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Antiproliferative, anti-inflammatory, antitumoral and proapoptotic effects of calcitriol on MCF-7 and MCF-10A cell lines

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Calcitriol, a biologically active metabolite of vitamin D, regulates signaling pathways related to proliferation, malignant transformation, invasion, metastasis, angiogenesis, and inflammation in human cells. In that context, it is crucial to determine anticancer effects of calcitriol in terms of preventing the development of breast cancer, which is the most common malignancy in women, and improving its prognosis. Here, we investigated possible anti-inflammatory, antitumoral, antiproliferative, and proapoptotic effects of various doses of calcitriol that we applied *in vitro* conditions on breast adenocarcinoma (MCF-7) and healthy breast epithelial cell lines (MCF-10A). We treated cell lines with 0.5, 1, 10, 25, 50, 100, 200, 500, 1000 and 10000 nM doses of calcitriol for 24 and 48 h. After exposure of 100 nM calcitriol to MCF-7 cell line for 24 h, a significant decrease in cell viability, a significant increase in apoptosis rate, a 13-fold increase in the vitamin D receptor (VDR), a 3-fold decrease in NF-κB, an 8-fold decrease in PGE_2, and a 1.5-fold decrease in COX-2 expression levels were detected compared to the control group. Further, it was determined that a 100 nM dose of calcitriol had antiproliferative, anti-inflammatory, apoptosis-inducing and tumor suppressive effects on MCF-7 breast adenocarcinoma cell line.

Keywords: Adenocarcinoma, Apoptotic, Breast cancer, COX-2, NF-κB, p53, PGE_2, Tumor, Vitamin D

Vitamin D is a steroid hormone synthesized either from 7-dehydrocholesterol in human epidermis layer by the effect of UVB light or through ergocalciferol (D_2), cholecalciferol (D_3) and vitamin D metabolite 25-hydroxy vitamin D in foods[1]. D_2 and D_3 are transported to the liver, which is the first hydroxylation site, and the main form of vitamin D in circulation called calcidiol $25(OH)D_3$ is synthesized there by CYP27A1 and CYP2R1 enzymes[2]. By a second hydroxylation reaction mainly in kidney mitochondria, the most active form of vitamin D in circulation called calcitriol [$1,25(OH)_2D_3$] is synthesized by CYP27B1 enzyme[2].

Calcitriol exerts its biological activity non-genomicly through $1,25D_3$-MARRS receptor or genomicly by binding to the vitamin D receptor (VDR) which is a member of nuclear receptor (NR) superfamily[3,4]. Once binding to VDR, it forms a dimer with retinoid-X receptor (RXR) and binds vitamin D response elements (VDREs) in nucleus, thus regulates transcription of target genes[5]. Studies showed that VDR complex can affect transcription of approximately 200-1000 target genes per cell type[6]. Some of these target genes affect signaling pathways related to proliferation, apoptosis, differentiation, inflammation, invasion, metastasis, and angiogenesis[7].

High circulating levels of vitamin D metabolites reduce incidence of atleast 12 types of cancers[8]. It is reported that the risk of breast cancer is lower in people living in areas that are constantly exposed to sunlight[9]. In that context, high circulating levels of $1,25(OH)_2D_3$ have protective effects against breast cancer, which is the most common malignancy among women in the US with an estimated 297,790 new cases in 2023 and the second leading cause of death[10,11]. There are also studies about protective effects of natural herbal compounds such as apigenin, berberine, curcumin, ellagic acid, luteolin, epigallocatechin, genistein, gingerol, icaritin, licochalcone E, noscapine, oxymatrine, piperine, pterostilbine, resveratrol, sulphorafane, and thymoquinone against breast cancer[12]. In the present study, we investigated anti-inflammatory, antitumoral,

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antiproliferative and apoptotic effects of certain doses of calcitriol on breast adenocarcinoma cell (MCF-7) and healthy breast epithelial (MCF-10A) cell lines and also evaluated its effect at cellular level.

Materials and Methods

Study design and preparation of calcitriol

The study was designed as: negative control (routinely used medium only), vehicle control (ethanol only), and calcitriol. Calcitriol (Item no. 71820, Cayman) was diluted with 5 µL of ethanol and 2.4 µM stock solution was obtained. Calcitriol dilutions at 0.5, 1, 10, 25, 50, 100, 200, 500, 1000 and 10 000 nM doses were prepared from the stock solution.

Cell culture

In this study, human breast adenocarcinoma MCF-7 (ATCC® HTB22™) and normal breast tissue MCF-10A (ATCC® CRL-10317™) cell lines were used. Cells were passaged into flasks containing Dulbecco's Modified Eagle Medium (Capricorn Scientific, Germany), 10% fetal bovine serum (FBS) (Capricorn Scientific, FBS-HI11B, Germany), 1% L-glutamine (Capricorn Scientific, GLN-B, Germany), 1% penicillin-streptomycin (Capricorn Scientific, PS-B, Germany), and incubated at 37°C and 5% CO_2.

Evaluation of cell viability

Approximately, 2×10^4 cells were seeded in each well of the 96-well U-bottom microplate and incubated at 37°C and 5% CO_2 for 24 h. Calcitriol doses at final concentrations of 0.5, 1, 10, 25, 50, 100, 200, 500, 1000 and 10000 nM were added to the wells individually and incubated for 24 and 48 hours at 37°C and 5% CO_2. After the incubation was completed, cell viability was determined by thiazolyl blue tetrazolium bromide (MTT) assay (Goldbio St. Louis MO, USA). Measurements were made with a spectrophotometer (Biotek Synergy HTX, USA) at a wavelength of 570 nm and the effective concentrations of calcitriol were calculated. The experiment was conducted in three replicates.

Detection of apoptosis by Flow cytometry

For apoptosis analysis, PE Annexin V Apoptosis Detection Kit I (BD Bioscience, USA) and propidium iodide (PI) staining were used. Cell suspensions of 1×10^5 cells in 500 µL of PBS were prepared using cultures supplemented with effective doses of calcitriol based on MTT results. Annexin (5 µL) and PI (20 µL) were added to the suspension and left in the dark at room temperature (21°C) for 30 min. Results were analyzed using flow cytometry (BD Accuri™ C6 Plus, USA) with Kaluza software version 1.3 (Beckman Coulter, Inc.).

RNA isolation and cDNA synthesis

Total RNA Extraction kit (ELK Biotechnology RNA, China) was used in accordance with the manufacturer's recommendations. cDNA was obtained using Biorad T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) according to the protocol of EntiLink™ 1st Strand cDNA Synthesis Kit (ELK Biotechnology, China).

RT-qPCR

Vitamin D receptor (VDR), NF-κB, PGE_2, COX-2, and p53 expression levels were determined by RT-qPCR using EnTurbo™ SYBR Green PCR SuperMix kit (ELK Biotechnology, China) according to manufacturer's recommendations. Reactions were performed on ABI 7500 RT-PCR device (Applied Biosystem, USA). GAPDH was used as a housekeeping gene. Expression levels of investigated genes in calcitriol group were compared with the control group and determined by the $2^{-\Delta\Delta}$Ct method. Designs of primers used in the study (Table 1) were made using Primer-BLAST (Basic Local Alignment Search Tool) and Primer3 Viewer program with reference to gene sequences in National Center for Biotechnology Information (NCBI) gene bank. Thirty-five cycles were carried out and heat cycle was adjusted to be 1 minute (initial denaturation) at 95°C, 15 seconds (denaturation) at 95°C, 1 min (binding) at 60°C and 30 s (synthesis) at 72°C.

Statistical analysis

Data were analyzed with IBM SPSS 21 (IBM SPSS Inc, Chicago, IL). Mean and standard deviation were used for continuous data as descriptive statistics. Conformity of continuous data to normal distribution

Table 1 — Primer sequences used in the study

Gene	Primers
GAPDH	F: GAAGGTGAAGGTCGGAGTCAAC
	R: CAGAGTTAAAAGCAGCCCTGGT
VDR	F: TCTCCTGCCTACTCACGATAA
	R: GCTACTGCCCGTGAGAATATAA
NF-κB	F: TGGCCCCTATGTGGAGATCA
	R: AGGGGTTGTTGTTGGTCTGG
PGE_2	F: CTGCTACCCACGCAGAGC
	R: CATACACCGCCAAATCAGCG
COX-2	F: CAATATCACAGCAGGCCACC
	R: GCGTGAGTTTGGTGTGTCAT
p53	F: TTCTCATCACCGGCATCACG
	R: GCTATCACAAACTGCAAGACG

was checked with Kolmogorov-Smirnov test. Effects of group-time and group-intervention binary independent variables on outcome variables were determined by one-way analysis of variance (One-way ANOVA). When interaction effect was found to be statistically significant as a result of the analysis, Bonferroni corrected Simple Effect analysis was used to find the source of the difference. In cases where interaction effect was not significant, marginal means of the main effects were compared and in case of significant difference, Bonferroni correction was used as the post-hoc test. The statistical significance level was taken as P <0.05.

Results

MTT test results with $1,25(OH)_2D_3$

After exposure to 100, 200, 500, 1000 and 10 000 nM calcitriol for 24 h, MCF-7 cell viability levels statistically significantly decreased from 100% to an average of 84% (P <0.001), 82% (P <0.001), 71% (P <0.001), 68% (P <0.001), and 70% (P <0.001), respectively. After 48 h, the decreases in order of doses reached 84% (P <0.001), 79% (P <0.001), 78% (P <0.001), 70% (P <0.001), and 65% (P <0.001, Fig. 1A). After exposure to doses of 1000 and 10000 nM, MCF-10A cell viability levels statistically significantly decreased from 100% to an average of 74% (P <0.005) and 58% (P <0.001), respectively (Fig. 1B). Antiproliferative effects of calcitriol on cancer and healthy breast cell lines were also evaluated with invert microscopy and microscopic images consistent with doses were obtained (Fig. 2).

Apoptosis analysis with annexin V/propidium iodide

For MCF-7 cell lines, doses of 100, 500, 1000 and 10 000 nM calcitriol were found to induce apoptosis at rates of 17.8, 18.9, 27.8 and 26.4%, respectively. For MCF-10A cell lines, these rates were calculated as 0.5, 7.2, 14.6 and 23.3% (Fig. 3).

RT-qPCR gene expression findings

VDR expression levels for the evaluation of calcitriol's genomic effect, NF-kappa B, PGE_2 and COX-2 expression levels for its anti-inflammatory effect, and p53 expression levels for its antitumoral effect were investigated. In MCF-7 cells treated with 100, 1000 and 10 000 nM doses of calcitriol,

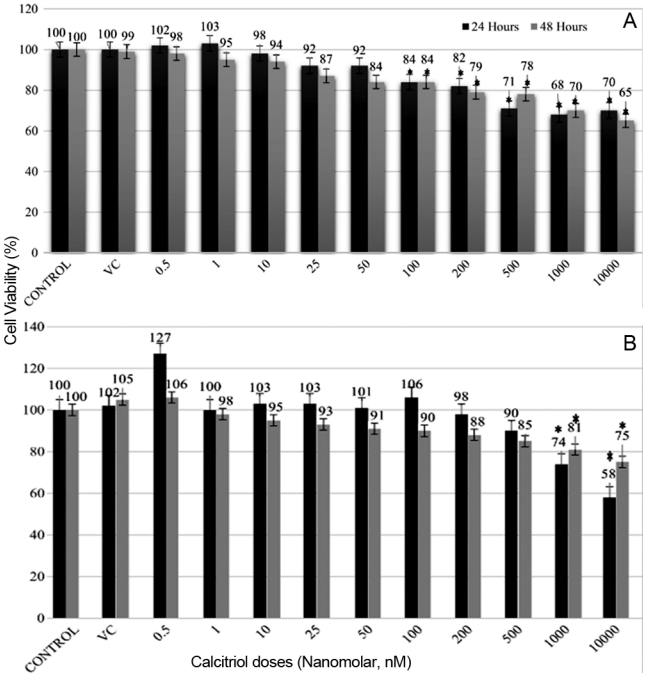

Fig. 1 — MTT results after 24 and 48 h of calcitriol application to (A) MCF-7; and (B) MCF-10A cells. [The doses were prepared as nanomolar. (A: *F test statistic value; **Statistical significance level compared to control group (P <0.05); ***Simple effect analysis with Bonferroni correction (n=24). B: *Statistical significance level compared to the control group (P <0.05); ** Simple Effect analysis with Bonferroni correction (n=24). VC, The amount of ethanol that was used when dissolving calcitriol and was applied directly on the cell]

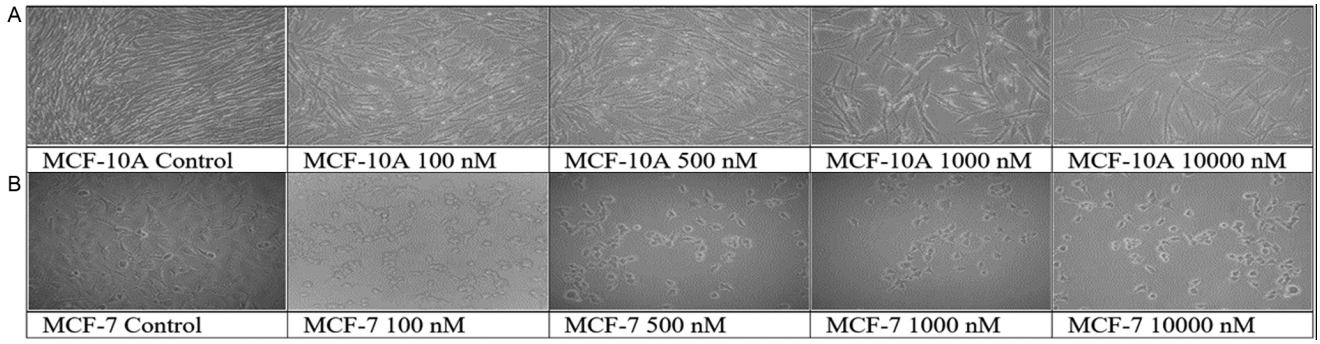

Fig. 2 — Inverted microscope images of (A) MCF-7; and (B) MCF-10A cell lines of control and calcitriol-treated group at the end of the 24th hour. [Control group; 100, 500, 1000 and 10000 nM concentrations of calcitriol applied group]

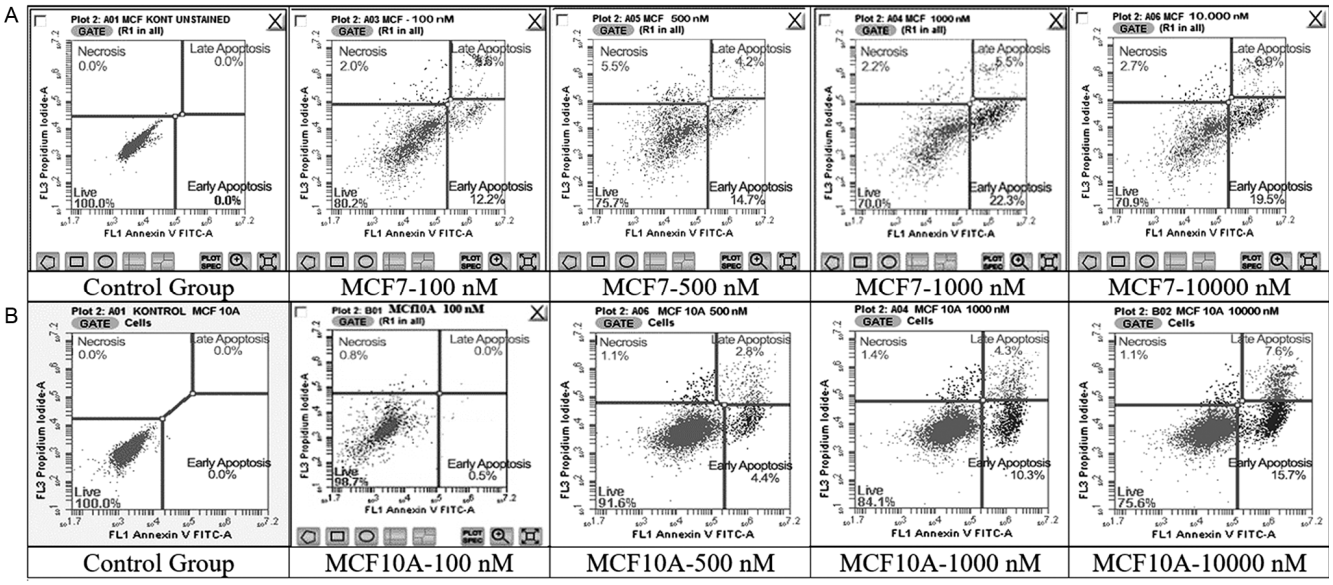

Fig. 3 — Apoptosis assay by fluorescein isothiocyanate (FITC)-labeled Annexin V/Propidium iodide (PI) flow cytometry of MCF-7 and MCF-10A cells treated with different doses of calcitriol. [Different labeling patterns discriminate different cell groups. Necrotic cells: Annexin, PI +; Late apoptosis: Annexin+, PI+; Living cells: Annexin, PI-; Early apoptosis: Annexin+, PI-.]

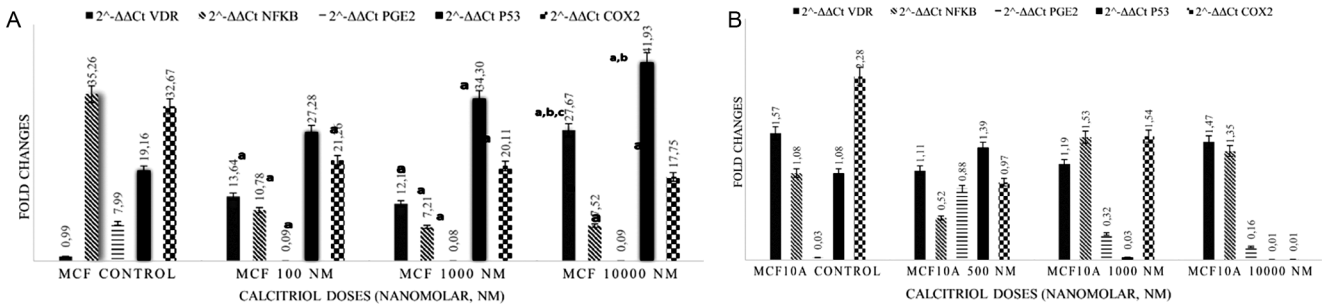

Fig. 4 — VDR, NF-*kappa* B, PGE_2, p53 and COX-2 mRNA expression levels in (A) MCF-7; and (B) MCF-10A cells after 24 h of calcitriol exposure. Expression levels of target genes were normalized based on GAPDH mRNA expression level. P <0.05 Significance level obtained from comparing (i) the control group and the cells treated with 100, 1000, and 10 000 nM doses of calcitriol; (ii) cells treated with 100, 1000, and 10000 nM doses of calcitriol; and (iii) comparing cells treated with 1000 and 10 000 nM dose of calcitriol. [Data are expressed as mean ± S.H, One Way ANOVA Post hoc LSD test (n=8)]

expression levels of VDR mRNA increased by an average of 13, 12 and 27 times (P <0.05), NF-κB mRNA decreased by an average of 3.5, 5, and 5 times (P <0.001), COX-2 mRNA decreased by an average of 1.5, 1.6, and 1.9 times (P <0.005), and PGE_2 mRNA decreased by an average of 8,8 and 8 times (P <0.001), compared to the control group, respectively (Fig. 4A). There were no statistically significant differences in p53 mRNA expression levels in MCF-7 cells treated with 100 nM dose of calcitriol, but for 1000 and 10 000 nM doses expression levels were increased by an average of 1.7 and 2 times (P <0.05), respectively. No statistically significant difference was observed between applied calcitriol doses in terms of changing NF-κB mRNA expression levels (P >0.05). No statistically significant differences were observed between applied calcitriol doses in terms of changing PGE_2 and COX-2 mRNA expression levels (P >0.05). When p53 expression levels in MCF-7 cells treated with different doses of calcitriol were compared, a statistically significant difference was found only between cells treated with 100 and 10000 nM doses. A 1.5-fold increase was found in p53 mRNA expression level in cells treated with 10000 nM dose compared to cells treated with 100 nM dose (P <0.05). There were no statistically significant differences in VDR, NF-κB, PGE_2, COX-2, and p53 mRNA expression levels in MCF-10A cells treated with calcitriol (P >0.05, Fig. 4B).

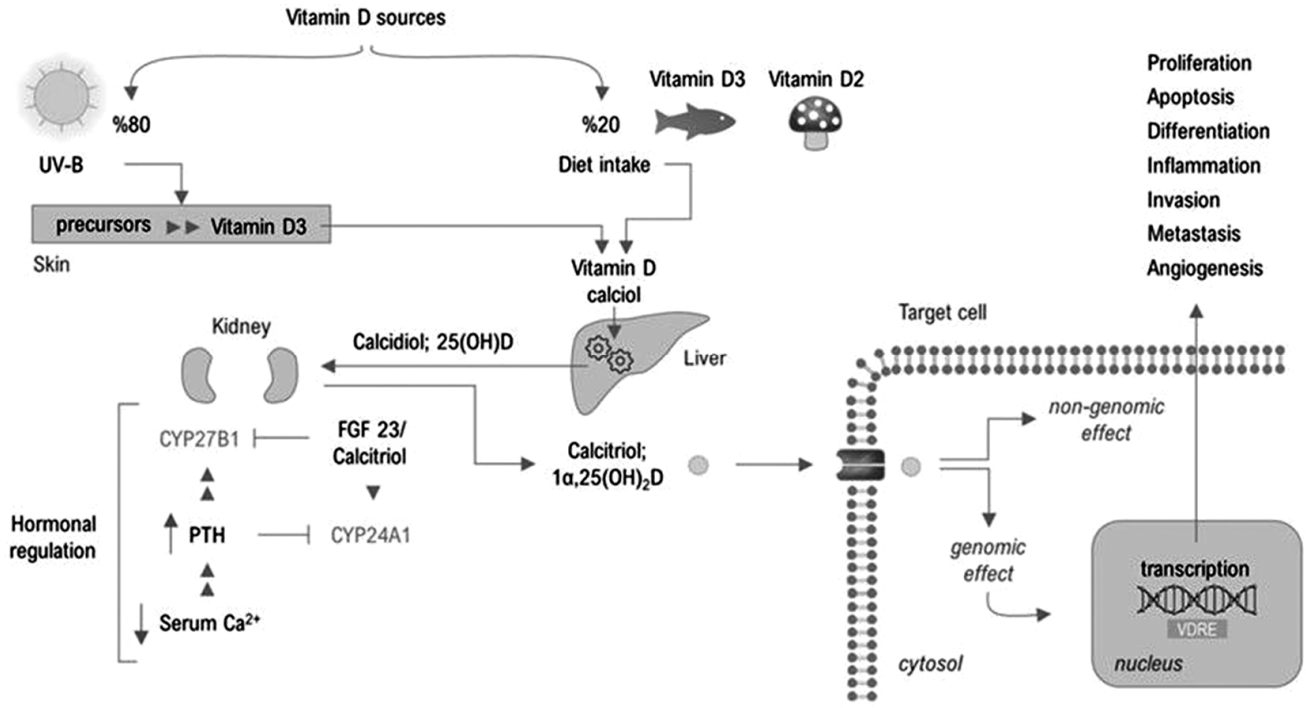

Fig. 5 — Mechanism of calcitriol action.

Discussion

Vitamin D is a prohormone that can be found in oily fish, egg yolk, mushrooms and organ meats, and can also be synthesized through the skin in the human body[13]. Calcitriol, a biologically active hormonal metabolite of vitamin D, shows biological activity by interacting with its steroid structured receptor called VDR[14]. VDR is found in many tissue types such as colon, breast, lung, ovary, bone, kidney, parathyroid gland, cancer cells, pancreatic β cells, monocytes, T lymphocytes, melanocytes, and keratinocytes[15]. Binding of calcitriol with its receptor causes a series of changes in the transcription of many specific genes involved in cell growth, migration and differentiation as shown in Fig. 5[16] High circulating vitamin D levels are thought to be associated with a reduced risk of bladder, breast, colorectal, stomach, ovarian, kidney, hematological, lung, prostate, head and neck, pancreatic, liver, and skin cancers[17].

Breast cancer is the most common malignancy in women and is the second leading cause of cancer-related deaths[18]. According to American Cancer Society report in 2023, the incidence of invasive breast cancer is increasing every year due to the reasons such as advancing gestational age and increasing average body weight of women[11]. Despite being at an early stage, 30% of patients die due to bone, lung, liver, brain, and lymph node metastases[19]. Although there are many treatments such as surgery, radiotherapy, chemotherapy, and immunotherapy, the incidence (11.7%) and mortality (6.9%) of breast cancer is still high[20]. The prevalence of vitamin D deficiency (45%) in this patient group suggested that high vitamin D intake may also be effective in the treatment[21]. Although studies have shown that breast cancer cells are heterogeneous in terms of VDR, 50% of patients can benefit from vitamin D therapy[22]. Recent studies have also reported that fatty acids such as Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA), and Linoleic acid (LA), medicinal herbs such as *Neurada procumbens* L. and *Erythrina variegata* L., and edible mushrooms such as *Lepista nuda* have antiproliferative effects on MCF-7 cells[23-26]. The contribution of the use of natural compounds to breast cancer treatment appears promising and is a valuable topic for further experimental studies.

In our study, cell viability gradually decreased as a result of applying 100, 1000 and 10000 nM calcitriol doses to MCF-7 cell line for 24 and 48 h. Our results showed that viability percentages after 24 and 48 h of exposure were close to each other. In other words, the effect of calcitriol continues for 48 hours. When the effects of 500, 1000 and 10 000 nM calcitriol doses on

MCF-10A cell line were examined after 24 and 48 h, it was observed that cell viability gradually decreased with increasing calcitriol doses. According to MTT results, cell viability level reached with 100 nM calcitriol dose application in MCF-7 cell line was reached with 500 nM dose in MCF-10A cell line. For this reason, while proliferation of healthy cells is not affected at 100 nM dose, proliferation of cancerous cells is reduced. In a similar study conducted in 2019, a 100 nM dose of calcitriol was found to significantly decrease MCF-7 cell proliferation[27]. It is described that due to the rapid metabolism of MCF-7 cells, it can only be inhibited at a concentration as high as 100 nM[27].

The cell cycle requires phosphorylation of retinoblastoma protein (Rb) by cyclin dependent kinase (CDK) and activation of transcription factor E2F[28]. It is known that calcitriol can stop the cell cycle by inhibiting the cyclin-dependent kinase inhibitor (CDKI) p21 and p27 proteins. Different mechanisms have also been reported, such as suppression of the MYC proto-oncogene and a number of intracellular kinase pathways such as p38 mitogen activated protein kinase (MAPK)[28].

Apoptosis is a well-designed natural process in multicellular organisms that removes damaged cells in a non-inflammatory manner[29]. One of the main mechanisms in the development of breast cancer is the changes in the regulation of apoptosis[30]. Bcl-2 family proteins regulate the release of apoptosis-related death factors from mitochondria. This family includes antiapoptotic Bcl-2, proapoptotic Bax, and BH3 only proteins[30]. Studies have reported that VDR agonists effectively reduce Bcl-2 and increase Bax expression, and increase the expression of the CHOP transcription factor, which has an important role in apoptosis and the regulation of Bcl-2 and Bax proteins on MCF-7 cells[31,32]. In our study, after the administration of 100, 500 and 1000 nM calcitriol doses to MCF-7 cells for 24 h, it was determined that the apoptosis levels of the cells increased in direct proportion to the doses. However, it was determined that the apoptosis level in cells treated with 10000 nM calcitriol dose was lower than in cells with 1000 nM dose. This showed that calcitriol induced apoptosis in MCF-7 cell lines, but there was no increase in this effect at higher doses. In our study, when apoptosis levels in cells were evaluated after application of 100, 500, 1000 and 10000 nM calcitriol doses to MCF-10A cell lines for 24 h, lower apoptosis levels were detected compared to MCF-7 cell lines. This showed us that a 100 nanomolar dose of calcitriol did not have a toxic effect on MCF-10A cells.

Since the intracellular response of calcitriol is mediated by the VDR, measurement of VDR expression has been prioritized instead of measurement of circulating levels of vitamin D in cancer cells[33]. In fact, VDR expression is quite high in breast cancer and papillary thyroid carcinoma[34]. It is reported that high VDR expression leads to an improvement in breast cancer prognosis and associated with small size, low grade, estrogen receptor (ER) and progesterone receptor (PgR) positivity[35]. In particular, lower VDR expression was observed in triple negative cancers, and ER negative tumors are resistant to vitamin D[33]. Especially for ER negative tumors, synthetic vitamin D analogues (inecalcitriol, seocalcitol) with stronger anti-proliferative effect could be effective in treatment[33]. There is also evidence that VDR gene poly-morphisms are associated with an increased risk of breast cancer[36]. In this study, VDR expression levels in MCF-7 cells did not increase in direct proportion with the increase in dose levels. This might be due to the fact that the VDR reached sufficient saturation at the receptor level with 100 nM calcitriol. Similar studies have found increased levels of VDR mRNA expression in MCF-7 cell lines treated with vitamin D or its agonists[37]. Elevated expression levels increased cell sensitivity to ER antagonist tamoxifen by inhibiting Wnt/β-catenin signaling, which is responsible for the cell growth and proliferation[37].

It is known that chronic inflammation plays an important role in the development of various types of cancer[38]. Especially in the breast microenvironment, inflammatory mediators such as cyclooxygenase 2 enzyme (COX-2) and consequently accumulated prostaglandin E_2 (PGE_2), and signaling pathways such as mitogen activated protein kinase (MAPK) p38, c-Jun N-terminal kinase (JNK) and NF-kappa B (NF-κB) contribute to the development of invasive types of cancer[38]. NF-κB is an inducible transcription factor that is frequently activated in tumor cells[39]. As a result of its activation, it passes from the cytoplasm to the nucleus together with the p65 subunit and plays a role in the immune response and inflammation[39]. Calcitriol has been shown to inhibit NF-κB mediated

inflammation in MCF-7 cells[40]. NF-kB also regulates the transcription of COX-2 in the case of cancer-induced inflammation[41]. COX-2 is an inducible isoform of the COX enzyme activated by stimulants such as bacteria, viruses, alcohol, trauma, and smoking, and its overexpression is associated with tumor aggressiveness and angiogenesis[42]. Considering that its expression is decreased in cancer patients responding to treatment, COX-2 expression levels can be used to determine prognosis, especially in breast cancer patients[42]. Among the COX-2 derived products, PGE_2 is known to contribute the most to tumorigenesis and immunosuppression[43]. PGE_2, through its G protein coupled receptor EP2, enhances Wnt signaling and consequently inhibits glycogen synthase kinase (GSK), and increases expression of protooncogenes via peroxisome proliferator activated receptor (PPAR) δ. It also induces Bcl-2 through RAS-MAPK, increases the expression of VEGF and its receptors, and can also affect differentiation and maturation of immune cells[43].

p53 is a tumor suppressor protein that acts as a transcription factor and has a major role in cell cycle control and induction of apoptosis in DNA damaged cell[44]. It is known that p53 activity is impaired in more than half of breast tumors[44]. Transcription factors of the NF-κB family appear to be major functional antagonists of p53, and p53 can suppress the expression of some NF-κB -dependent genes[44]. Studies have shown that the expression of the VDR gene is directly controlled by the p53 protein and that mutant p53 can deregulate the antiproliferative effects of the VDR pathway[28].

Chiang *et al*.[45] who applied 10^{-7} and 10^{-8} M calcitriol and MART-10 (active form of vitamin D) molecules to MCF-7 cells observed decreased vascular endothelial growth factor-A (VEGF-A), which are associated with angiogenesis and breast cancer metastasis, and NF-κB expression levels. In another study, calcitriol and the COX-2 inhibitor celecoxib were applied separately to MCF-7 cells[46]. It was observed that after the treatment the decrease in COX-2 mRNA expression levels was very similar[46]. They suggested that the combined use of these two drugs in breast cancer patients may be beneficial in the treatment[46]. Our results of evaluating mRNA expression levels of NF-κB, PGE_2 and COX-2 in calcitriol treated MCF-7 cells here in this study corroborate with their findings.

Conclusion

In the above study, after exposure to certain doses of calcitriol to the MCF-7 cell line, a decrease in the ability of cells to proliferate and increase the cell number, apoptotic effect consistent with the increase in dose, an increase in VDR and p53 mRNA expression levels, and a decrease in NF-κB, COX-2 and PGE_2 expression levels were detected. As a result, it was observed that the MCF-7 cell line responded effectively to calcitriol, while the same doses did not show a toxic effect in the MCF-10A healthy cell line. In this context, the anticancer action of calcitriol through genomically regulated signaling pathways throw more light on the key processes involved in carcinogenesis and potentially help developing treatment strategies tailored to tumor status.

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Conflicts of Interest

Authors have no competing interests.

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