanthin regulates the expression levels of ER-stress-related proteins. (C) Expression levels of GRP78 and CHOP proteins in cells after fucoxanthin treatment. (D) Changes in ATF4 protein expression and phosphorylation levels of PERK and EIF2A proteins in cells after fucoxanthin treatment. (E) Following fucoxanthin treatment, a significant increase in reactive oxygen species(ROS) was observed in Nalm6 cells. (F) After fucoxanthin treatment, calcium ion concentrations in Nalm6 cells markedly elevated. (G) The calcium chelator BAPTA-AM reduced the ROS elevation induced by phycocyanin treatment. (n=3; *P <0.05, **P <0.01, ***P <0.001)

Supplementary Files

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