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The Investigation of the Effects of Calcitriol on Human Ovarian Carcinoma Cells

Kalsitriol'ün İnsan Ovaryum Kanser Hücrelerine Etkisinin Araştırılması

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ÖZ

Amaç: Over kanseri jinekolojik malignitelerden ölümlerin önde gelen nedenlerindendir. Kadınlarda kansere bağlı ölümlerde beşinci sırada yer almaktadır. D Vitaminin aktif formu olan kalsitirol, vitamin D reseptörüne (VDR) bağlanarak fonksiyon göstermektedir. Kalsitriol, proliferasyon, apoptoz, diferansiasyon, inflamasyon, invazyon, anjiyogenez ve metastaz ile ilgili çoklu sinyal yollarını düzenleyerek kanser gelişimini ve büyümesini etkileme potansiyeline sahiptir. Kolon, meme ve prostat kanseri büyümesini sağlayan spesifik sinyal yollarının kalsitriol ile düzenlemesi incelendiğinde kalsitriolün birçok farklı kanser türünde kanser hücreleri üzerinde geniş bir etkiye sahip olduğu görülmüştür. Çalışmamızın amacı kalsitriolün ovaryum kanser hücrelerine karşı etkisini araştırmaktır.
Araçlar ve Yöntem: Çalışmamızda MDAH-2774 insan ovaryum kanseri hücre hattı kullanılmıştır. Hücreler farklı dozlarda kalsitriole 24 ve 48 saat maruz bırakıldıktan sonra MTT testi ve kantitatif gerçek zamanlı PCR yöntemi uygulanmıştır.
Bulgular: MTT testi sonucunda, kalsitirolün ovaryum kanser hücrelerinin canlılığını azalttığı tespit edildi. Kalsitriol uygulanan grupta kontrol grubuna kıyasla VDR ve p53 gen ekspresyonlarında artış saptandı. Bunlara ek olarak, kalsitriol uygulamasının proapoptotik belirteç Bax'ın gen expresyonunda artışa ve anti-apoptotik Bcl-2 ekspresyonunda azalmaya neden olduğu tespit edildi.
Sonuç: Sonuç olarak kalsitriol tedavisinin, ovaryum kanser hücrelerinin proliferasyonunu azalttığı ve apoptozu indüklediği saptanmış olup, kasitriolün tek başına veya kemoterapi ilaçlarıyla kombinasyon halinde kullanılmasının ovaryum kanser tedavilerinde potansiyel bir rolü olabileceği düşünülmektedir.

Anahtar Kelimeler: apoptoz; kalsitriol; mtt analizi; ovaryum kanseri; qrt-pcr

ABSTRACT

Purpose: Ovarian cancer is the fifth leading cause of cancer death in women, leading cause of death from gynecologic malignancies, and the second most commonly diagnosed gynecologic malignancy, however the underlying pathophysiology is not clearly understood. Calcitriol, the active form of vitamin D serves its activity by binding to the vitamin D receptor (VDR). Calcitriol regulates multiple signaling pathways such as proliferation, apoptosis, differentiation, inflammation, invasion, angiogenesis and metastasis. It has been found to have a broad effect on several cancer types such as colon, breast and prostate cancer. Therefore, the study aimed to investigate the effects of calcitriol on human ovarian cancer cells.
Material and Methods: The human MDAH-2774 ovarian carcinoma cells were exposed to different dose ranges of calcitriol for 24 and 48 hours. Cultured cells were evaluated in terms of MTT assay and quantitative Real time PCR.
Results: As evidenced by the MTT assay, calcitriol treatment resulted in the reduction of cell viability in human MDAH-2774 cells. The gene expressions of VDR and p53 were increased with the calcitriol treatment compared to control. Additionally the gene expression of proapoptotic marker Bax increased and the anti-apoptotic marker Bcl-2 decreased with the presence of the calcitriol.
Conclusion: In conclusion calcitriol treatment decreased cell proliferation and induced apoptosis in ovarian cancer cells, therefore we can suggest that calcitriol, either by itself or in combination with chemotherapy drugs, may be effective in treating ovarian cancer.

Keywords: apoptosis; calcitriol; mtt assay; ovarian cancer; qrt-pcr

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INTRODUCTION

The process of ovarian carcinogenesis is complex. Obvious symptoms including weight loss, bloating, and discomfort are not present in ovarian cancer.[1] It frequently occurs just before or after menopause and is associated with breast, cervical, colorectal, lung, stomach, and endometrial cancer.[2] Progression and metastasis of ovarian cancer are equal to those of other solid tumors require additional processes, such as the destruction of intercellular structures, changes in cellular adhesion, cell migration and invasion.[3] Patients with ovarian cancer receive treatment that combines surgery and chemotherapy. The main surgical procedure's objectives are to confirm the diagnosis, establish the lesion's stage, and reduce the tumor.[4] A lipid-soluble hormone called vitamin D is typically produced by exposure to sunshine. Vitamin D comes in two different forms: vitamin D3 and vitamin D2.[5] The primary form of vitamin D that is stored and circulated is 25-hydroxyvitamin D, which is produced by the liver from both forms of the vitamin. The serum levels of this form are thought to be the best indicator of the vitamin D status of the entire body.[6] Kidney receives it after the initial hydroxylation and transforms it into calcitriol, the active form of vitamin D.[7]

Calcitriol regulates a wide range of biological activities, mostly via the nuclear vitamin D receptor (VDR).[8] In Additionally, to modulating biological processes like cell proliferation, wound healing, neuromuscular activity, and immunological response, calcitriol is essential for preserving the body's calcium balance. Additionally, through controlling signaling pathways involved in cell proliferation, differentiation, apoptosis, inflammation, invasion, and metastasis, calcitriol may have an anti-cancer effect.[9,10]

While calcitriol's anti-tumor effects have been extensively studied in a number of in vitro and in vivo human and murine tumor models, including leukemia,[11] squamous cell carcinoma,[12] prostate,[13] breast,[14] and colon cancer,[15] there is little information on ovarian cancer. As a result, the current study's objective is to assess how calcitriol affects the human ovarian cancer cell line.

MATERIALS and METHODS

Cell Culture

MDAH-2774 ovarian cancer cells (CRL No: 10303; ATCC, USA) were grown in RPMI-1640 media (Biological Industries, Israel) with 10% fetal calf serum (GIBCO, Invitrogen Co.,UK), 100 units/mL penicillin and 100 g/mL streptomycin (Sigma Chemical Co., MO, USA).

After adding RPMI-1640 to inactivate the trypsin and harvesting semi-confluent cells from flasks with 0.05% trypsin (Sigma Chemical Co., MO, USA), the cells were resuspended in culture media**.**

MTT Assay

MDAH cells were seeded in 96-well plates at a density of (2×10^3 cells/well) and maintained for 24 and 48 hours with calcitriol (71820, Cayman ChemicalCo, USA) at concentrations of 0.5nM, 1nM, 10nM, 50nM, 100nM, 200nM, 500nM and 1000nM.[16] GroupI(control): nontreated, groupII: (0.5nM, 1nM, 10nM, 50nM, 100nM, 200nM, 500nM and 1000nM) calcitriol treatment for 24h and group III: (0.5nM, 1nM, 10nM, 50nM, 100nM, 200nM, 500nM and 1000 nM) calcitriol treatment for 48 hours. Following incubation, cell viability was assessed using the MTT assay (Sigma-Aldrich, St. Louis, USA), in compliance with the guidelines provided by the manufacturer. On a microplate reader (Enspire, PerkinElmer, USA), the absorbance was measured between 570 and 630 nanometers. Cell viability was evaluated as a percentage of untreated cells.

Quantitative Real-Time PCR

qRT-PCR was utilized to assess the levels of VDR expression for the evaluation of calcitriol's genomic effect, BAX expression levels for its pro-apoptotic effects, BCL2 expression levels for its anti-apoptotic and. P53 expression levels for its antitumor impact.

The cultured MDAH-2774 cells were treated with 1nM, 100nM and 1000nM calcitriol for 24h. RNeasy Mini Kit (QIAGEN Brand Cat.No.74104) was utilized to perform mRNA isolation according to defined guidelines. 1 mg of

RNA and 2mL of 5xPrimerScript RT Master Mix (TaKaRa, Japan) made up the reverse transcription reactions, which had 10 mL total in volume. RNA primer sequences were given in Table 1. The C100 PCR System (Bio-Rad, CA, USA) was used to conduct reactions for 15 minutes at 37°C. The internal control was GAPDH. On the ABI 7500 PCR equipment, the qPCR was carried out using the SYBR Green (Roche, Basel, Switzerland) dye detection method.

Table 1. The primer sequences of RNA.

GAPDH	**F:**GAAGGTGAAGGTCGGAGTCAAC	**R:**CAGAGTTAAAAGCAGCCCTGGT
VDR	**F:**TCTCCTGCCTACTCACGATAA	**R:**GCTACTGCCCGTGAGAATATAA
BCL-2	**F:**TTCTTTGAGTTCGGTGGGGTC	**R:**TGCATATTTGTTTGGGGCAGG
BAX	**F:**ATGGACGGGTCCGGGGAG	**R:**TCAGAAAACATGTCAGCTGCC
P53	**F:**TTCTCATCACCGGCATCACG	**R:**GCTATCACAAACTGCAAGACG

Statistical Analysis

The statistical analyzes were done by applying two-tailed student's t-test and analysis of variance (ANOVA) by using GraphPad Prism® V.5.00 software (GraphPadsoftware Inc., La Jolla, USA). The data was presented as means ± SEM. The free Relative Expression Software Tool (REST 2009, Qiagen) was used to determine fold changes in gene expression, the comparative CT technique, and statistical analysis. The tests considered significance level of $p<0.05$.

RESULTS

MTT Assay Results

We examined the potential of calcitriol at various concentrations 0.5nM, 1nM, 10nM, 50nM, 100nM, 200nM, 500nM and 1000 nM for 24, and 48 hours. The MTT assay data analysis revealed that 50nM, 200nM and 500nMcalcitiriol treatment for 24 hours reduced the cell proliferation at %90, %93, and %94 respectively, compared to control. 1nM, 100nM and 1000nM doses of calcitriol treatment for 24h reduced the MDAH cell proliferation at %82,%86 and %94 respectively (Figure 1A).

On the other hand, treatment with the same amounts of calcitriol, particularly 100nM and 1000nM for 48 hours led to an increase in cell proliferation (Figure 1B). According to these observations, calcitriol showed its inhibitory effects for 24 hours.

For the ensuing studies, the concentrations of 1 nM, 100 nM, and 1000 nM were chosen because they had the best inhibitory effects on both cell viability and proliferation.

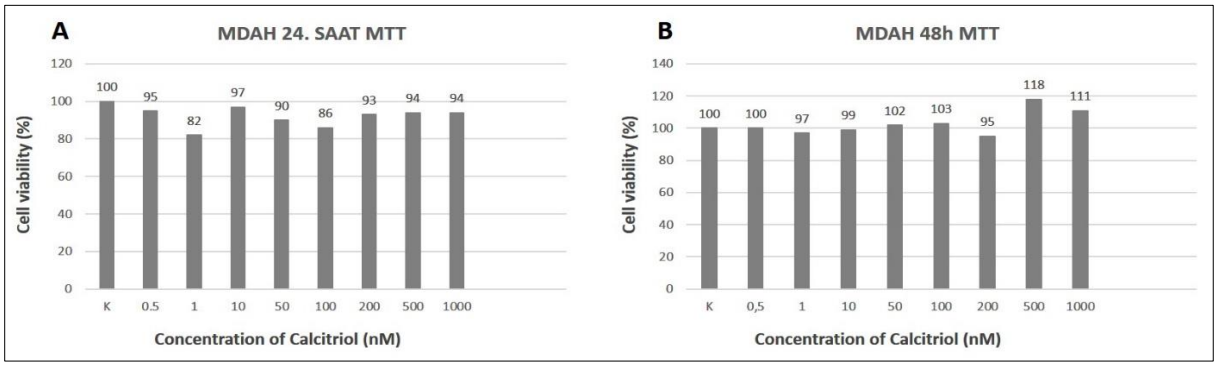

Figure 1. Analyses of cell viability.

QRT-PCR Results

The gene expressions of the human MDAH-2274 ovarian cancer cells treated with calcitriol at 1 nM, 100 nM and 1000 nM were presented in (Figure 2). This translated to the up-regulated expression of VDR, P53, and proapoptotic protein Bax and down-regulated antiapoptotic protein Bcl-2 due to culturing in the presence of 1000 nM calcitriol for 24 hours (Figure 2).

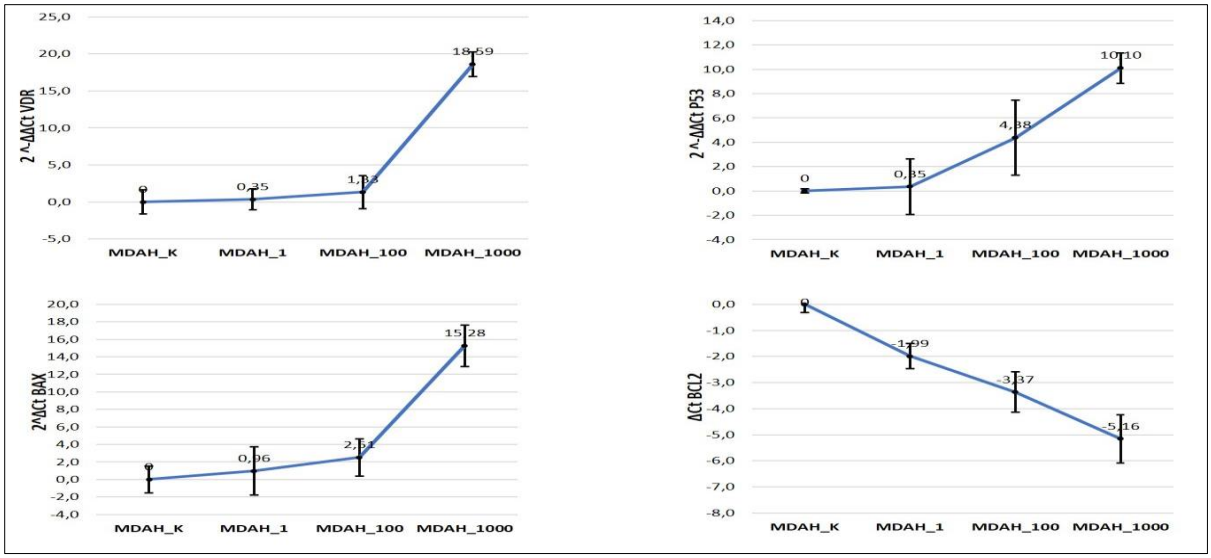

Figure 2. Quantitative RT-PCR Analyses.

DISCUSSION

A powerful steroid hormone, calcitriol is an active metabolite of vitamin D. By controlling numerous signaling pathways, calcitriol has demonstrated anti-tumor benefits in a variety of malignancies. It is crucial in prostate and other malignancies and is biologically active. According to studies, populations residing at higher latitudes have greater risks of various malignancies due to low vitamin D levels.[17]

Calcitriol, the biologically active derivative of vitamin D, has anticancer properties both independently and in conjunction with chemotherapeutic medicines, in a variety of cell types. The aggressive tumor known as malignant pleural mesothelioma (MPM) is uncommon. It has a poor prognosis and few therapies are available. The researchers investigated calcitriol's potential anticancer function. The findings demonstrated calcitriol decreased cell survival and proliferation in human MPM cells.[18]

Numerous cancer cell models have been used in studies to show the effects of calcitriol on cell differentiation and anti-proliferation. In addition, in cancer animal models, calcitriol and its analogs demonstrated the capacity to postpone tumor development and suppress tumor progression, either alone or in combination with anticancer medicines.[19,20,21]

Sunlight exposure, nutrition, and supplements all affect vitamin D levels. There was mounting evidence that ovarian cancer risk was elevated in people with low vitamin D levels. Yin et al.'s meta-analysis of 10 cohort studies on the incidence of ovarian cancer discovered an average rise in 25(OH)2D3 of 20 ng/ml.[22] Higher 25(OH)D concentrations are linked to prolonged survival rates, according to a case-control study of 1631 women with epithelial ovarian cancer (adjusted HR: 0.93; 95%CI: 0.88-0.99 per 10 nmol/L).[23]

Wong et al. researched into the connection between postmenopausal women's serum 25(OH)D) concentrations and cancer-specific mortality. Women who had lower serum concentrations of 25 (OH) D is less than the average value of 64 nmol/L; had a greater risk of dying from cancer. For every 30 nmol/L drop in serum 25 (OH) D level, resulted in a 30% increase in the overall risk of cancer-related death.[24] According to epidemiological data, vitamin D supplementation is linked to a lower cancer mortality rate and lower circulating vitamin D levels are linked to to a higher chance of developing ovarian cancer.[25]

In Lagos, Nigeria, women were studied by E Sajo et al. to ascertain the connection between the risk of ovarian cancer and serum vitamin D levels. Each participant's venous blood was taken to measure the serum 25-hydroxyvitamin D [25(OH)D] level using a vitamin D ELISA kit. Researchers discovered that the vitamin D levels in ovarian

cancer patients were lower than those in healthy individuals. Epithelial ovarian cancer risk was four times higher in people with vitamin D deficiency.[26]

In one study, the impact of calcitriol and calcidiol on the growth of melanoma cells and the way they responded to radiation from proton beams was evaluated. Melanoma cell lines (human SKMEL-188, hamster BHM Ma, and hamster BHM Ab) were given calcitriol as pretreatment at graduated doses (0, 10, and 100 nM) and then exposed to radiation of 0 to 5 Gy. They discovered that that at 10 nM, calcitriol inhibited the growth of human melanoma, yet just calcidiol did the same at 10 and 100 nM levels for hamster lines. Melanoma cells were made more sensitive to modest doses of proton beam radiation after receiving either 1.25(OH)2D3 or 25(OH)D3.[27]

We used the MTT test to analyze different calcitriol concentrations and treatment durations to identify the effects of calcitriol on cell viability. The MTT assay showed that treatment with calcitriol decreased the viability of human MDAH-2774 ovarian cancer cells in a concentration- and time-dependent manner.The MTT assay data analysis revealed that calcitriol therapy at concentrations of 1 nM, 100 nM, and 1000 nM for 24 hours reduced the proliferation of MDAH-2774 cells.

Virtually every type of human cell contains VDR, but it is primarily found in metabolic organs. Recent research has identified VDR as a mitochondrial localization, and it has been discovered that in cancer cell lines, keratinocytes, and adipocytes, calcitriol suppresses mitochondrial respiration. This has an impact on lipid metabolism, cell differentiation, and proliferation.[28] Being a fat-soluble substance, calcitriol easily interacts with VDR, where it has an impact on biological processes.[29]

Reduced fibrosis and inflammation in both acute and chronic murine pancreatitis are two advantages of VDR-directed treatment. Genomic investigations have also revealed a link between tumor growth and shorter disease-free life periods and low-to-absent expression of VDR and cytochrome p450.[30]

Tumor suppressor gene (TSG) inactivation is a molecular target for the emergence of neoplasia. One of the TSGs in a range of malignant tumors is p53. Advanced-stage and high grade tumors have been linked to endometrial cancer that expresses mutant P53 protein strongly.[31]

Bcl-2, an anti-apoptotic protein, prevents cell death by controlling the activity of the mitochondrial membrane. The pro-apoptotic proteins that are responsible for causing cell death are encoded by the Bcl-2-associated X protein (BAX). Excessive Bcl-2 expression suppresses apoptosis while excess of Bax causes cell death. It has been hypothesized that the P53/BCL2/BAX apoptotic signaling pathway is dysfunctional during the development and progression of tumors.[32]

A melanoma cell line was utilized in one study to investigate the anti-proliferative impact of calcitriol using a cell viability assay. PCR, expression of apoptosis-related genes, and western blot analysis of apoptosis protein levels. They discovered that the apoptosis-related proteins caspase-3, caspase-8, and caspase-9 could all be activated by calcitriol. These calcitriol side effects highlight the drug's potential as a strong adjuvant therapy for melanoma.[33,34]

By using qRT-PCR, we examined the gene expression of VDR, p53, Bax, and Bcl-2. We discovered that treatment of MDAH-2274 ovarian cancer cells for 24 hours with 1000nM calcitriol raised the expression of VDR, p53, and Bax and decreased Bcl-2.

Ohnishi et al. demonstrated that vitamin D-induced cell cycle arrest is caused by the inhibition of numerous essential proteins that control the G1/S phase and up-regulate the expression of P53.[35] In accordance with the literature, we discovered enhanced p53 expression. The current study's findings further demonstrated that the decreased expression of Bcl-2 and elevated Bax expression in calcitriol-treated MDAH cells are necessary for calcitriol's anti-apoptotic and anti-proliferative actions.

In addition to inhibiting the epithelial-to-mesenchymal transition, calcitriol has been demonstrated to enhance VDR expression in a variety of cancer cells such as bronchial epithelial and peritoneal mesothelial cells.[36]

In the current study, we also discovered elevated VDR expression in MDAH cells that had received calcitriol treatment. We demonstrated the antiproliferative and apoptotic effects of calcitriol on human ovarian cancer cells. Thus, we can propose that vitamin D might be helpful as an adjunctive therapy for ovarian cancer.

Conflict of Interest

The authors declare that there is not any conflict of interest regarding the publication of this manuscript.

Ethics Committee Permission

Since a cell line was used in this study, ethics committee approval is not required.

Authors' Contributions

Concept/Design: BK. Data Collection and/or Processing: EA. Data analysis and interpretation: EA. Literature Search: BK. Drafting manuscript: BK. Critical revision of manuscript: EA. Supervisor: BK.

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