RESEARCH ARTICLE
Bull Biotechnol (2025) 6(2):53-60
https://doi.org/10.51539/biotech.1765261 Journal homepage: www.dergipark.org.tr/biotech

Bulletin of Biotechnology

Cholecalciferol modulates autophagy and endoplasmic reticulum stress to inhibit colon cancer cell proliferation

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Received : 18/08/2025
Accepted : 26/09/2025

To Cite: Tanrıverdi E, Avcı V, Güçlü E, Vural H (2025) Cholecalciferol modulates autophagy and endoplasmic reticulum stress to inhibit colon cancer cell proliferation. Bull Biotechnol 6(2):53-60 https://doi.org/10.51539/biotech.1765261

Abstract: Cholecalciferol is a type of vitamin D and has significant effects on the human body. Among its many roles, recent studies have shown that cholecalciferol can inhibit the proliferation of various cancer cells. In this study, we aimed to investigate the impact of cholecalciferol treatment on colon cancer cells through endoplasmic reticulum stress and autophagy. First, the cytotoxicity of cholecalciferol was evaluated by XTT. Accordingly, in further analysis cholecalciferol was applied to cells at concentrations of 150 and 250 µM for 48 hours. Colony formation capacity of cells, autophagy and ER-stress associated GRP78/BiP levels were evaluated after cholecalciferol treatment. And, the expression levels of genes linked to autophagy, ER stress, and apoptosis were examined using qRT-PCR. Results show that cholecalciferol suppresses the capacity of colony formation of colon cancer cells while raising ER stress marker GRP78/BiP level and lowering autophagy level. And, it was observed that cholecalciferol decreased the expression level of autophagy-related genes and increased the expression level of ER stress and apoptosis-related genes. These findings suggest that cholecalciferol operates its anticancer effect by suppressing autophagy and enhancing apoptosis through the induction of ER stress in colon cancer cells.

Keywords: Autophagy; Cholecalciferol; Colon cancer; Endoplasmic reticulum stress.

1 Introduction

Colon cancer also known as colorectal cancer (CRC) ranks third in terms of cancer-related deaths and is the second most frequent malignancy globally (Siegel et al., 2023). It is a multifactorial disorder with an etiology that includes genetic factors, environmental factors such as diet, and inflammatory conditions of the digestive system (Rawla et al., 2019). Although surgical resection, chemotherapy, radiation, and immunotherapy are some of the commonly used treatment strategies to treat CRC, there is a need to explore novel therapeutic approaches and agents to prevent it and increase the survival rate (Ahmed 2020).

Vitamin D compounds have been found to exert anticancer effects on a variety of cancer cells through mechanisms including apoptosis, cell cycle regulation, inflammation, and immune response (Charoenngam and Holick, 2020; Muñoz et al., 2022; Carlberg and Muñoz, 2022). Vitamin D3, known as cholecalciferol, is biologically inert and it is converted into its active form, calcitriol (1,25-(OH)2D3), through a two-step process in the liver and kidneys (Bikle 2014). Most of the 1,25-dihydroxycholecalciferol activity is considered to be mediated by its interaction with VDR (vitamin D receptor). A nuclear transcription factor VDR binds specific regions in DNA and initiates reactions that modulate the transcription of a lot of genes (Pike and Meyer, 2010). Vitamin D, primarily in the active form of 1,25-dihydroxycholecalciferol has taken attention for its potential anticancer properties. When colon cancer risk factors are considered, low vitamin D levels are a significant risk factor for colon cancer (Byers et al., 2012). Researchers have examined the effect of vitamin D on the development of colon cancer, and the primary findings have been that vitamin D suppresses angiogenesis, decreases cell proliferation, supports cell differentiation, and promotes apoptosis (Díaz et al., 2000; Pálmer et al., 2001; Wu et al., 2019). 1,25-dihydroxycholecalciferol exerts its major effect by inhibiting cell cycle phases G1/S (Bhoora and Punchoo, 2020). VDR and 1,25-dihydroxycholecalciferol decrease the

proliferative effect, increase differentiation, and alter the transcription of genes that inhibit carcinogenesis in colon cancer < autophagic vesicles.

2.5 Determination of GRP78 level

GRP78/BiP level in relation to endoplasmic reticulum stress in control and dose groups was determined using an ELISA-based kit (Enzo Life Sciences, ADI-900-214). Cells from the control and dosage groups that were seeded in 6-well plates were lysed using the kit's extraction buffer. Cell lysates were transferred to eppendorf tubes and supernatants were collected after centrifugation. Diluted standards were prepared with the GRP78 standard included in the kit for analysis. 100 µL of supernatant was added to the sample wells, 100 µL of standard was applied to the standard wells, and 50 µL of antibody solution was added. Following an hour of room temperature incubation, 50 microliters of conjugate solution were added to the wells, and another hour of room temperature incubation was carried out. Following the incubation period, each well had 200 µL of TMB solution, which is made up of 3,3'5,5' tetramethylbenzidine (TMB) and hydrogen peroxide. The washing solution that came with the kit was used for this purpose. Following a 30-minute incubation period at room temperature, 50 µL of stop solution was added to each well, and the absorbance values were analyzed using a microplate reader set at 450 nm.

2.6 RNA isolation and qPCR analysis

To measure the levels of gene expression, total RNA was collected from the dosage and control groups using the RiboEx (GeneAll, 301–001). After that, reverse transcription was carried out using a cDNA synthesis kit (Bio-Rad, 170–8891) in consistence with the manufacturer's instructions.The effects of cholecalciferol on *CASP3*, *CASP7*, *CASP8*, *EIF2A*, *CASP9*, *BAX*, *BCL2, CYCS*, *FADD*, *MAP1LC3A*, *ATF4*, *MAP1LC3B*, *GRP78*, *ATG16L1*, *CHOP*, *SQSTM1*, *PERK* gene expression levels were analyzed by qRT-PCR analysis. To start the reaction, prepare the tube with 5 µL of 2X qPCR MasterMix (ABM, MasterMix-R), 5 pMol of forward primer, 5 pMol of reverse primer, and 2 µL of cDNA. Add nuclease-free water to get the volume up to 10 µL. Next, using a Real-time PCR System (Bio-Rad, CFX Connect), the reaction was carried out in 40 cycles of 15 minutes at 95˚C and 60 seconds at 60˚C for the PCR prtocol. Melting curve analysis was also performed to evaluate primer dimer/misbinding. For this purpose, at the end of 40 cycles, after incubation at 95˚C for 10 seconds, the temperature was reduced to 65˚C and gradually increased to 95˚C in 0.5˚C increments. For analysis, threshold cycle values (Ct) were identified. Using the web-based "RT² Profiler™ PCR Array Data Analysis" application, the $2^{(-\Delta\Delta Ct)}$ method was applied to determine the quantitative analysis of gene expression. The reference gene chosen was *ACTB*.

2.7 Statistical analysis

The groups were compared using one-way analysis of variance (One-way ANOVA) using GraphPad Prism program (Version 8.0.2, San Diego, CA). Every experiment was carried out in triplicate, and the mean ± standard deviation is shown for each outcome. When *$p < 0.05$, it was determined statistically significant.

3 Results

3.1 Cholecalciferol inhibits the proliferation of colon cancer cells

The effect of cholecalciferol treatment on cell viability in HCT 116 and HT-29 cells was evaluated by XTT assay. For this purpose, cells were applied with cholecalciferol at concentrations of 50, 100, 150, 200, 250, 250, 300, 400, 400, 500, 750, and 1000 µM for 24, 48 and 72 hours. The XTT analysis revealed that cholecalciferol administration prevented colon cancer cell growth in a time- and dose-dependent manner (Fig 1a). In further analysis, cells were administered for 48 h with cholecalciferol at concentrations of 150 and 250 µM, which gave viability values of 66.26% and 50.78% for HCT 116 cells and 62.38% and 48.42% for HT-29 cells, respectively.

By using the colony formation test, the impact of cholecalciferol administration on cell growth in HCT 116 and HT-29 cells was also assessed. Accordingly, the colony formation capacity of the cells decreased significantly after cholecalciferol treatment at concentrations of 150 and 250 µM in both cell lines compared to the control group (Fig 1b) ($p < 0.05$).

The cells were treated with different concentrations of cholecalciferol for 24, 48 and 72 h and cell viability (%) was assessed by XTT analysis. (b) The effect of cholecalciferol on the colony formation capacity of colon cancer cells was assessed by colony formation analysis. The cells were treated with 150 and 250 µM cholecalciferol for 48 h and cultured for 10 days. Then, staining was done with crystal violet and colonies were counted. ***$p < 0.001$.

3.2 Cholecalciferol decreases the autophagy level in colon cancer cells

Using a fluorescence-based kit based on the marking of autophagic compartments with Cyto-ID dye, the impact of cholecalciferol administration on autophagy in colon cancer cells was assessed. The autophagy analysis results showed that when cholecalciferol at 150 and 250 µM was administered to HCT 116 and HT-29 cells for 48 hours, there was a significant decrease in autophagy as compared to the control group (Fig 2) ($p < 0.05$).

3.3 Cholecalciferol increases the GRP78/BiP level in colon cancer cells

The impact of cholecalciferol on ER stress in colon cancer cells was calculated by ELISA-based kit that enables the determination of GRP78/BiP level, one of the important markers of ER stress. Accordingly, administration of cholecalciferol at concentrations of 150 and 250 µM significantly increased GRP78/BiP in both cell lines compared to the control group (Fig 3) ($p < 0.05$).

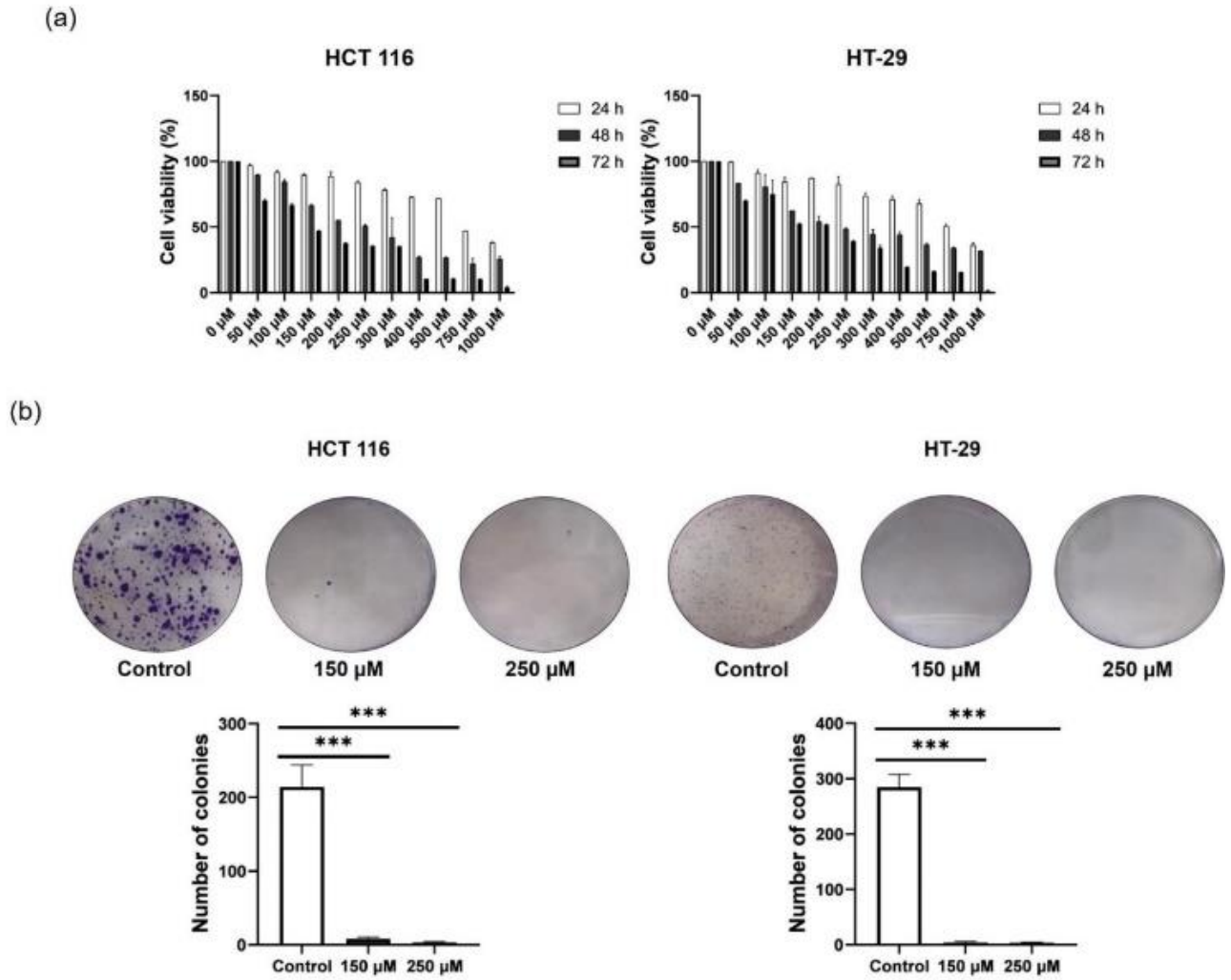

Fig. 1 Impact of cholecalciferol on growth of colon cancer cells. (a)

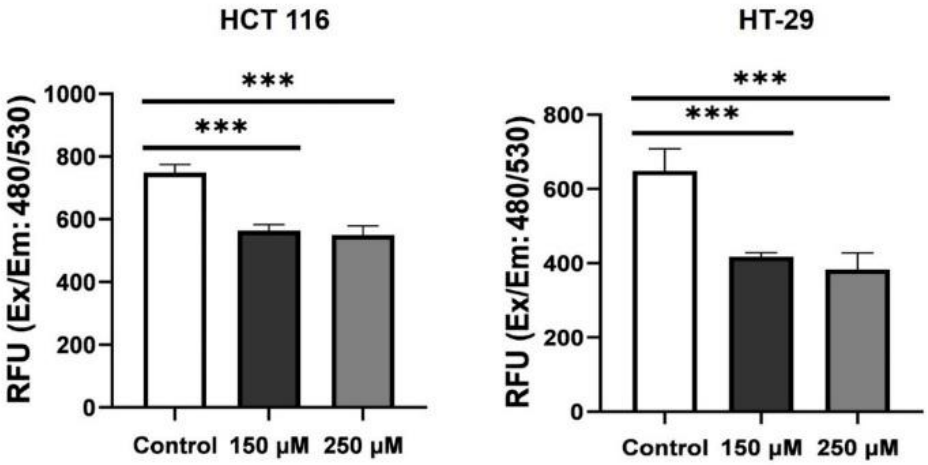

Fig. 2 Impact of cholecalciferol on autophagy in colon cancer cells. The cells were treated with 150 and 250 µM cholecalciferol for 48 h and fluorescent based-autophagy analysis was performed. ***$p < 0.001$.

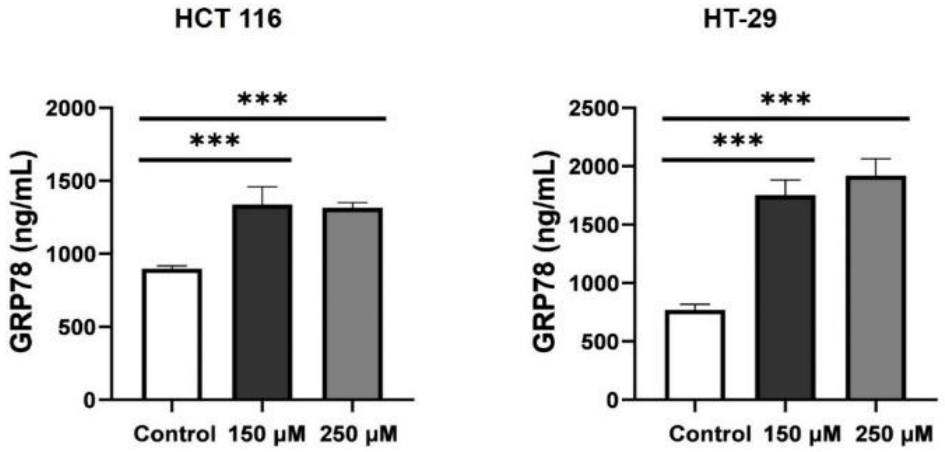

Fig. 3 Impact of cholecalciferol on GRP78 level in colon cancer cells. The cells were treated with 150 and 250 µM cholecalciferol for 48 h. Cells were lysed and GRP78 level was assessed with an ELISA-based kit. ***$p < 0.001$.

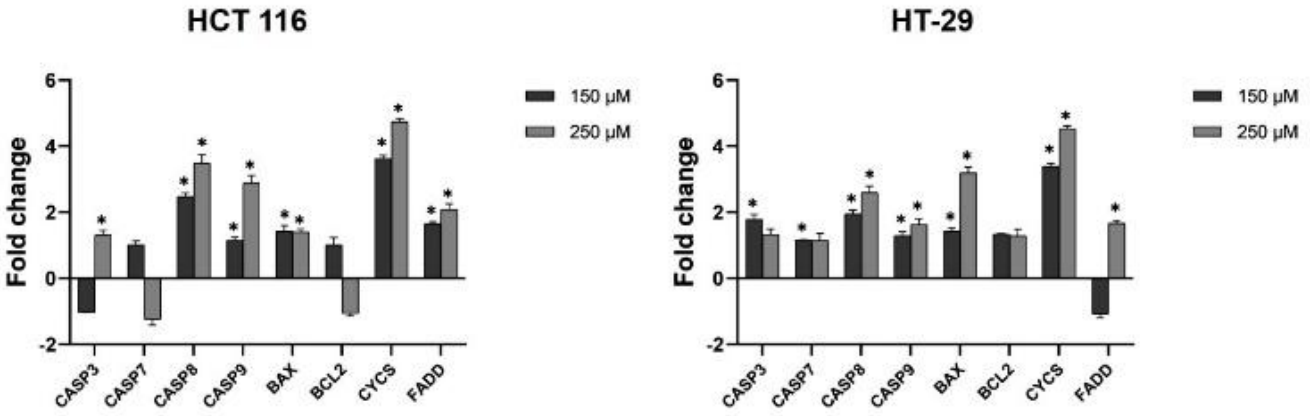

Fig. 4 Impact of cholecalciferol on expression level of genes associated with apoptosis in colon cancer cells. The cells were treated with 150 and 250 µM cholecalciferol for 48 h. Expression differences of target genes compared to the control group were analyzed by qRT-PCR. *$p < 0.05$.

3.4 Cholecalciferol affects the expression levels of apoptosis, ER stress and autophagy-associated genes in colon cancer cells

Using qRT-PCR analysis, the impact of cholecalciferol administration at 150 and 250 µM for 48 hours on the expression rates of genes related with autophagy, ER stress, and apoptosis in colon cancer cells was assessed. Accordingly, expression levels of apoptosis-related *CASP8*, *CASP9*, *BAX*, *CYCS* and *FADD* genes were significantly increased by 2.47, 1.15, 1.43, 3.62 and 1.65 fold, respectively, after cholecalciferol treatment at a concentration of 150 µM in HCT 116 cells compared to the control group. After 250 µM cholecalciferol treatment, 1.31, 3.48, 2.89, 1.4, 4.74 and 2.08 fold significant increases were detected in the expression levels of *CASP3*, *CASP8*, *CASP9*, *BAX*, *CYCS* and *FADD* genes, respectively, compared to the control group (Fig 4) ($p < 0.05$). In HT-29 cells, cholecalciferol treatment at a concentration of 150 µM significantly increased the expression levels of *CASP3*, *CASP7*, *CASP8*, *CASP9* and *CYCS* genes by 1.78, 1.16, 1.94, 1.29, 1.42 and 3.39 fold, respectively, compared to the control group. After administration of cholecalciferol at a concentration of 150 µM to HT-29 cells, the expression levels of *CASP8*, *CASP9*, *BAX*, *CYCS* and *FADD* genes were significantly increased by 2.6, 1.63, 3.19, 4.53 and 1.66 fold, respectively (Fig 4) ($p < 0.05$).

Regarding the genes associated with ER stress, a significant 2.89, 2.42, 4.56 and 1.2-fold increase was detected in the expression levels of *ATF4*, *GRP78*, *CHOP* and *ERN1* genes, respectively, after 150 µM concentration of cholecalciferol treatment in HCT 116 cells compared to the control group. At a concentration of 250 µM, cholecalciferol treatment significantly elevated the expression levels of these genes by 3.4, 1.95, 4.84 and 1.45 fold compared to the control group (Fig 5) ($p < 0.05$). In HT-29 cells, cholecalciferol treatment at a concentration of 150 µM significantly increased the expression levels of *ATF4*, *GRP78*, *CHOP* and *ERN1* genes by 2.78, 2.62, 16.03 and 1.65 fold, respectively, compared to the control group. After 250 µM cholecalciferol treatment, the expression levels of these genes increased significantly by 3.53, 5.31, 36.38 and 2.11 folds compared to the control group (Fig 5) ($p < 0.05$).

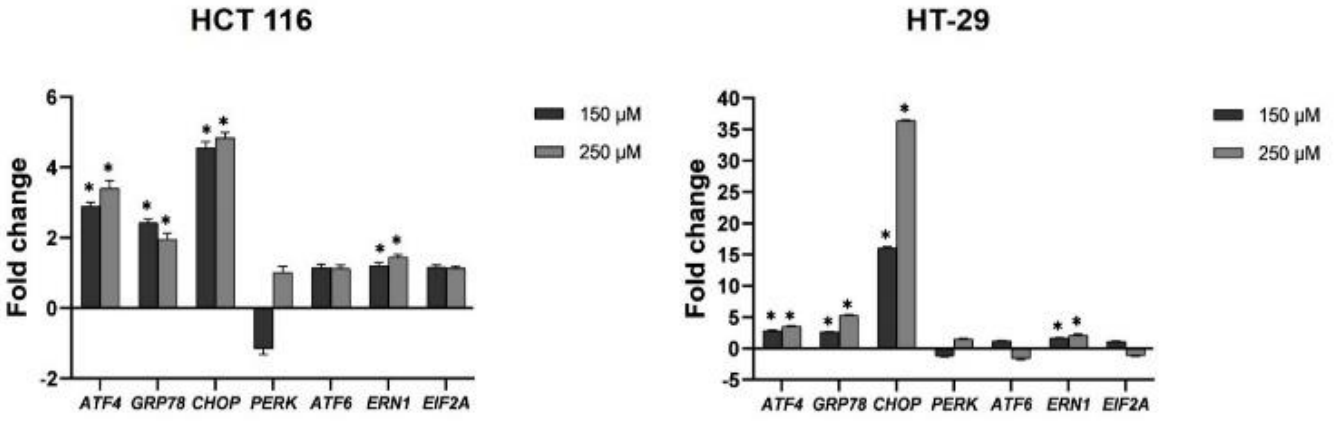

Fig. 5 Impact of cholecalciferol on expression level of genes associated with ER stress in colon cancer cells. The cells were treated with 150 and 250 µM cholecalciferol for 48 h. Expression differences of target genes compared to the control group were analyzed by qRT-PCR. *p < 0.05.

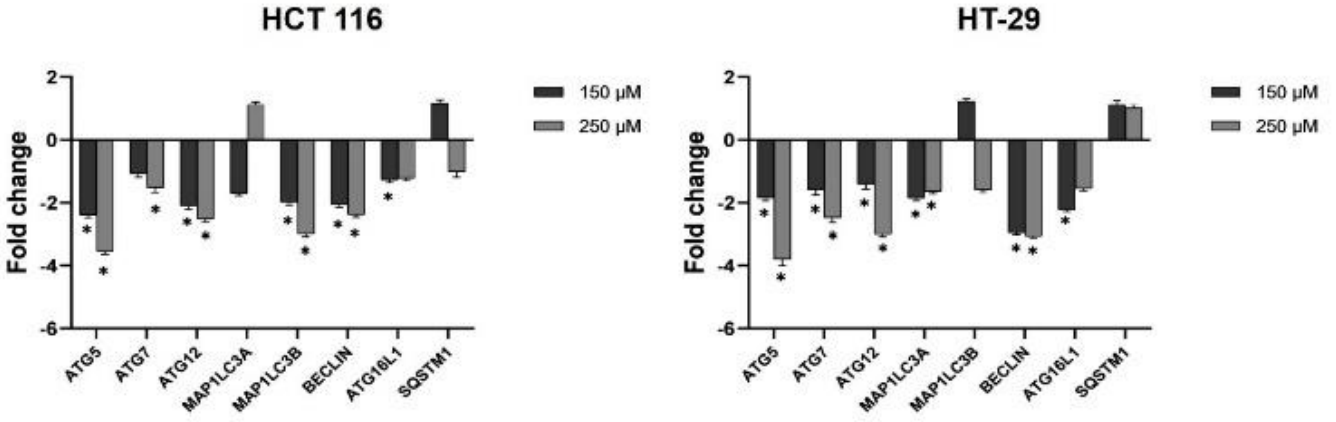

Fig. 6 Impact of cholecalciferol on expression level of genes associated with autophagy in colon cancer cells. The cells were treated with 150 and 250 µM cholecalciferol for 48 h. Expression differences of target genes compared to the control group were analyzed by qRT-PCR. *p < 0.05.

In HCT 116 cells, administration of cholecalciferol at a concentration of 150 µM significantly decreased the expression levels of autophagy-related *ATG5*, *ATG12*, *MAP1LC3B*, *BECLIN* and *ATG16L1* genes by 2.39, 2.1, 1.98, 2.06 and 1.27 fold, respectively, compared to the control group. After 250 µM cholecalciferol treatment, the expression levels of *ATG5*, *ATG7*, *ATG12*, *MAP1LC3B* and *BECLIN* genes decreased significantly by 3.55, 1.52, 2.51, 2.99 and 2.38 fold, respectively, compared to the control group (Fig 6) ($p < 0.05$). In HT-29 cells, a significant 1.84, 1.59, 1.42, 1.85, 2.95, and 2.23 fold decrease was detected in the expression levels of *ATG5*, *ATG7*, *ATG12*, *MAP1LC3A*, *BECLIN* and *ATG16L1* genes, respectively, after cholecalciferol treatment at a concentration of 150 µM compared to the control group. And, 250 µM cholecalciferol treatment significantly decreased the expression levels of *ATG5*, *ATG7*, *ATG12*, *MAP1LC3A*, and *BECLIN* genes by 3.8, 2.48, 3, 1.64, and 3.06 fold, respectively, compared to the control group (Fig 6) ($p < 0.05$).

4 Discussion

CRC is an important public health problem due to its increasing incidence, resistance to treatments and complicated molecular pathogenesis (Papamichael et al., 2013). Cholecalciferol, or vitamin D3, has a potential medicinal agent cause cholecalciferol, especially in its active form, shown as inhibits colon cancer by reducing angiogenesis, encouraging apoptosis and differentiation, and decreasing cell proliferation (Díaz et al., 2000; Pálmer et al., 2001; Bhoora et al., 2020). Investigating how cholecalciferol modulates autophagy and ER stress, two critical cellular processes intricately linked to cancer development and progression, offers a promising approach to understand its anticancer properties. For this reason, endoplasmic reticulum stress and autophagy were used to assess cholecalciferol's potential anticancer effect in HT-29 and HCT 116 colon cancer cells. In the experimental part, cytotoxicity analysis was performed with XTT, evaluation of autophagy and GRP78/BiP levels were analyzed and for gene level analysis, qRT-PCR was used.

It is generally accepted that cholecalciferol tends to suppress cell growth and/or induce differentiation in human CRC cells. Khriesha et al. (2021) used CaCo II as a human CRC cell line to study the potentially anticancer effect of cholecalciferol and find out IC_{50} values 92 µM for 24h and 76 µM for 48 h. In a study conducted with 1,25(OH)2D3, the active form of cholecalciferol, IC_{50} values were found to be 168 nM for LoVo, 57 nM for HT29 and 47 nM for HCT116 colon cancer cells (Wierzbicka et al., 2015). Pálmer et al. (2001) found that 1,25(OH)2D3 promoted differentiation in SW480 cells.

Our study demonstrates that cholecalciferol treatment has a significant inhibitory effect on the growth of colon cancer cells, as evidenced by the XTT assay and colony formation analysis. The inhibitory effect was dose- and time-dependent, with concentrations of 150 and 250 µM showing the most marked reduction in cell viability. Studies examining the impact of vitamin D in cervical cancer cell lines have shown inhibition of cell growth. Calcitriol has been demonstrated to promote G1 cell cycle arrest and reduce cell proliferation in HeLa cells in a dose-and time-dependent manner (Wang et al., 2015). In another study, for the HCT116 cell line, 50 µM concentration resulted in 50% growth suppression at all time periods; however, for the HeLa and MCF-7 cell lines, a concentration of about 1250 µM was required to achieve 50% growth inhibition (Shruthi et al., 2017).

It's crucial to analyse the function of autophagy under various mutational burdens and stages in order to precisely target malignancies. In this study, the efficacy of cholecalciferol in colon cancer cells was analyzed in relation to autophagy. Both the results of autophagy analysis and qRT-PCR analysis, in which we evaluated the expression levels of autophagy-related genes, revealed that cholecalciferol treatment inhibited autophagy in colon cancer cells. Although autophagy has a controversial role in cancer, studies have shown that autophagy may act as a protective mechanism for cancer cells by inducing tumor progression in CRC. In a clinical study, increased expression of ATG5 correlated with enhanced incidence of invasion in CRC patients (Cho et al., 2012). Similarly, LC3B and SQSTM1 expression was found to be associated with poor prognosis (Niklaus et al., 2017). In mouse models lacking the necessary autophagy genes *Atg5*, *Atg14*, or *Atg16L1* exhibit a marked reduction in tumor growth (DeVorkin et al., 2019). Furthermore, in CRC, autophagy was stimulated and proliferation was encouraged while apoptosis was inhibited by activated protein kinase C receptors, a mutation frequently observed in cancer (Xiao et al., 2018). In CRC, autophagy also affected the transcription factor FOXO3a degradation. Autophagy inhibition increased FOXO3a levels and increased the transcription of genes that promote apoptosis (Fitzwalter et al., 2018). In CRC cells, autophagy was suppressed after p53 activation and endoplasmic reticulum stress, and this also enhanced apoptosis (Sakitani et al., 2015).

In this study, the effects of cholecalciferol administration in colon cancer cells were also evaluated through ER stress. According to our results, an increase in the amount of GRP78/BiP, a marker of ER stress, was detected after cholecalciferol administration in colon cancer cells. In addition, the results of qRT-PCR analysis revealed that cholecalciferol treatment increased the expression levels of ER-stress-related genes in colon cancer cells compared to the control group. However, the role of ER stress in cancer is as controversial as autophagy. ER stress and UPR are critical for cancer cells. The UPR is triggered in response to ER stress and regulates cell fate in cancer, including apoptosis, adaptation, and survival. In some cases, the UPR can be activated by cancer cells to adapt to the stress and promote their survival. This adaptation allows cancer cells to continue growing and resisting apoptosis by upregulating chaperone proteins, which help in proper protein folding and reduce ER stress (Bonsignore et al., 2023). According to some studies, UPR increases the survival of tumors in the following ways: mouse fibroblasts transformed with Ras were less able to grow into tumors in mice when PERK was knockout, and tumor growth potential was decreased in human fibrosarcoma cells that had partial knockdown of the ER chaperone immunoglobulin-binding protein GRP78/BiP. Likewise, XBP1 knockdown prevents mice from developing transplantable tumors (Jamora et al., 1996; Ron and Walter, 2007). Taken together, these findings highlight the possible advantages of targeting UPR in the treatment of cancer. The PERK inhibitors, GSK2606414 and GSK2656157, inhibit the growth of tumors in human xenograft models of various cancers (Axten et al., 2012; Axten et al., 2013). Possible explanations for the inhibitors' reported anticancer efficacy include changed amino acid metabolism, reduced blood vessel density, and reduced vascular perfusion. PERK inhibition also sensitizes CRC cells to 5-fluorouracil (5-FU) chemotherapy (Shi et al., 2019). However, it is also known that increased ER stress in cancer cells can trigger intrinsic and extrinsic pathways of apoptosis (Sano and Reed, 2013). According to our qRT-PCR analysis results, cholecalciferol treatment caused an enhance in the expression levels of apoptosis-related genes in colon cancer cells compared to the control group. When every outcome is assessed collectively, it is thought that cholecalciferol induces ER stress while inhibiting autophagy, which may act as a protective mechanism for cancer cells, and thus has anticancer activity by regulating apoptosis in colon cancer cells.

5 Conclusion

In conclusion, our study revealed that cholecalciferol caused cytotoxic effects in HCT 116 and HT-29 human colon cancer cells in a dose and time dependent manner. In addition, the colony forming ability of the cells was suppressed after administration of cholecalciferol at concentrations of 150 and 250 µM. Cholecalciferol inhibited autophagy and increased ER stress in colon cancer cells. As a consequence, the expression levels of apoptosis-related genes increased. However, further investigations are required to fully characterize the local metabolism of cholecalciferol in HCT 116 and HT-29 cell lines. In addition, the mechanisms of autophagy, ER stress, and apoptotic induction by cholecalciferol in HCT 116 and HT-29 cell lines need to be further elucidated.

Acknowledgements This study was produced from the MSc thesis of Esra Tanrıverdi and was supported by Necmettin Erbakan University, Scientific Research Projects (#221318002).

Authors' contributions: ET:Investigation, methodology, formal analysis, writing-original draft. VA: Investigation, methodology,formal analysis, writing-original draft, EG: Investigation, methodology, formal analysis, writing-review&editing, supervision. HV: Investigation, writing-review&editing, supervision.

Conflict of interest disclosure:

The authors declare that they have no conflicts of interest.

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