



Investigation of the Effect of Calcitriol on Radiofrequency Radiation-Exposed Breast Cancer Cell Lines

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ABSTRACT

Objectives: Radiofrequency (RF) fields have been classified as probable human carcinogens and nonthermal. RF radiation can increase oxidative stress and inflammation in tissues. Vitamin D has been studied for many years for its anti-cancer properties and its ability to suppress cancer cell growth. In this study, we aimed to examine the effects of RF exposure on cell viability, free radical formation, and inflammation, as well as the therapeutic effect of vitamin D against such effects. MCF-7 and MDA-MB-231 cells were exposed to 2.1-GHz W-CDMA-modulated RF radiation.

Materials and Methods: The MTT was used for cell viability, and the FITC-conjugated annexin-V/PI was applied for apoptosis. The vitamin D receptor (VDR), NF- κ B, cyclooxygenase-2 (COX-2), and p53 gene expression levels were studied using real-time PCR. Reactive oxygen species (ROS) analysis was performed using flow cytometry.

Results: A statistically significant decrease in MCF-7 cell viability ($p < 0.001$) was observed with increasing doses of calcitriol compared to the control group; no significant difference in MDA-MB-231 cell viability was observed ($p > 0.05$). ROS analysis revealed that calcitriol caused significant decreases in cell viability in both cell types. Both cell lines showed a statistically significant increase in p53 expression with increasing calcitriol doses. An increase in the expression of VDR, NF- κ B, and COX-2 was also observed in MCF-7 cells.

Conclusion: We observed that while calcitriol administered after RF exposure did not cause statistically significant changes in ROS levels, we observed that it caused an increase in messenger RNA expression of apoptosis and inflammation markers, which could be evaluated in cancer treatment or prevention.

Keywords: Radiofrequency radiation, breast cancer, calcitriol, ROS, apoptosis

INTRODUCTION

Mobile devices emit and receive low-intensity radiofrequency (RF) signals during operation. This RF radiation (RFR) can cause a series of changes and damage to biological tissues, which can be explained by thermal or non-thermal mechanisms.^{1,2} Thermal effects occur when electromagnetic energy absorbed

by the body raises the temperature of the body or of its tissues. This temperature increase persists until it is stabilized by blood circulation. Thermoregulatory mechanisms can counteract the elevated body temperature caused by RF sources such as mobile phones. The non-thermal effects of RF radiation are observed at energy levels that do not raise tissue or whole-

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body temperature to levels that damage tissue or the whole body. At these energy levels, free radical production has been reported to increase in tissues exposed to RFR.³

The effects of electromagnetic fields on biological tissues have become a topic of increasing research interest, especially as the use of communication systems such as cell phones, base stations, and wireless internet has increased rapidly. Previous studies have shown that non-thermal RF radiation can induce changes at the molecular or cellular levels.^{4,5} As a result of epidemiological studies, RF fields have been classified as probable human carcinogens (Group 2B) by IARC.⁶ Despite epidemiological studies indicating an increased risk of cancer, experimental investigations provide limited information on the effects and mechanisms of RF, and findings in this area remain controversial.

Another important phenomenon that should be emphasized in cancer studies is the role of free radicals. Reactive oxygen species (ROS) are continuously produced as by-products of cellular processes. The most important source of endogenous ROS is the mitochondrion.⁷ Low- and moderate-intensity ROS are effective in protecting cells against infectious agents, regulating signal transduction pathways, and initiating responses to mitogenic stimuli. Due to overproduction of ROS, the oxidant/antioxidant balance in cells may shift toward oxidants, leading to oxidative stress.⁸ Cancer cells exhibit high ROS production due to their rapid metabolism and impaired cellular signaling. Cancer cells exhibit greater growth and proliferation than normal cells. They activate growth factor pathways to achieve this. This triggers excessive production of ROS in organelles such as the mitochondria and endoplasmic reticulum of cancer cells, as well as increased activity of NADPH oxidases.^{9,10} Another important metabolic process associated with cancer and ROS production is inflammation. Inflammation is an important immune defense mechanism in which the body recruits immune cells to defend against an irritant or pathogen. Inflammation can develop either acutely or chronically. When hemostasis is impaired in tissues for any reason, various bioactive stimuli are released from cells, which causes leukocyte mobilization towards the trauma site.¹¹ The long-term persistence of these responses can cause chronic inflammation and many diseases, especially cancer.¹²

Currently, cancer research has shifted its focus to the causes and treatment methods of the disease. One of the substances investigated in this context is vitamin D. It has been studied for many years for its anti-cancer and suppressive effects on cancer cell growth.¹³ It has also been suggested that vitamin D has antioxidant properties, including inducing the expression of antioxidant defense system components, such as glutathione (GSH), GSH peroxidase, and superoxide dismutase, and suppressing the expression of NADPH oxidase. However, there are few studies on its antioxidant properties, and it is unclear whether it is an antioxidant or a pro-oxidant.^{14,15} Cholecalciferol (vitamin D₃) is formed in the skin by the interaction between 7-dehydrocholesterol and sunlight. Vitamin D₃ and oral vitamin D are metabolized in the liver to 25-hydroxycholecalciferol [calcifediol; 25(OH)D₃], and this compound is then hydroxylated

at the 1 α -position in the kidney to convert it into the active form, 1,25-dihydroxycholecalciferol [calcitriol; 1,25(OH)₂D₃]. The well-known function of 1,25(OH)₂D₃ is to help regulate serum calcium concentrations.¹⁶ The biomarkers of vitamin D in humans are mostly 25(OH)D₃ and, less frequently, 1,25(OH)₂D₃.¹⁷ Studies are based on these indicators of serum vitamin D levels. Vitamin D deficiency is defined as a serum vitamin D level of less than 20 ng/mL (50 nmol/L). Epidemiologic studies on vitamin D levels in cancer patients show that these levels are lower than normal.¹⁸ Active vitamin D interacts with the vitamin D receptor (VDR) on the target cell. This structure must be activated for vitamin D to exert its effects inside the cell. The lower-than-normal serum vitamin D levels in individuals who have not been exposed to sufficient sunlight may be attributed to VDR inactivation. Especially in individuals with cancer, it can be seen that vitamin D cannot fulfill its anti-cancer effects due to the disruption of the VDR structure as a result of the effects of cancerous cells.¹⁹

Breast cancer is one of the leading causes of cancer-related mortality among women worldwide. In addition to conventional anticancer therapies, calcitriol has been investigated in combination with various treatment modalities, including chemotherapy, radiotherapy, and supportive interventions, because of its antiproliferative and immunomodulatory properties. However, to the best of our knowledge, no previous study has evaluated the effects of calcitriol administered following RF exposure in breast cancer cell lines. Although breast cancer patients are continuously exposed to non-thermal RF radiation in daily life, it remains unclear whether calcitriol can modulate RF-induced cellular responses. Therefore, the present study aimed to evaluate the effects of calcitriol, administered after RF exposure, on cell viability, oxidative stress, apoptosis, and inflammation-related gene expression in MCF-7 and MDA-MB-231 breast cancer cells (Figure 1).

MATERIALS AND METHODS

Cell culture

MCF-7 (ATCC® HTB22™) and MDA-MB-231 (ATCC® HTB-26™) cell lines were used in our study. Cell lines were incubated at 37 °C in a 5% CO₂ incubator and passaged when they reached 80% confluence. DMEM (Capricorn Scientific, Germany) supplemented with 10% fetal bovine serum (FBS) (Capricorn Scientific, FBS-HI11B, Germany), 100 IU/mL penicillin, and 100 µg/mL streptomycin (Capricorn Scientific, PS-B, Germany) was used as the culture medium.

RF exposure

The RF exposure system consisted of an RF exposure cabinet with a half-wave dipole antenna mounted at its center. The exposure cabinet had air holes in the upper and lower parts. A fan was placed under the exposure cabinet to ensure homogeneous temperature distribution within the exposure system (TR2016 12016B). Wideband code division multiple access (W-CDMA)-modulated RF radiation at 2.1 GHz was produced by a vector signal generator (Rohde & Schwarz, Model SMBV100A, Munich, Germany). The system was placed inside a 5% CO₂

incubator with a 95% humidified environment. The incubator temperature was maintained at 37 °C. An isotropic probe and an R&S®FSH handheld spectrum analyzer, manufactured by Rohde & Schwarz (Munich, Germany), were used to measure the electric field generated by the antenna. Two 12-well plates containing MCF-7 and MDA MB 231 cells were placed around the dipole antenna and simultaneously exposed to 2.1 GHz W-CDMA modulated RF radiation for 24 h. Sham-control samples were maintained under the same conditions as RF-exposed samples, except that the signal generator was off. Specific absorption rate (SAR) was determined according to the formula $SAR = \sigma/\rho \cdot |ERMS|^2$, where σ is the electrical conductivity (S/m) of the medium, $|ERMS|$ is the root mean square magnitude of the E-field strength (V/m), and ρ is the mass density (kg/m³) of the medium. The average SAR was calculated to be 0.632 W/kg.

Experimental design

Cells were divided into five experimental groups: (i) negative control (NC; without RF exposure), (ii) positive control (PC; RF exposure without calcitriol treatment), (iii) RF + 0.1 µM calcitriol, (iv) RF + 1 µM calcitriol, and (v) RF + 10 µM calcitriol. Cells in the RF-treated groups were first exposed to RF for 24 h and subsequently treated with calcitriol at the indicated concentrations. The negative control group was maintained under identical culture conditions without RF exposure. All experiments were performed in triplicate (n = 3 independent biological replicates).

The cells were placed in the *in vitro* RF field exposure system and exposed to a 2.1 GHz W-CDMA-modulated RF field with an average SAR of 0.632 W/kg for 24 h. At the end of exposure, cells were simultaneously harvested from the system to measure cell proliferation, gene expression, and apoptosis.

Administration of calcitriol

Calcitriol (1,25-dihydroxycholecalciferol; Cayman Chemical, USA) was dissolved in ethanol to prepare a 1 mg/mL (2.4 mM) stock solution. Working solutions were subsequently prepared by serial dilution in DMEM to obtain the desired final concentrations (0.1, 1, and 10 µM). Fresh working solutions were prepared immediately before each experiment and added directly to the culture medium.

Cell viability assay

The cells were seeded in a 96-well plate at 200 cells per mm³ and incubated for 24 hours at 37 °C and 5% CO₂. Cells were then exposed to RF radiation and treated with calcitriol (0.1, 1, and 10 µM). After 24 hours of incubation, 20 µL of MTT (Sigma

M2128 St. Louis, MO, USA) solution was added to each well, and the plates were incubated for 3–4 hours. At the end of the incubation, the supernatant was carefully aspirated without touching the cells. One hundred µL of isopropyl alcohol was added to each well, and the plate was incubated for 2 hours at room temperature in the dark. At the end of the procedure, the plate was gently shaken and read at 570 nm (BioTek Synergy HTX, USA).

Annexin-V analysis

5 × 10⁵ cells were used for apoptosis analysis. Cells treated with various doses of calcitriol for 24 hours were collected by trypsinization; the cell pellet was resuspended in 100 µL of binding buffer, containing 5 × 10⁵ cells in 500 µL. Then, 5 µL of annexin and 20 µL of PI were added and mixed by pipetting. After incubation for 30 minutes at room temperature in the dark, 400 µL of 1X binding buffer was added, and the sample was analyzed using a flow cytometer (BD Accuri™ C6 Plus, USA).

ROS analysis

1 × 10⁵ cells were obtained in PBS, centrifuged at 1000 rpm for 5 minutes, and the supernatant was discarded. Cell pellets were resuspended in 200 µL of DCF-DA solution and incubated for 30 minutes at 37 °C in the dark. The cells were then centrifuged at 1000 rpm for 5 min, and the supernatant containing the DCF-DA solution was removed. Cells were resuspended in 100 µL/tube of PBS and analyzed on a flow cytometer (BD Accuri™ C6 Plus, USA).

Gene expression analysis

Quantitative analysis of messenger RNA (mRNA) expression levels of VDR, NF-κB, p53, and cyclooxygenase-2 (COX-2) was performed using Real-time PCR (Applied Biosystems, Foster City, CA, USA). After administration of doses of calcitriol following RF exposure, cells were incubated for 24 hours and then removed from the flasks by trypsinization. RNA extraction (ELK Biotechnology, Wuhan) was performed according to the manufacturer's instructions. Purity and quantity were measured using a multi-mode microplate reader (BioTek Synergy HTX). Complementary DNA synthesis (ELK Biotechnology EntiLink 1st strand, Wuhan) was performed according to the manufacturer's instructions. GAPDH was used as the housekeeping gene. The real-time PCR thermal cycling conditions were set as follows: 95 °C for 1 min, 95 °C for 15 sec, 60 °C for 1 min, and 72 °C for 30 sec, repeated for 35 cycles. Primary designs are generated using the National Center for Biotechnology Information (NCBI) and Primer3 sites. The primers used are given in Table 1.

Table 1. Forward and reverse primers of genes.

GAPDH	F: GAAGGTGAAGGTCGGAGTCAAC	R: CAGAGTTAAAGCAGCCCTGGT
VDR	F: TCTCCTGCCTACTCAGATAA	R: GCTACTGCCCCGTGAGAATATAA
NF-κB	F: TGGCCCTATGTGGAGATCA	R: AGGGGTTGTTGTTGGTCTGG
COX-2	F: CAATATCACAGCAGGCCACC	R: GCGTGAGTTTGGTGTGTCAT
p53	F: TTCTCATCACC GG CATCACG	R: GCTATCACAACTGCAAGACG

VDR, vitamin D receptor; COX-2, cyclooxygenase-2.

NCBI GenBank. Primer-Blast® (Basic Local Alignment Search Tool) was designed and evaluated by sequence-matching analysis.

Statistical analysis

Mean and standard deviation (SD) (mean $\pm$ SD) were used for continuous data as descriptive statistics. The distributions of variables in three or more groups were analyzed using the Kruskal–Wallis test, and the formula developed by Conover (1980) was used as the multiple comparison test. Data were analyzed using IBM SPSS 23 (IBM SPSS Inc., Chicago, IL) software. MTT test results were analyzed by GraphPad Prism (GraphPad Software Inc., La Jolla, USA). The significance level was set at $p < 0.05$.

RESULTS

MTT results

Cell proliferation was examined to determine the effect of RF exposure on breast cancer cells. For this purpose, cell cultures were subjected to 2.1 GHz RF at 0.632 W/kg for 24 hours. In the MDA-MB-231 cell line (Figure 2a), PC (RF-exposed cells without calcitriol treatment) were exposed for 24 hours, and cell viability decreased from 100% to 89% compared to the NC group. Similarly, in the MCF-7 cell line (Figure 2b), cell viability decreased from 100% to 69% compared with that of the NC group (Figure 2). After the MTT test, we selected appropriate doses of calcitriol (0.1 μ M, 1 μ M, and 10 μ M).

Figure 2 shows the cell viability of MDA-MB-231 and MCF-7 cell lines at the end of the 24-hour RF exposure following

application of various doses of calcitriol. Compared with the NC group, 1 μ M and 10 μ M calcitriol decreased cell viability from $100\% \pm 1.82$ ($p < 0.001$) to $91\% \pm 1.43\%$ ($p < 0.005$) and $85\% \pm 3.11\%$ ($p < 0.001$), respectively. When the 24-hour exposure of the MCF-7 cell line to calcitriol was evaluated using the MTT test, a significant decrease in proliferation was observed at a concentration of 0.1 μ M. Compared to the PC group, 0.1, 1, and 10 μ M calcitriol decreased MCF-7 cell viability from $100 \pm 2.32\%$ ($p < 0.001$) to $85 \pm 3.44\%$ ($p < 0.001$), to $68 \pm 1.97\%$ ($p < 0.001$), to 68 ± 3.14 ($p < 0.001$), respectively. Compared with the PC group, treatment with 0.1 μ M calcitriol was associated with increased cell viability, whereas higher calcitriol concentrations (1 and 10 μ M) produced a reduction in cell viability, indicating a biphasic, dose-dependent response in MCF-7 cells. No significant difference in MDA-MB-231 cell viability was observed with increasing doses of calcitriol compared with the control and PC ($p > 0.05$).

Apoptosis results

Percentages of apoptotic cells were determined by the annexin-V assay (Figure 3). The percentage of live MDA-MB-231 cells in the control group is 72.1% (Figure 3). After 24 hours of RF exposure and calcitriol application at 0.1 μ M, 1 μ M, and 10 μ M, cell viability percentages were 82.8%, 92.2%, and 90.2%, respectively (Figure 3). In MDA-MB-231 cells, although the percentage of viable cells increased following calcitriol treatment compared with the PC group, these differences were not statistically significant ($p > 0.05$).

According to the annexin-V assay, the percentage of live cells in the control group was 31.5%. After calcitriol application

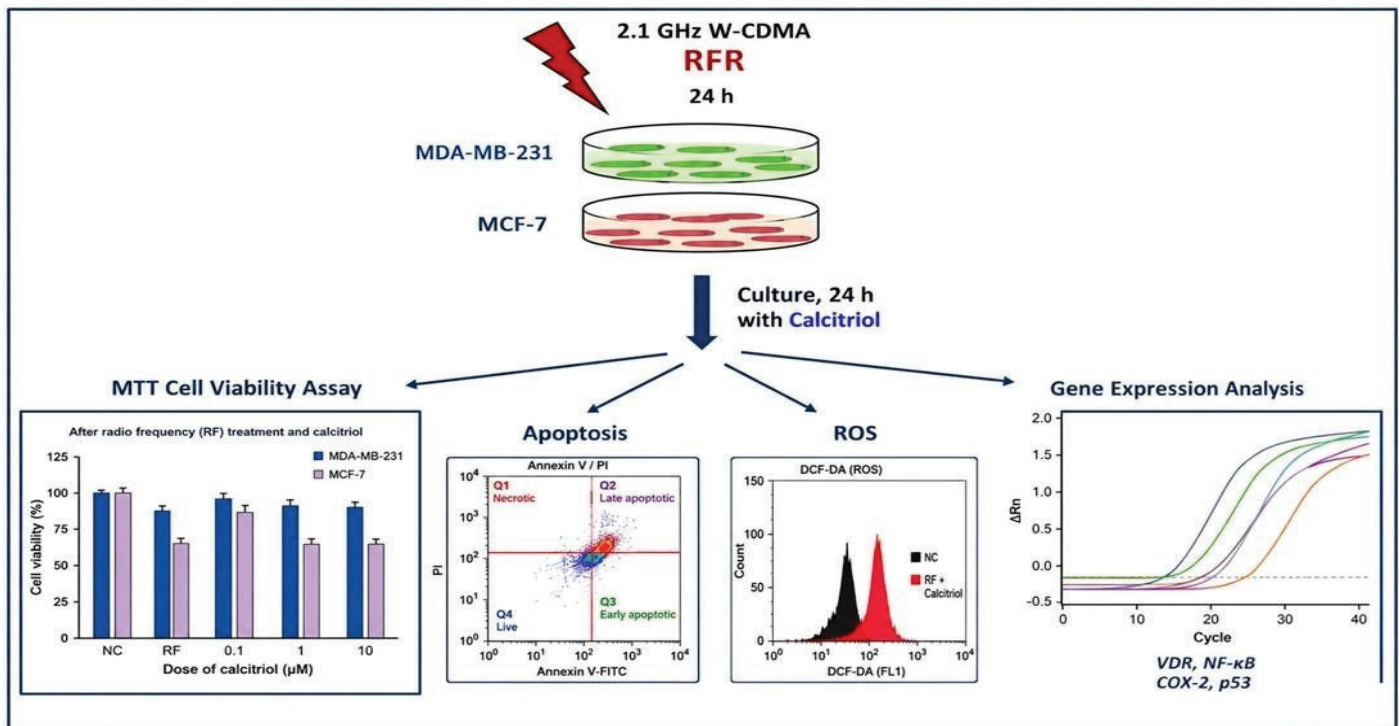


Figure 1. Schematic representation of study design.

ROS, reactive oxygen species; VDR, vitamin D receptor; COX-2, cyclooxygenase-2.

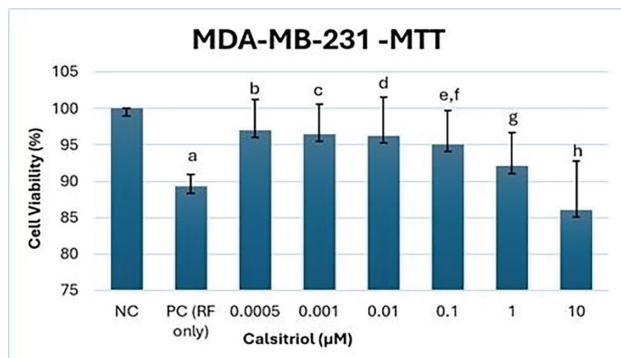
(0.1 μM , 1 μM , and 10 μM), the percentages of live cells were 97.6%, 94.1%, and 89.7%, respectively (Figure 4). In MCF-7 cells, there was no statistically significant increase or decrease in apoptosis levels at 0.1, 1, and 10 μM doses compared to the control group and between doses ($p > 0.05$).

ROS results

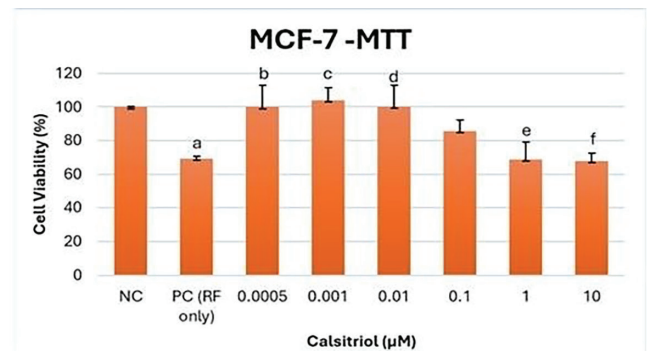
Intracellular ROS levels were determined following RF exposure and subsequent calcitriol treatment. In MDA-MB-231 cells, the relative ROS level in the PC was 76.3%, whereas levels of 76.2%, 65.7%, and 49.4% were observed after treatment with 0.1, 1, 10 μM calcitriol, respectively.

and 10 μM calcitriol, respectively (Figure 5). Although a dose-dependent decrease in ROS levels was observed compared with the PC group, these differences were not statistically significant ($p > 0.05$).

In MCF-7 cells, the relative ROS level in the positive control PC was 80.9%, whereas ROS levels following treatment with calcitriol at 0.1, 1, and 10 μM were 46.4%, 56.1%, and 39.2%, respectively (Figure 6). Although lower ROS levels were observed in the calcitriol-treated groups compared with the PC group, the differences did not reach statistical significance ($p > 0.05$).



(a)
^a $p < 0.001$ comparing the NC and PC.
^{b,c,d,e} $p < 0.005$, 0.005, 0.01, 0.05 comparing the PC with cells treated with 0.0005 μM , 0.001 μM , 0.01 μM , and 0.1 μM of calcitriol, respectively.
^{f,g,h} $p < 0.5$, 0.001, 0.005 comparing the NC with cells treated with 0.1 μM , 1 μM , 10.0 μM of calcitriol, respectively.



(b)
^a $p < 0.001$ comparing the NC and PC.
^{b,c,d} $p < 0.001$, 0.001, 0.005 Significance level obtained from comparing the PC and the cells treated with 0.0005 μM , 0.001 μM , and 0.01 μM of calcitriol (respectively).
^{e,f} $p < 0.005$, 0.001 Significance level obtained from comparing the NC group and the cells treated with 1.0 μM and 10.0 μM of calcitriol (respectively).

Figure 2. Cell viability of MDA-MB-231 (a) and MCF-7 (b) cells in the negative control (NC; cells without RF-exposure or calcitriol treatment), positive control (PC; RF-exposed cells without calcitriol treatment), and calcitriol-treated groups following 24 h of RF-exposure.

RF, radiofrequency.

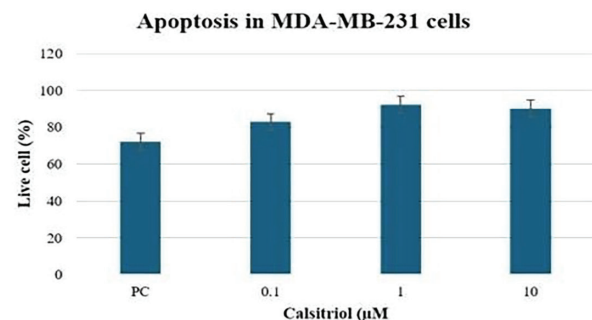
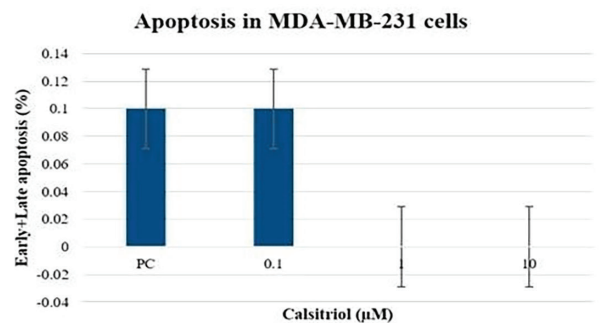
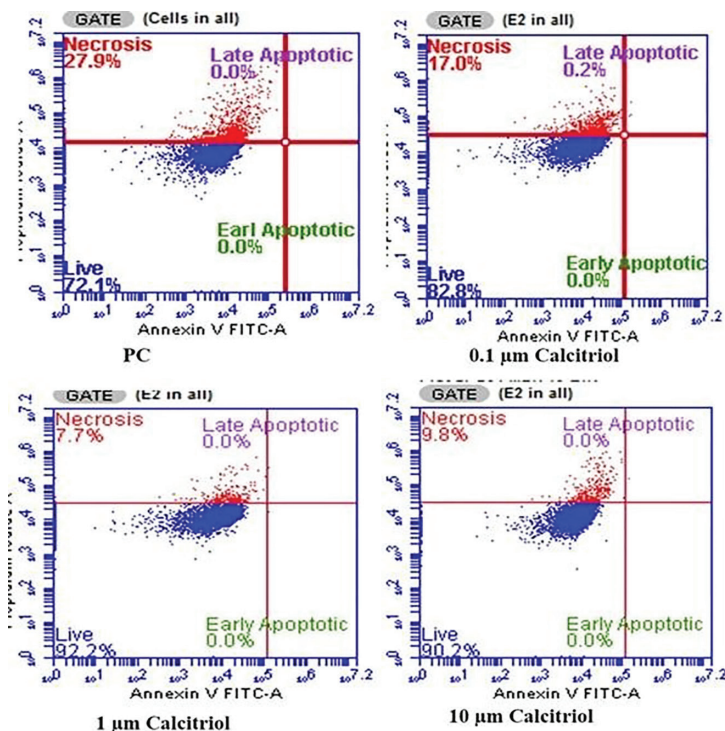


Figure 3. Percentage of apoptotic and viable MDA-MB-231 cells.

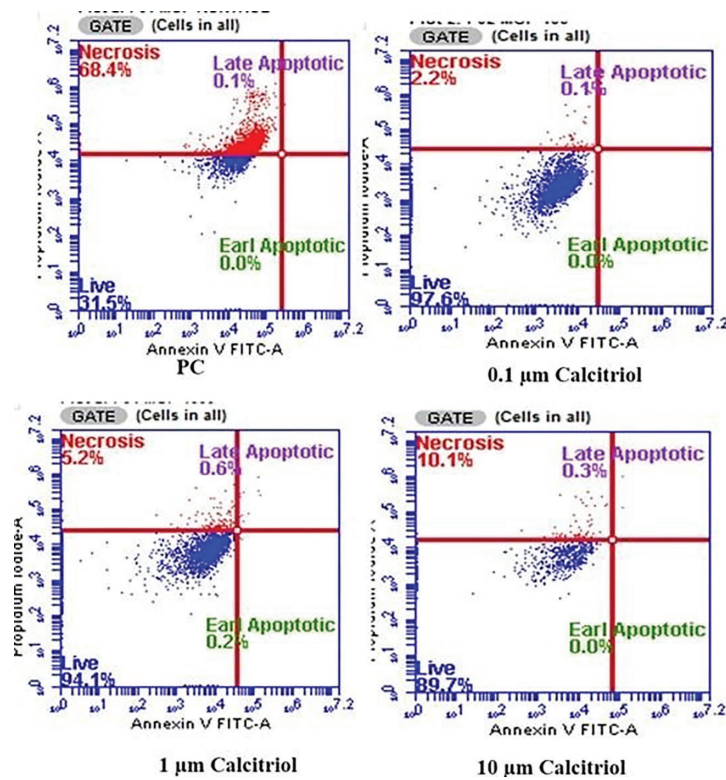


Figure 4. Percentage of apoptotic and viable MCF-7 cells.

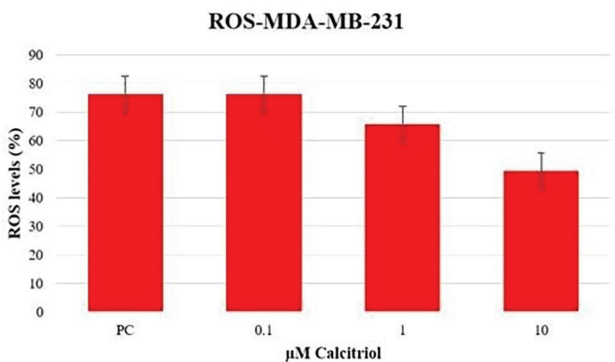
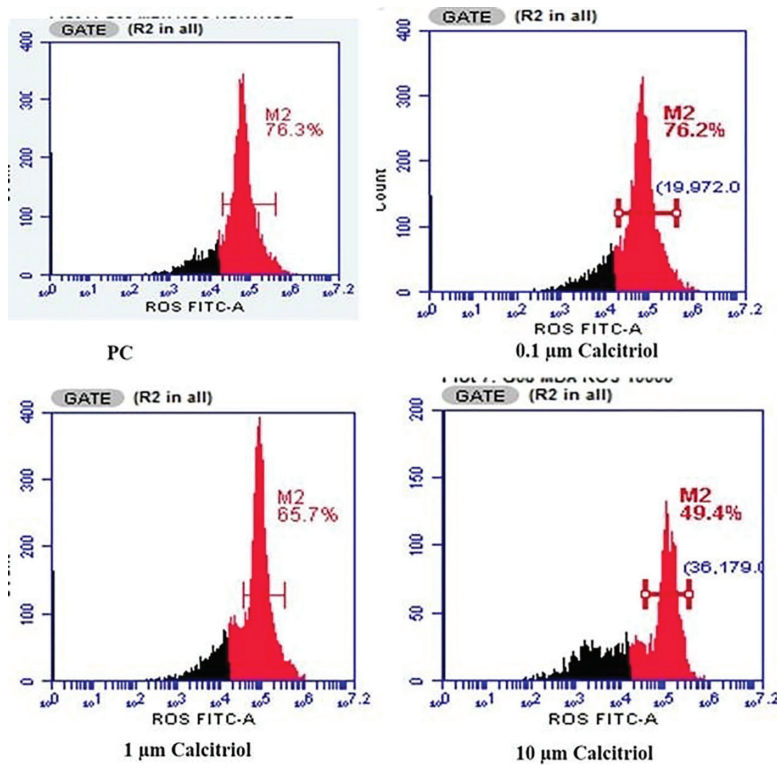
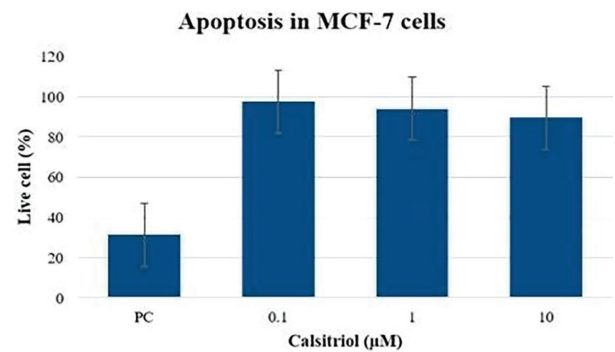
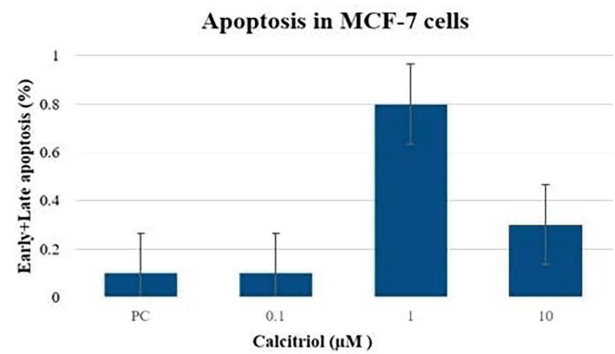


Figure 5. Intracellular ROS levels in MDA-MB-231 cells.

ROS, reactive oxygen species.

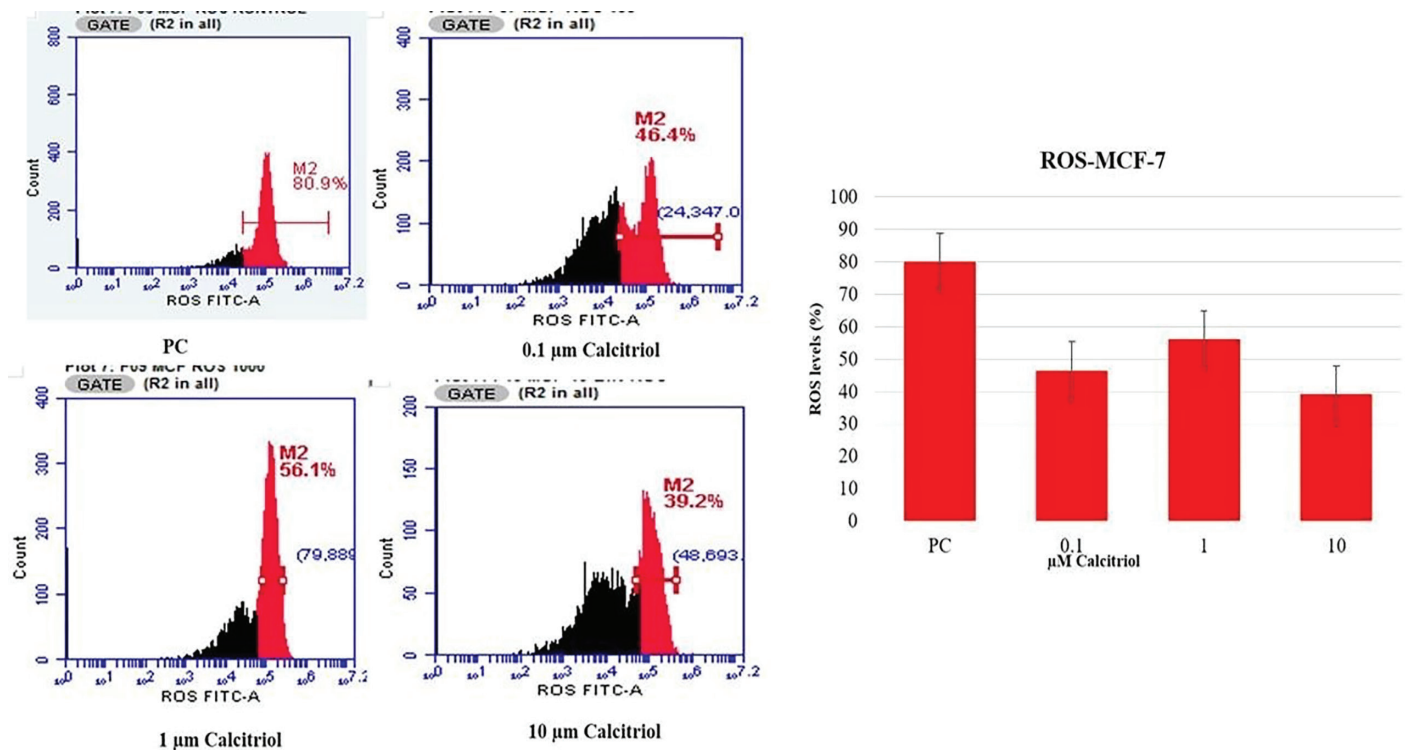


Figure 6. Intracellular ROS levels in MCF-7 cells. ROS, reactive oxygen species.

Gene expression results

To evaluate the genomic effects of calcitriol following RF exposure, the mRNA expression levels of VDR, NF- κ B, COX-2, and p53 were determined by RT-qPCR (Figure 7). In MDA-MB-231 cells, the mRNA expression levels of VDR, NF- κ B, and COX-2 did not differ significantly between the PC group and the calcitriol-treated groups (0.1, 1, and 10 μ M) or among the calcitriol doses ($p > 0.05$). Although NF- κ B expression tended to decrease and COX-2 expression tended to increase with increasing calcitriol concentrations, these changes did not reach statistical significance. Likewise, treatment with 0.1 μ M calcitriol did not significantly alter p53 mRNA expression

compared with the PC group ($p > 0.05$). However, 1 μ M and 10 μ M calcitriol significantly increased p53 mRNA expression by approximately 3.2- and 4.3-fold, respectively, compared with the PC group ($p < 0.05$) (Figure 7).

Expression levels of VDR, NF- κ B, COX-2, and p53 were also evaluated in MCF-7 cells following 24 h of RF exposure and subsequent 24 h calcitriol treatment (Figure 8). VDR mRNA expression increased 2.38-fold, 2.62-fold, and 2.25-fold following treatment with 0.1, 1, and 10 μ M calcitriol, respectively, compared with the PC group ($p < 0.05$) (Figure 8). However, no statistically significant differences were observed among the

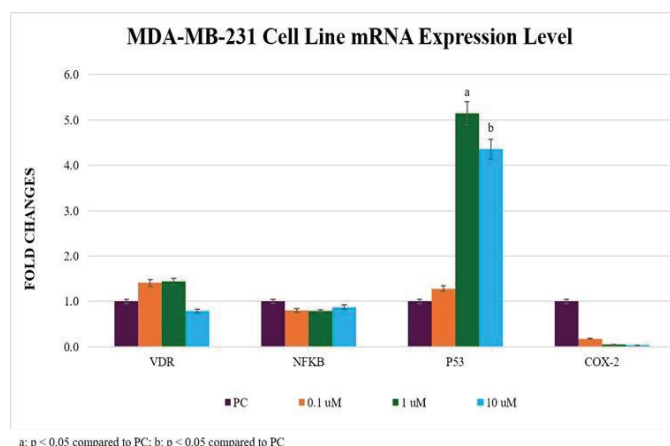


Figure 7. VDR, NF- κ B, COX-2, and p53 mRNA expression levels in the MDA-MB-231 cell line.

VDR, vitamin D receptor; COX-2, cyclooxygenase-2; mRNA, messenger RNA.

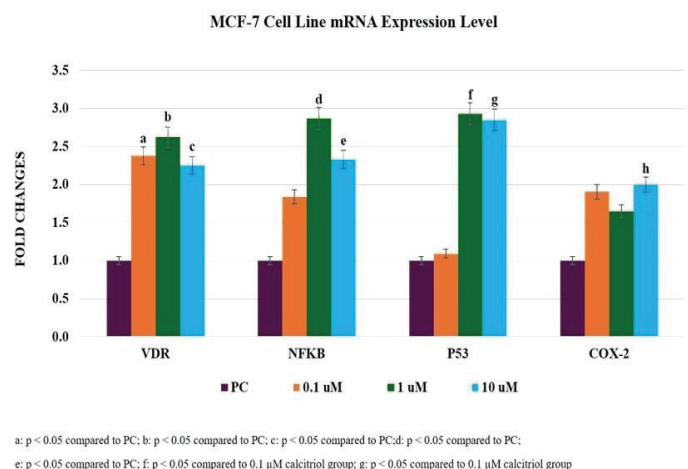


Figure 8. VDR, NF- κ B, COX-2, and p53 mRNA expression levels in the MCF-7 cell line.

VDR, vitamin D receptor; COX-2, cyclooxygenase-2; mRNA, messenger RNA.

calcitriol-treated groups ($p > 0.05$). NF- κ B mRNA expression was not significantly altered following treatment with 0.1 μ M calcitriol compared with the PC group ($p > 0.05$). In contrast, treatment with 1 μ M and 10 μ M calcitriol increased NF- κ B mRNA expression by 2.87- and 2.33-fold, respectively, compared with the PC group ($p < 0.05$). No statistically significant differences were observed between the 1 μ M and 10 μ M calcitriol-treated groups ($p > 0.05$). p53 mRNA expression was significantly higher in cells treated with 1 μ M and 10 μ M calcitriol than in those treated with 0.1 μ M calcitriol ($p < 0.05$). COX-2 mRNA expression increased approximately 2-fold following treatment with 10 μ M calcitriol compared with the PC group; however, this increase did not reach statistical significance ($p > 0.05$).

DISCUSSION

The present study investigated the cellular responses of breast cancer cells to calcitriol treatment following RF exposure. We evaluated whether post-RF administration of calcitriol modulates RF-associated changes in cell viability, oxidative stress, apoptosis, and inflammation-related gene expression in MDA-MB-231 and MCF-7 cells. To interpret these findings, it is important to consider the well-established anticancer properties of calcitriol reported in the literature.

Many published studies associate low vitamin D levels not only with ischemic diseases but also with non-ischemic cancers, metabolic syndrome, infections, and autoimmune diseases.²⁰ Studies show that calcitriol can potentially influence cancer development and growth by regulating multiple signaling pathways involved in proliferation, apoptosis, differentiation, inflammation, invasion, angiogenesis, and metastasis.^{21,22} Vitamin D and its metabolites reduce the incidence of many types of cancer by inhibiting tumor angiogenesis, enhancing cell-to-cell communication, and increasing contact inhibition of proliferation.

When calcitriol's regulation of specific signaling pathways involved in the growth of colon, breast, and prostate cancers was examined, calcitriol exerted a broad effect on cancer cells across multiple cancer types.²³ The anticancer effects of vitamin D can be analyzed in terms of antiproliferative, anti-inflammatory, and proapoptotic activities. The antiproliferative effect of vitamin D is achieved by interrupting cell cycle progression.²⁴ The cell cycle, which occurs in four stages, is regulated by cyclin-dependent kinases and their inhibitors, both of which are involved in specific processes such as cellular transcription. Vitamin D exerts this effect by suppressing kinase activity or inducing kinase inhibitors.²⁵

Vitamin D also exerts its anti-inflammatory effect by modulating prostaglandin metabolism, which is implicated in the development and progression of many cancers. This is accomplished by NAD⁺-dependent up-regulation of the *15-hydroxyprostaglandin dehydrogenase* gene and COX-2, thereby reducing prostaglandin levels.²⁴ Increased COX-2 expression has been reported to inhibit apoptosis, stimulate cell division, and inhibit antitumor immune responses and protein synthesis in cells, as well as inhibit cell adhesion and promote metastasis by stimulating

neovascularization, thereby affecting cancer development.²⁶ Vitamin D exerts its proapoptotic effect in many cancer cells by suppressing the expression of anti-apoptotic proteins or by inducing apoptosis through the expression of apoptosis-inducing proteins.²⁷ However, in our study, COX-2 expression was suppressed at low doses in MDA-MB-231 cells, whereas it was re-induced at higher doses. COX-2 expression in MCF7 cells was higher at all doses than in the PC. In a similar study, COX-2 expression increased in MCF-7 cells and decreased in MDA-MB-231 cells after application of 1 μ M calcitriol.²⁸ However, in MCF-7 cells the protein expression profile was generally consistent with mRNA expression, whereas in MDA-MB-231 cells protein expression appeared to increase despite relatively low mRNA expression.

The organism is constantly exposed to ROS that occur as byproducts of cellular processes and to exogenous ROS. Endogenous ROS at low and moderate concentrations effectively protect cells against infectious agents, regulate signal transduction pathways, and initiate responses to mitogenic stimuli. On the other hand, the oxidant/antioxidant imbalance resulting from excessive ROS production may favor oxidants, leading to oxidative stress.⁸ Cancer cells produce elevated ROS levels due to their rapid metabolism and impaired cellular signaling mechanisms. Following RF exposure, calcitriol treatment showed a dose-dependent tendency toward lower ROS levels in both cell lines. However, these decreases did not reach statistical significance ($p > 0.05$). Although the observed trend may suggest an antioxidant effect of calcitriol, the present data do not provide sufficient evidence to conclude that it significantly reduced ROS levels under the experimental conditions used in this study. Although calcitriol treatment showed a tendency to reduce ROS levels following RF exposure, these changes did not reach statistical significance. Therefore, alterations in ROS should be interpreted cautiously, as the biological role of ROS in cancer is highly context-dependent. Depending on the cellular environment, reduced ROS levels may contribute to either enhanced cell survival or modulation of signaling pathways involved in apoptosis. In the present study, the tendency toward lower ROS levels was accompanied by increased apoptosis, particularly in MCF-7 cells, suggesting that the observed cellular responses cannot be explained solely by changes in oxidative stress but may also involve other apoptosis- and inflammation-related molecular pathways. Numerous studies have investigated the effects of vitamin D and its analogs, such as tacalcitol and calcipotriol, in cancer treatment. In a study by Milczarek et al.²⁹ in rats, vitamin D analogs were reported to inhibit tumor cell growth by enhancing anticancer effects. In another study by Ajibade et al.² in mice, vitamin D analogs reduced cancer cell numbers, suggesting that vitamin D is a reversible risk factor for cancer. 1 α ,25(OH)₂D₃ inhibits mitosis and induces apoptosis in breast cancer cells.³⁰ Following RF exposure, calcitriol treatment was associated with increased apoptosis, particularly in MCF-7 cells.

Activation of NF- κ B in immune cells leads to the production of cytokines that activate NF- κ B in cancer cells. NF- κ B is

involved in angiogenesis, invasiveness, motility, chemokine and cytokine production, as well as in the regulation of genes that control cell survival, proliferation, and growth. The sustained activation of key inflammatory signaling pathways, such as NF- κ B, COX-2, and Janus kinase/signal transducer and activator of the transcription, has been shown to contribute to the initiation of inflammatory breast cancer (IBC). Some inflammatory molecules, such as interleukin (IL)-6, tumor necrosis factor- α (TNF- α), and γ -interferon, function mainly in the process of malignant transformation of IBC, while transforming growth factor- β , IL-8, IL-1 β , and TNF- α are involved in cancer cell proliferation, survival, epithelial-to-mesenchymal transition, invasion, and metastasis.³¹ NF- κ B appears to have a complex role in cancer. Although NF- κ B is a critical molecule in the initiation and progression of cancer, it is also an essential player in the immune response to cancer. While inhibition of NF- κ B is undesirable during the tumor elimination phase of the immune response, it is expected to have positive effects during the chronic inflammation phase of tumor progression, when the immune system targets transformed cells.³² Following RF exposure, we observed a slight increase in NF- κ B mRNA expression in MDA-MB-231 cells and a significant decrease at the 10 μ M dose. In MCF-7 cells, NF- κ B levels increased significantly and then showed slight dose-dependent declines.

p53 is a transcription factor that regulates the cell cycle. It is a protein that plays a key role in cancer suppression across many organisms. It is also involved in cell growth arrest, programmed cell death, cell differentiation, and initiation of DNA repair. It has been emphasized that p53 activity is impaired in cancerous cells and that its expression is decreased. Calcitriol has been shown to induce p53 expression.³³ Following RF exposure, calcitriol treatment significantly increased p53 expression in both cell lines, particularly at doses above 1 μ M.

An additional finding of the present study was that the two breast cancer cell lines differed in their responses to calcitriol. While MCF-7 cells exhibited a more pronounced increase in apoptosis and a biphasic pattern of cell viability, MDA-MB-231 cells showed more modest changes in these parameters. These differences may reflect the distinct molecular characteristics of the two cell lines. Gx1A notable finding of the present study was a biphasic response in MCF-7 cells. Lower-dose calcitriol partially improved cell viability following RF exposure, whereas higher concentrations reduced viability. This pattern may reflect concentration-dependent cellular responses to calcitriol, suggesting that different signaling pathways may predominate at different doses. However, the underlying mechanisms were not investigated in the present study and should be explored in future studies.

Study limitations

A limitation of this study is that calcitriol-only treatment groups (without RF exposure), a separate vehicle (ethanol) control group, and non-malignant breast epithelial cells were not included. Therefore, the direct effects of calcitriol could not be determined based on its effects observed following RF exposure;

the potential contribution of the vehicle could not be excluded; and the findings could not be directly extrapolated to normal breast epithelial cells. Future studies incorporating additional control groups may clarify the respective contributions of calcitriol and RF exposure under the experimental conditions used.

CONCLUSION

The present findings suggest that calcitriol modulates cellular and transcriptional responses in RF-exposed breast cancer cells. Following RF exposure, calcitriol treatment was associated with increased apoptosis and p53 and NF- κ B mRNA expression levels, whereas ROS levels showed a decreasing trend that did not reach statistical significance. These findings indicate that calcitriol may modulate apoptosis- and inflammation-related pathways following RF exposure. However, additional studies, particularly those including calcitriol-only treatment groups and protein-level analyses, are required to further clarify the underlying mechanisms.

Ethics

Ethics Committee Approval: Not applicable. This study involved only *in vitro* cell culture experiments and did not include human participants or animals. Therefore, ethics committee approval was not required.

Informed Consent: Not applicable.

Footnotes

Authorship Contributions

Concept: E.M.S., M.P.E., S.Y., Design: E.M.S., M.P.E., S.Y., Data Collection or Processing: E.M.S., M.P.E., T.F., S.Ö., M.E., Analysis or Interpretation: E.M.S., M.P.E., S.Y., Literature Search: E.M.S., M.P.E., S.Y., Writing: E.M.S., M.P.E., S.Y.

Conflict of Interest: The authors declare no conflicts of interest.

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