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# Vitamin D can be used as a supplement against cancer stem cells

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**Abstract:** Cancer is standing like a bottomless pit or a black hole in front of mankind. Scientists are trying all possible ways to find a solution against to cancer. As known, cancer is a phenomenon fed from internal dynamics. One of internal dynamic is cancer stem cells that are involved in the formation and development of cancer. Because of these dynamics, scientists began to search solution inside of the body. Another internal dynamic is vitamin D and it is not only important in calcium homeostasis but also it is important for cell proliferation, differentiation, and apoptosis. In this study, we investigated the effect of vitamin D on cancer stem cells that sorted from MCF-7 cell line and on HEK293 cell line as control. Our results showed that calcitriol treatment reduced the number of CSC (Cancer Stem Cell) in the MCF-7 cell while increased in HEK293 cell population. Gene expression analyses showed that effect of calcitriol on apoptosis plays an important role in this reduction. Deficiency or unavailability of vitamin D may take a role in the pathogenesis of breast cancer.

***Key words:*** Calcitriol; Vitamin D; Cancer stem cell; MCF-7; HEK293.

## Introduction

Cancer continues to be one of the major health problems as the most common cause of death, after cardiovascular diseases in the world. Despite significant improvements in the development of effective treatment protocols against some cancer types over the past 30 years, no significant progress has been achieved in terms of overall success in five-year survival rate (1). Cancer stem cells (CSC) are one of the obstacles to the complete elimination of cancer. Some studies showed that CSC were contributing to the development and maintenance of the tumor (2,3). Presence of CSC in almost all solid tumors, especially in the breast and brain tumors reported in last years. (4,5). It has been found that CSC quantities differ between tumor types and also between the same originated tumors (6). CSC exhibit a dynamic behavior within the tissue rather than a static state and this dynamism is caused by extrinsic factors as well as intrinsic factors (6,7).

Vitamin D is a steroid-based prohormone produced mainly by the action of ultraviolet light from the 7-dehydrocholesterol (8). About 95% of the needed vitamin D is synthesized directly under the influence of sunlight (8). The potential anticancer effect of active vitamin D (calcitriol) has been evaluated in animal and cell culture studies for approximately 30 years (9,10). In vitro cell culture and in vivo animal studies have shown that active vitamin D enhances cell differentiation, inhibits cancer cell proliferation, exhibits anti-inflammatory effect, proapoptotic and antiangiogenic properties (11). The list of malignant cells that express Vitamin D Receptor (VDR) is quite extensive and laboratory studies have shown that active vitamin D binds to VDR and inhibits the growth of cancer cells by regulating various genes responsible for cell proliferation and also differentiation (11). The effect of vitamin D on differentiation may also be effective in the transformation of CSC.

Active vitamin D (calcitriol) stimulates the expression of p21, p27 (cell cycle inhibitors), e-cadherin (cell adhesion molecule) and inhibits the transcriptional activity of catenin (11). It has shown that in keratinocytes, active vitamin D repairs DNA damage that caused by UVR and increases the p53 (12). Calcitriol may be an important target in treatment approaches for malign tumors and also for elimination of CSC. Although there have been numerous studies in the literature that investigate the effect of calcitriol on different cancers, there is not too much study that investigates the effect of calcitriol on CSC. Based on this data, especially the effect of Vitamin D on cell differentiation, we have studied the effects of calcitriol on breast CSC.

## Materials and Methods

### Cells and cell culture

MCF-7 and HEK293 cells were cultured in RPMI (Biochrome, Germany) culture medium containing 10% FBS (Fetal Bovine Serum) (Biochrome, Germany) and 1% penicillin/streptomycin (100 U / ml penicillin and 100 µg / ml streptomycin). After the cells were filled to 80% of the flask, cells were washed with 1xPBS (Gibco, USA) and removed with an enzymatic solution of 0.25% trypsin-EDTA (Biochrome, Germany). Subsequently, the enzymatic activity was inactivated with an equal volume of medium. The dead and alive cells were counted by using a hemacytometer.

**Identification of IC50 dosage and cell viability analyzes**

Approximately $5x10^3$ cells were seeded in a 96 well plate and incubated for 24 h in a 5% $CO_2$ humidified incubator at 37 °C. The old medium was removed after incubation, and after the cells washed with PBS, 100 µl of media containing calcitriol in different concentrations were treated for 24, 48, 72 hours. Subsequently, 10 µl of 12 mM Thiazolyl Blue Tetrazolium Bromide solution was added to each well and incubated for 4 hours at 37 °C, 5% $CO_2$. At the end of the incubation, the medium was removed from the wells and 50 µl DMSO was added and incubated for 10 min at 37 °C, 5% $CO_2$. After incubation, the mixture was pipetted and the absorbance values were measured on a micro-plate reader at 490 nm wavelength.

**Stem cell separation by flow cytometry**

In order to demonstrate the effect of calcitriol on stem cells, total HEK293 and MCF-7 cells were divided into four groups and one group of each cell was cultured for 48 h in a medium that contains 100 nM calcitriol, other two groups were cultured without calcitriol and evaluated as the control group. When the cell number reached to $1x10^8$ cells, cells were digested and dispersed into single cell solution and prepared for cell sorting. Cell sorting was carried out in a FACS instrument (FACS Aria III - BD Pharmingen, USA) with CD44-APC-A and CD24-FITC-A antibodies. (CD44-APC, Sigma-Aldrich Corporation, Germany; CD24-FITC, Sigma-Aldrich Corporation, Germany).

**Determination of colony formation of cancer stem cells via soft agar assay**

Colony formation was determined by soft agar assay to confirm the stem cells obtained from HEK293 and MCF-7 cell lines by flow cytometry. Basal agar was prepared as 0.8% with 2xDMEM / RPMI + 10% FBS media and 1 ml of this mixture was taken into a 6-well petri dish. Cultured cells were removed by trypsinization and transferred to wells with at least 2500 cells per well. Another type of agar, top agar, was prepared to be in a concentration of 0.6% in the same manner as the basal agar. 1 ml of this mixture was transferred to wells to form agar. Wells were then incubated at 37 °C for 10-14 days. Top agar medium was changed every 4-5 days. Finally, colony formation was observed by dyeing wells with Crystal violet.

**Total RNA extraction and cDNA synthesis**

Five hundred µl TRI Reagent® (Sigma, USA, # MKBP6595V) solution was added and pipetted onto the stem cells isolated by flow cytometry. The pellets were lysed by pipetting in trizol and homogenized using QIAshredder Columns (Qiagen, Hilden, Germany). Then, total RNA was extracted by the trizol-chloroform method in the direction of the manufacturer's instructions. The RNA preparations were cleaned up by using RNeasy Mini columns (Qiagen), and the intactness and concentration of the isolated RNA were determined via NanoDrop™ 2000/2000c Spectrophotometers (Thermo Fisher Scientific, MA USA) and agarose gel electrophoresis. cDNA synthesis from total RNA was performed using the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, USA, # K1622) following the manufacturer's instructions.

**Quantitative RT-PCR analysis**

Quantitative RT-PCR analysis was performed in triplicate with LightCycler 480 instrument (Roche Diagnostics,). GeNorm analysis (PrimerDesign Ltd, Southampton UK) was performed to decide a housekeeping gene. The housekeeping genes including Ubiquitin C (UBC), Eukaryotic initiation factor 4A2 (eIF4A2), 18S ribosomal RNA, Glyceraldehyde 3-Phosphate dehydrogenase (GAPDH), β-actin (ACTB), β2 Microglobulin (B2 M), Glucose-6 phosphate dehydrogenase (G6PDH) were tested for consistent expression. Finally, Glucose-6 phosphate dehydrogenase (G6PDH) was chosen as a stably expressed gene for normalization. Expression of Oct4, SOX2 and Nanog genes, which are stem cell markers and p53, Bax and Bcl-2 as apoptosis markers, were assessed by using real-time quantitative PCR (qPCR) assay.

Specific primers for *p53, Bax, Bcl-2, Oct4, Sox2, Nanog* and *Gapdh* genes were designed by using the Integrated DNA Technology (IDT) PrimerQuest online program. Primer sequences are given in Table 1. The

**Table 1.** Primer sequences used in real time qPCR gene expression assays.

| Primer name | Primer Sequences |
|---|---|
| BAX - forward | 5'- CCCGAGAGGTCTTTTTCCGAG -3' |
| BAX - reverse | 5'- CCAGCCCATGATGGTTCTGAT -3' |
| BCL-2 – forward | 5'- GGTGGGGTCATGTGTGTGG-3' |
| BCL-2 – reverse | 5'- CGGTTCAGGTACTCAGTCATCC -3' |
| OCT4 – forward | 5'- CTGGGTTGATCCTCGGACCT -3' |
| OCT4 – reverse | 5' CCATCGGAGTTGCTCTCCA-3' |
| SOX2 – forward | 5'- TACAGCATGTCCTACTCGCAG -3' |
| SOX2 – reverse | 5'- GAGGAAGAGGTAACCACAGGG -3' |
| NANOG – forward | 5'- AAGGTCCCGGTCAAGAAACAG -3' |
| NANOG – reverse | 5'- CTTCTGCGTCACACCATTGC -3' |
| TP53 – forward | 5'-CAGCACATGACGGAGGTTGT-3' |
| TP53 – revers | 5'- TCATCCAAATACTCCACACGC-3' |
| GAPDH – forward | 5'- GGAGCGAGATCCCTCCAAAAT -3' |
| GAPDH – reverse | 5'- GGCTGTTGTCATACTTCTCATGG -3' |

primers were synthesized by Biomers Company (Ulm, Germany).

### Statistical analysis

Data were expressed as means ± SEM. Fold changes in gene expression, comparative CT method (comparative -ΔΔCT method) (13) and statistical analysis were determined by using the freely available Relative Expression Software Tool (REST 2009, Qiagen).

## Results

### IC50 values of calcitriol

The IC50 value for calcitriol was determined by MTT assay. As a result of the analysis, this value was found to be 100 nM for 48 hours (Figure 1), and the study continued with this dose and duration.

### Determination of stem cell quantities by flow cytometry

A statistically significant decrease in CD44 (+) / CD24 (-) cells were detected in MCF7 cells and a minimal increase in the amount of stem cells in the HEK293 cells that treated with calcitriol was observed, when results compared with the control group. However, the increase in HEK293 cells was not statistically significant when compared with the control group (Figure 2).

### Annexin V analysis

Annexin V analysis was performed to evaluate the apoptotic effects of dose determined by MTT assay. As a result of this analysis, a statistically significant decrease determined in calcitriol-treated HEK293 cells when compared with control group. However, the MCF7 group treated with calcitriol showed a statistically significant increase in apoptosis when compared to the untreated group (Figure 3).

### Gene expression analysis

When calcitriol was applied at appropriate dose and period to HEK293 cells, the expression levels of the genes accepted as stem cell markers were significantly increased. These increases were found as 3.742±0.411, 6.316±0.283, 5.213±0.293 fold for *OCT4*, *SOX2* and *Nanog* genes respectively (Figure 4). In contrast, these gene expressions were significantly reduced in MCF7 cells that were treated with calcitriol. These reductions were calculated as 4.294±0.283, -4.91±0.324 and 6.05±0.372 fold for *OCT4*, *SOX2* and *Nanog* genes (Figure 4), respectively.

There was a significant increase in *Bax* (7,8±3,18) and *p53* (6,3±0,291) gene expression levels in MCF7-treated cells with calcitriol when compared with control group (Figure 4), but no significant increase in *Bcl-2* (0,83±0,278) expression was observed. Beside this, *Bax* (-4,287±0,324) and *p53* (-4,567±0,216) gene expression levels were significantly decreased in HEK293 obtained stem cells, while *Bcl-2* gene expression (3,671±0,382) levels were increased (Figure 4).

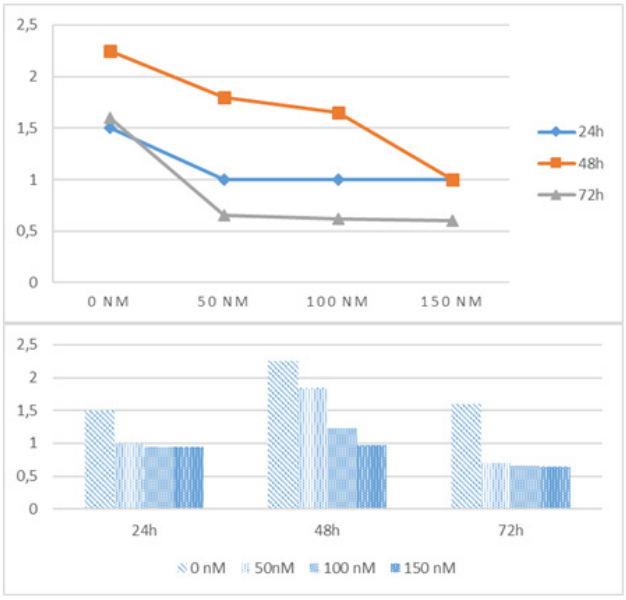


**Figure 1.** The dose-time graphic of the MTT analysis results.

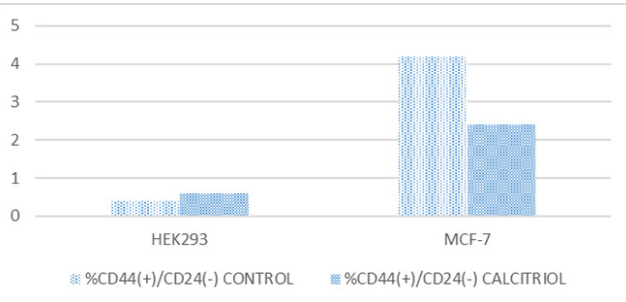


**Figure 2.** Graphical representations of stem cell numbers obtained from calsitriol treated and untreated cell lines.

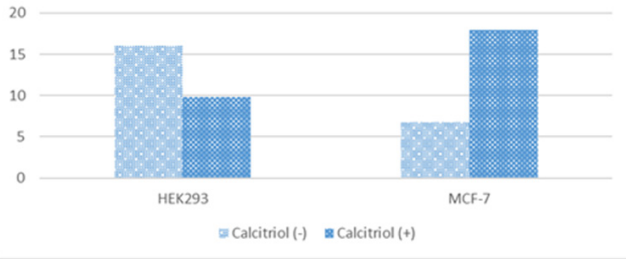


**Figure 3.** Graphical and figural display of Annexin V analysis results in HEK293 and MCF7 cells.

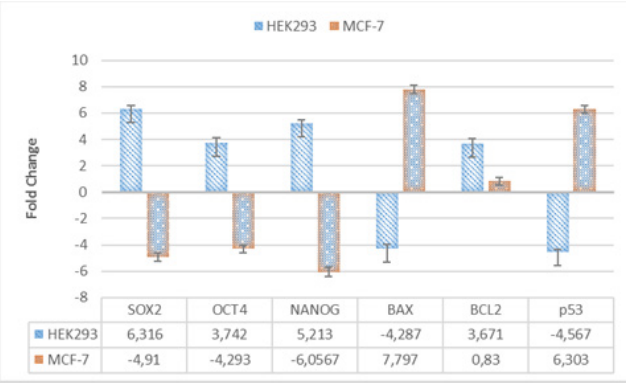


**Figure 4.** Gene expression levels of HEK293 and MCF-7 sorted stem cells. Expression levels obtained as fold change by comparison of treated group with calcitriol and untreated control group. The fold changes were determined by the -ΔΔCT method.

## Discussion

Numerous studies have been reported so far on the relationship between vitamin D and cancer, and various books related to this topic have been published (1-6). Firstly, in 1981, Colson et al. (14) demonstrated that calcitriol inhibited the growth of malignant melanoma

cells. After this date, the antineoplastic effects of calcitriol have shown in both in vitro and in vivo studies in different cancer types (15-21). In some of these studies, it was determined that calcitriol makes this effect by triggering different pathways in different types of cancer (22,23).

Active vitamin D (calcitriol) stimulates the expression of p21, p27 (cell cycle inhibitors), e-cadherin (cell adhesion molecule) and inhibits the transcriptional activity of catenin (11). It has shown that in keratinocytes, active vitamin D repairs DNA damage that caused by UVR and increases the apoptosis via p53 (12). In another study carried out on postmenopausal women, 1100 IU vitamin D3 and 1400 - 1500 mg calcium were given to the patients for 4 years. At the end of the first year, the cancer incidence was significantly lower in the vitamin D3 and calcium receiving group compared to the placebo group (24). VDR and 1-alpha-hydroxylase enzyme expression were studied in the colorectal tissue. It has shown that active vitamin D and analogous regulate the proliferation and differentiation of the human colon cancer cells (25). In another study, tumor growth was assessed after the injection of colon cancer cells into the normal and vitamin D deficient mice, tumor growth was observed significantly greater in vitamin D deficient mice (26). Active vitamin D can affect colon cancer progression and development by increasing the intracellular calcium influx that changed calcium balance (24). Observational studies have shown an inverse relationship between 25 (OH) D levels and the incidence of colorectal cancer (27).

Dietary vitamin D3 is not only a vitamin, it is also an indispensable precursor of the effective steroid hormone 1,25(OH)2D3 or calcitriol which has anti-inflammatory, antiproliferative, pro-differentiating and pro-apoptotic activities in various cancers including breast cancer (28). An opposite association has also been presented between breast cancer risk and the levels of serum 25-hydroxyvitamin D [25(OH)D] (28). Vitamin D signaling presents an essential role in the development of the normal mammary gland (29). Studies performed in vitamin D receptor (VDR) knock out mice showed that calcitriol signaling inhibits estrogen driven proliferation of mammary epithelial cells and supports normal differentiation (29). Recent studies have shown that calcitriol inhibits cell proliferation in both ER (+) and ER (-) human breast cancer cell lines (28-30). There are some studies about vitamin D on cancer tissue and cancer cell lines but studies related to the effect of calcitriol on stem cell and CSC are so limited.

In a research carried out to enrich mammospheres for CSC, it was determined that knocking down VDR increased the mammosphere formation, potentially by increasing the proliferation (31). However, high-dose calcitriol has been shown to have a minimal effect on the mammospheres (31). In vitro and xenograft experiments, demonstrated that the calcitriol analog BXL0124 inhibited the tumor proliferation and reduced the tumor CSC levels, and this increases the chance of the therapy in term of the elimination of the CSC population (32).

In a study carried out in mouse prostate stem cells by Maund et al., it has shown that calcitriol inhibits cell proliferation in prostate stem cells by inducing cell cycle arrest and senescence (33). The data revealed by Maund et al. showed that interleukin-1α is important for the genes targeted by VDR and regulates senescence in this way (33). It has been determined that cancer stem cells are in contact with normal stem cells (33).

In our study, we found a significant decrease in CD44 (+) / CD24 (-) cells in the MCF7 cell line treated with calcitriol. This was not consistent with the results obtained in prostate stem cells by Maund et al. (33). This data is consistent with the results of Annexin V analysis, which suggests that apoptosis mechanisms may be involved in this decrease. Gene expression analysis showed the increased expression of *Bax* gene that has apoptotic effect, however, expression of Bcl-2 that has pro-apoptotic effect was reduced in a manner consistent with Annexin V results. These results are consistent with the literal knowledge that calcitriol has pro-apoptotic effects on breast cancer cells (28-30). We have not made any comparison in this respect since there is no publication related to the effect of calcitriol on breast cancer stem cells. According to expression results of genes that are accepted as stem cell markers, calcitriol increased the number of stem cells in the HEK293 population and these results are consistent with the FACS and Annexin V analysis results. Analysis of Annexin V showed that calcitriol reduced apoptosis in HEK293 cells. This was not consistent with the results obtained by Maund et al. in relation to prostate stem cells (33). This may be related with the behavioral differences of normal prostate and HEK293 stem cells against to calcitriol.

In conclusion, our results indicate that calcitriol causes a decrease in the number of breast CSC whereas increases the number of stem cell in HEK293. This can be evaluated as a positive outcome in terms of treatment approaches. Annexin V analysis indicates that these changes may be related to apoptosis mechanisms. Because of the limited number of studies available, it is necessary to identify these mechanisms in further studies. Considering these effects of calcitriol, vitamin D deficiencies should be considered to be effective in cancer pathogenesis and progression. The use of vitamin D supplements in the treatment of cancer patients may be beneficial. In addition, the detection of vitamin D deficiencies at the prevention of cancer formation may be beneficial.

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