Journal of Shahrekord University of Medical Sciences
doi:10.34172/jsums.2022.22 2022;24(3):133-138 http://j.skums.ac.ir

Original Article

An investigation of the anti-cancer effects of vitamin D on the expression of HE4 gene and the viability of ovarian cancer cell line A2780S

Maryam Yahyaie[1], Elham Salehi[1], Majid Morovati-Sharifabad[1*], Fatemeh Sarkargar[2], Mohammad Saeed Heydarnejad[3], Gholamhosein Pourghanbari[4]

[1]Department of Basic Sciences, Faculty of Veterinary Medicine, Ardakan University, Ardakan, Iran
[2]Meybod Genetic Research Center, Meybod, Iran
[3]Department of Animal Sciences, Faculty of Basic Sciences, Shahrekord University, Shahrekord, Iran
[4]Department of Clinical Sciences, Faculty of Veterinary Medicine, Ardakan University, Ardakan, Iran

*Corresponding Author: Majid Morovati-Sharifabad, Email: mmorovati@ardakan.ac.ir

Abstract

Background and aims: Ovarian cancer is the second most common cause of death in Iran. A reduction in vitamin D production has been associated with an increased risk for ovarian cancer in many studies. Human epididymis protein 4 (HE4) is known as one of the most accurate tumor markers for the analysis of ovarian epithelial cancer illness or progression. The expression of the gene increases in many types of ovarian cancer. The aim of this study was to find whether vitamin D has anticancer effects on the viability of HE4 gene expression.

Methods: The human ovarian cancer cell line (A2780S) was cultured in an RPMI-1640 medium. To determine the inhibitory concentration (IC50), the cells were treated with various concentrations of vitamin D and then incubated for 24, 48, and 72 hours. The effect of drugs on the expression of HE4 gene modification was measured and evaluated using the real-time polymerase chain reaction and a 2-ΔΔCT method, respectively.

Results: The IC50 value for vitamin D was 0.359 µM, and the maximum death rates were observed after 24 hours (56.20 ± 5.79). The HE4 gene expression treated with vitamin D increased compared with the cells in the control group (Fold change > 1).

Conclusion: Vitamin D decreases the viability of A2780S cells, whereas the expression of the HE4 gene is improved in cells treated with vitamin D compared to control cells, indicating that vitamin D may be unable to modify A2780S cells.

Keywords: Vitamin D, Ovarian cancer, HE4 gene, A2780S cell line

Received: 23 October 2021, Accepted: 31 May 2022, ePublished: 18 August 2022

Introduction

It is known that ovarian cancer is one of the major causes of mortality as a gynecologic cancer. Ovarian cancer ranks eighth among prevalent gynecologic cancers and is the second leading cause of cancer mortality among Iranian females (1). Epithelial ovarian tumors are more prevalent among women in their sixth decade of life. Serous carcinoma is the most frequent epithelial ovarian tumor (2). Considering the high frequency of ovarian cancer, it is critical to conduct more research on the identification of new anticancer compounds with high therapeutic values and minimal side effects. Vitamin D is a fat-soluble steroidal prohormone. The vitamin D endocrine system regulates over 3% of human genomes (3). Calcitriol is a valuable marker for vitamin D3 diagnosis. Calcitriol has been proven to have a variety of anticancer effects in different malignant cells and animal models, including anti-proliferative impacts, the inhibition of cell detachment, and anti-the inflammatory influence of apoptosis promotion (4). Alterations in a nucleotide of the vitamin D-encoding gene raise people's chances of developing ovarian cancer (5). Numerous ecological studies have shown that the risk of ovarian cancer incidence and death is inversely associated with UV-B radiation, which is necessary for vitamin D generation in the skin. Women living in places with high levels of ambient ultraviolet radiation have a decreased chance of developing epithelial ovarian cancer (6). The mRNA expression on vitamin D receptors (VDRs) increases by an increase in estrogen levels. The VDR and its ligand regulate the cell cycle through controlling the P21 and P27 proteins (7). This receptor is very moderately expressed in normal ovarian cells, while it is highly expressed in tumor tissues and ovarian cancer cells (6). On the other hand, some studies demonstrated that there is no significant relationship between serum vitamin D concentrations and the risk of ovarian cancer (8).

WFDC2 expresses multiple WAP proteins on 20q12-13.1, including HE4. The use of molecular tools to quantify the HE4 protein is a relatively new non-invasive diagnostic method for determining the degree of disease invasion, early diagnosis of cancer, and follow-up on the recurrence and progression of ovarian epithelial cancer in affected individuals (9). The Roma test, which simultaneously

evaluates HE4 and CA125 levels in a patient's blood, allows doctors to diagnose ovarian cancer in women before menopause (10). HE4 expression is elevated in the urine, serum, and neoplastic tissue of the ovaries.

The HE4 gene expression occurs in 100% of endometrioid carcinoma, 50% of transparent cell adenoma, and 93% of serious adenocarcinoma, helping in differentiating tumor subtypes (11). The high sensitivity and specificity of HE4, as well as a strong expression in the serum and tissues of patients with ovarian cancer, were the justification for the choice of this tumor marker (12). As a result of the increasing occurrence of ovarian cancer, more research is required to find novel anticancer agents with high treatment efficacy and minimal side effects. Considering that earlier research on the influence of vitamin D viability on ovarian cancer cells yielded inconsistent results, this study focused on exploring the anticancer effect of vitamin D on HE4 gene expression and vitality of A2780S ovarian cancer cells.

Materials and Methods

Cell culture

The Pastor Institute of Iran provided the human ovarian cancer cell line A2780S for this investigation. The cells were grown and cultured in the RPMI1640 medium (Iran-made Inoclon Company) with 10% fetal bovine serum (Biochrom GmbH Company, Germany) and 1% Pen-Strep antibiotic (Iran-made Inoclon Company) and then incubated at appropriate conditions. Adherent cultures should be passaged after cell density has achieved 70-80% in the log phase.

Drug

From the pharmacy, a 300,000 IU/mL vitamin D solution was purchased from Iran Hormone Pharmaceutical Company. Vitamin D was dissolved in DMSO (Iran-made Inoclon Company) for serial dilutions 1 and 10 λ.

Cellular part

IC50 assay and time calculation

To determine the IC50, the cells were cultured in a plate and then separated from the flask floor after reaching the necessary cell density. The cells were then placed on a 12-well plate and incubated in ideal circumstances. Different amounts of vitamin D were applied after each well had reached the necessary density. The cells were separated after trypsinization. Following centrifuging the cell suspension, the cells were counted using a Neubauer slide. Next, the time calculation was conducted in the same manner as the IC50 technique, except that the appropriate dose from the IC50 method was administered to the wells, and the cells were subsequently removed at various intervals of 24, 48, and 72 hours.

Cell culture

The cells were placed in the 24-well plates and incubated under optimal circumstances. After obtaining the necessary density, 1 μM vitamin D was added to the wells, and cell harvesting was completed between 24 and 48 hours. When the cell suspension was centrifuged, cell deposition immediately reached the nitrogen tank. The control group (without vitamin D) was included in all tests.

Molecular part

RNA was extracted according to the protocol of the High Pure RNA Isolation Kit (Germany-made Roche Company). RNA electrophoresis on an agarose gel was used to assess the quality of isolated RNA. On the gel, high purity RNA formed two distinct bands. In addition, a nanodrop spectrophotometer was applied to quantify the extracted RNA, and optical absorption was evaluated at wavelengths 230, 260, and 280 nm.

Lithuania-made Thermo Scientific kit was employed for performing cDNA, and after combining materials, was implanted in a thermal cycler for 10, 60, and 10 minutes at 25°C, 42°C, and 65 °C, respectively. The nanodrop was then employed for evaluating cDNA sample absorption.

The primers used in this study were extracted from the relevant article (13). Pishgam Biotech Company provided the lyophilized primers (Table 1).

At the final 25 Ml volume, real-time polymerase chain reactions (RT-PCR) were performed in Applied Biosystems StepOnePlus and StepOne™ Company of USA. Initially, a RealQ Plus 2x Master Mix (Denmark-made Ampliqon Company) and water were prepared, followed by adding HE4 and GAPDH primers to their master mix solution and inserting cDNA. The temperature-time protocol was run in three steps using the RT-PCR. The first step, resulting in pattern DNA denaturation and polymerase enzyme activation, lasted 15 minutes at 95°C. The DNA proliferation process was repeated 40 cycles in the second step and at 95°C, 72°C, and 62°C for 20, 30, and 15 seconds, respectively. The final stage was to test the product's specificity by plotting the melting curve in the range of 73-95°C.

Statistical analysis

All experiments presented in this article were performed in triplicate. Data were statistically evaluated by the one-way ANOVA and Tukey's tests in Graph Pad PRISM software (Version 8). Further, the $2^{-\Delta\Delta CT}$ method was used to change the expression of the HE4 gene. All data were presented as the mean ± standard deviation (SD). $P<0.05$ was considered statistically significant.

Table 1. The sequence of the applied primers

Primer name	Sequence (5´to 3´)	Tm	%GC	Product size (bp)
HE4	F: CGGCTTCACCCTAGTCTCAG	59.54	60	164
	R: CATTGGGCAGAGAGCAGAAG	58.62	55	
GAPDH	F: TCCTCCACCTTTGACGCTG	59.63	57.89	102
	R: CACCACCCTGTTGCTGTAGC	61.24	60	

Results

The results obtained after 24 hours of incubation showed a decrease in the survival rate after vitamin D treatment according to a dose-dependent manner; therefore, the percentage of living cells in the control decreased significantly from 47.44 ± 3.05 (at a concentration of 1 µM) to 41.75 ± 2.73 (at a concentration of 10 µM) ($P<0.05$). According to Figure 1, the IC50 for vitamin D was 0.3592 µM, which has been treated with a concentration of 1 µM.

According to the results of the statistical analysis (ANOVA test), $P=0.0007$ and F (2,3) = 187.5 indicated that the mean viability of A2780S cells at different concentrations of vitamin D has a significant difference. The Tukey's test results revealed that there was no significant difference between the two doses of 1 µM and 10 µM ($P=0.3343$); however, a significant difference existed between these treatments and the $P<0.05$ control group (1 µM vs. Control = 0.0012, 10 µM vs. Control = 0.0009).

Based on the results (Figure 2), significant differences occurred at different times ($P<0.05$), and survival declined over time; however, the highest fatality in vitamin D was found 24 hours after treatment (56.20 ± 5.79), and the lowest cell mortality was observed in 72 hours after treatment (46.30 ± 3.38). According to the results of statistical analysis (ANOVA test), $P=0.0006$ and F (3,4) = 73.06 represented that the mean viability of A2780S cells at a different time of vitamin D diagnosis has a significant difference. Based on Tukey's test results, there was no significant association between treatment groups. However, vitamin D therapy at 24, 48, and 72 hours was significant in the $P<0.05$ group compared to the control group (Table 2).

As shown in Figure 3, HE4 gene expression was increased in vitamin D-treated A2780S cell line compared with control cells (Fold change >1); in other words, when vitamin D is used in cell treatment, it shows its negative effect on A2780S ovarian cancer cells (Table 3).

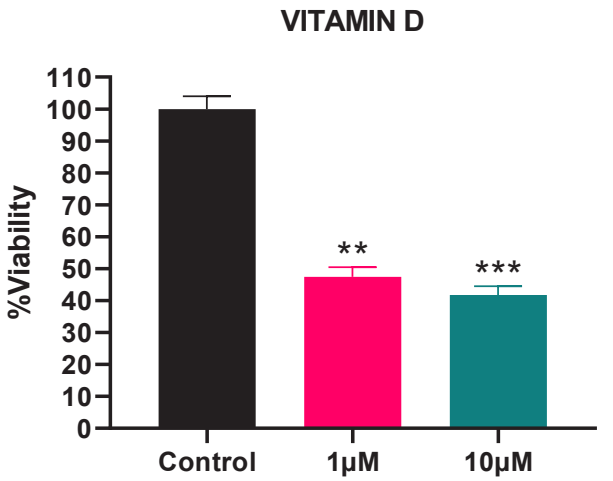

Figure 1. Significance of A2780S cell line cell viability at different concentrations of vitamin D for 24 hours of treatment compared with controls. *Note*. *$P<0.01$ and $P<0.01$ vs. control group.

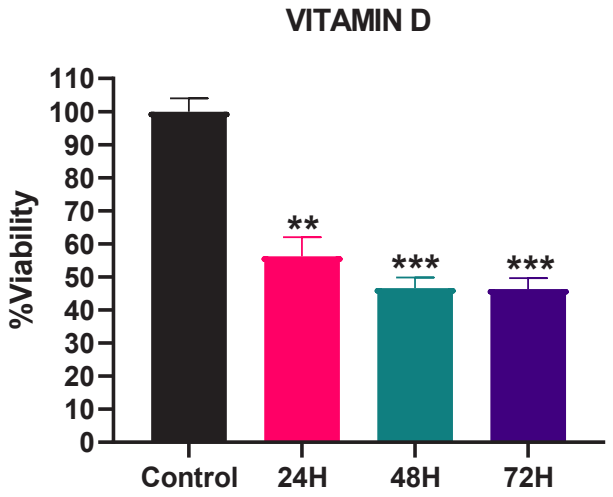

Figure 2. Cell viability of the A2780S cell line at different times. The graph is based on the percentage of viable cells 24, 48, and 72 hours after vitamin D treatment at a dose of 1 µM compared to the control group. It indicates the greatest reduction in the cell count after 24 hours. *$P<0.01$ vs. control group

Discussion

Ovary cancer is one of the most common cancers for women. This cancer is treatable in the early stages, while it is rarely curable in the more advanced stages. The use of specific tumor markers with diagnostic potential is a useful measure to improve therapeutic results. If the ovarian cancer is diagnosed and treated, which has not yet penetrated outside the ovary, the percentage of survivors will reach 95% within five years of starting treatment, while only 25% of ovarian cancers are identified at an early stage (14). This study sought to examine the impact of vitamin D on the viability of A2780 ovarian cancer cells. By affecting different concentrations of vitamin D in the cell line, vitamin D was observed at the minimum concentration of 1 µM, causing 50% viability of A2780S

Table 2. Comparison of the effect of vitamin D treatment using the Tukey's test

Tukey's test	Mean Dif.	95.00% CI of Dif.	Summary	Adjusted *P* value
24 h vs. Control	-43.80	-61.01 to -26.59	**	0.0017
48 h vs. Control	-53.36	-70.57 to -36.14	***	0.0008
72 h vs. Control	-53.70	-70.91 to -36.49	***	0.0008
48 h vs. 24 h	-9.555	-26.77 to 7.659	ns	0.2505
72 h vs. 24 h	-9.900	-27.11 to 7.314	ns	0.2315
72 h vs. 48 h	-0.3450	-17.56 to 16.87	ns	0.9998

* $P<0.05$
** $P<0.01$
*** $P<0.001$

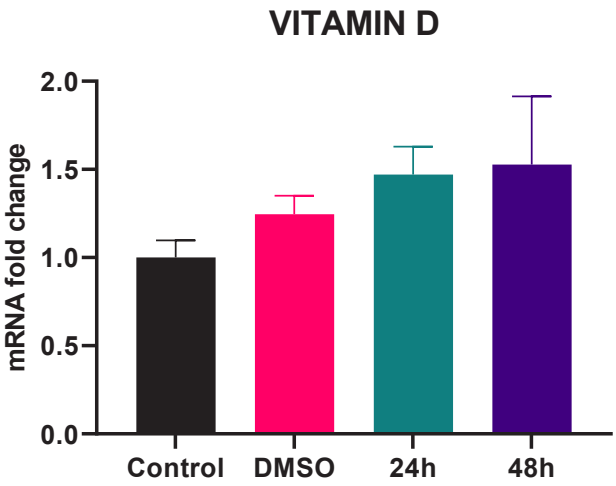

Figure 3. Changes in HE4 gene expression in A2780S cell line with a concentration of 1 µm for vitamin D in 24- and 48-hour time points. *Note*. No significant difference is observed compared to the control group. * $P<0.05$, ** $P<0.01$, and $P<0.001$.

cells (47.44±3.05). The highest concentration of vitamin D used in this study was 10 µM with the cell viability of 41.75±2.73. In this study, vitamin D affected cell lines at 24, 48, and 72 hours, and the survival rate of A2780S cancer cells decreased over time, but the highest mortality rate of vitamin D-treated cancer cells occurred in the first 24 hours (56.20±5.79). Probably one of the reasons is the presence of enough space for the effect of this vitamin on cells; cell layers are added, and the level of effectiveness of the drug is reduced over time and because of the proliferation of cells. Another cause includes the drug's initial shock on cells, which is why it will have the highest lethality (15). An increase in HE4 gene expression was observed given the effect of vitamin D on A2780S cell lines. Accordingly, the destructive and toxic effects of DMSO as a vitamin D solvent prevent the positive anticancer effect of this vitamin (16,17). The other reason for this result is the maintenance substances in vitamin D, which can be used to justify the negative effect of vitamin D on the anticancer properties of this vitamin on A2780S ovarian cancer cells.

The findings of a recent meta-analysis demonstrated that vitamin D supplements are associated with a 13% reduction in cancer deaths (18). Carlberg found a relationship between high levels of vitamin D in circulation and a reduction in the risk of certain types of cancer (breast, colon, stomach, blood, head and neck, kidney, lung, ovarian, liver, pancreas, prostate, and skin) so that vitamin D prevents tumor proliferation and differentiates cancer cells in in vitro and in vivo conditions (19). The results of Liu et al indicated that 1, 25 $(OH)_2D_3$ in ovarian cancer amplified the expression of E-cadherin and VDR genes; on the other hand, they reported that it attenuates the expression of a ß-catenin gene by inducing DMBA and plays the key role in inhibiting tumor metastasis in tumors (20). In another study, Lungchukiet et al suggested that 1, 25 (OH)2D3 and its analogs may prevent the release of cancer cells into the Omentule region by VDR binding into epithelial cancer cells and stromal cells. Omentum is the most common tissue affected by ovarian cancer metastasis because it contains a large number of adipocytes, immune cells, capillary cells, and fibroblasts that provide a good space for the growth of cancer cells (21). Target genes 1,25 $(OH)_2D_3$ go through a variety of molecular pathways for vitamin D apoptosis activity in ovarian cancer, two of which include strengthening P21 and P27 inhibition with CDK weakening and subsequent phase inhibition of G1/S and phase inhibition of G2/M by 1,25 $(OH)_2D_3$ by the independent induction of GADD45 by P53 (22). Various studies have presented several mechanisms to inhibit tumor growth by VDR, including genetic and nongenetic transmission pathways. The genetic pathway is that 1,25 $(OH)_2D_3$ is attached to VDR and transferred to the nucleus of the cell; then, it regulates gene transcription by forming the VDR/retinoid X receptor complex into vitamin D. In addition, vitamin D exerts other nongenetic effects such as regulating the homeostasis of phosphate and calcium, as well as activating protein kinase C, protein kinase A, phosphoinositide3-kinase, and phospholipase C (23). Van Etten et al also reported that VDR leads to a decrease in NF-κB transcription, resulting in a decreased IL-12 expression and a weakened immune system (24). Likewise, Reichrath et al concluded that there is a special relationship between P53 and VDR; the VDR gene is a member of the P53 family that plays a role in the induction of apoptosis and cell cycle stoppage and is the main target of P53 (25). Kasiappan et al demonstrated that in ovarian cancer, the induction of cellular apoptosis occurs by the suppression of hTERT mRNA transcription because the miR-498 gene is the primary target of 1, 25 $(OH)_2D_3$ (26). All these results are inconsistent with those of the present study. Moreover, epidemiological evidence suggests that decreased levels of vitamin D in circulation are associated with a higher risk of ovarian cancer, which contradicts the results of this study.

Table 3. Comparison of the effect of vitamin D treatment using the Tukey's test

Tukey's test	Mean Dif.	Summary	Adjusted *P* value	Fold change	Interpretation
DMSO vs. control	0.3174	ns	0.1997	1.246083	Up: 1.246 fold
24 h vs. control	0.5567	ns	0.05197	1.471	Up: 1.471 fold
48 h vs. control	0.7027	ns	0.0553	1.628	Up: 1.628 fold
24 h vs. DMSO	0.2389	ns	0.3999	1.180	Up: 1.558 fold
48 h vs. DMSO	0.3849	ns	0.1046	1.306	Up: 2.121 fold
48 h vs. 24 h	0.1460	ns	0.7452	1.106	Up: 1.362 fold

There is strong evidence that vitamin D levels are not related to ovarian cancer risk. Cook et al found that there is no coherent or strong evidence to support the claims made in many review articles, indicating that exposure to vitamin D reduces the risk in the cancer of the ovaries or its death (27). Toriola et al concluded that there was no significant relationship between the serum concentration of vitamin D and risk of ovarian cancer (8). Ecological studies and empirical data represent that vitamin D may reduce the risk of ovarian cancer. Tworoger et al investigated the association between the plasma concentrations of 25-hydroxy vitamin and the risk of epithelial ovarian cancer in a case-control study and found that plasma vitamin D levels were not significantly correlated with the risk of ovarian cancer (28). In a case-control study, Arslan et al measured the serum or plasma level 25(OH)D in 170 acute cases of epithelial ovarian cancer and indicated that the level of 25(OH)D was not associated with EOC risk, and there was no indication of interaction between the genotype or haplotype SNP in the VDR and the level of 25(OH)D associated with the risk of ovarian cancer; nonetheless, they suggested there may be complex gene and environment interactions (29). Based on the reports of Zheng et al (30), there was an inverse relationship between 25(OH)D and the risk of ovarian cancer in women with a body mass index of ≥ 25 kg/m^2, and generally, their research does not support the blood levels of 25(OH)D and risk of ovarian cancer (excluding among overweight women), which is in line with the results of the present study.

Conclusion

The results of this study revealed that vitamin D increases the expression of the HE4 gene so that vitamin D cannot affect ovarian cancer at the cell level, although epidemiological and preliminary clinical trials are inconsistent; furthermore, randomized control tests on humans are not yet available to highlight the beneficial role of vitamin D as a key nutrient to prevent attack and metastasis of ovarian cancer. However, further cellular and molecular aspects of vitamin D studies are recommended due to the lack of sufficient statistical reasons. Maybe vitamin D is a suitable drug delivery approach to increase the efficiency of treatment in ovarian cancer.

Acknowledgments

This study was derived from a thesis in Animal Biology-Cell Biology and Development approved by the Department of Basic Sciences, Faculty of Veterinary Medicine, Ardakan University, Ardakan, Iran in October 2020. The authors of this article express their gratitude to colleagues who helped us in this research.

Authors' contributions

MY, ES, MM, and FS, contributed to the conception and design of experimental work. GP participated in data and statistical analysis.

Conflict of interests

None.

Ethical approval

This experimental study was conducted on ovarian A2780s cancer cell lines at the Cell and Developmental Laboratory of the Basic Sciences Department, Faculty of Veterinary Sciences, Ardakan University (Ethics code: IR.YAZD.REC.1399.036).

Funding/Support

The present study was conducted with the financial support of Ardakan University.

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