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Original Article

Effects of vitamin D on proliferation, invasion and energy metabolism of MCF-7 breast cancer cell line

Emine Nedime Korucu[1], Ali Unlu[2]

[1]Necmettin Erbakan University, Faculty of Science, Department of Molecular Biology and Genetics, Konya, Turkiye
[2]Selcuk University, Faculty of Medicine, Department of Medical Biochemistry, Konya, Turkiye

ORCID IDs of the authors: E.N.K. 0000-0001-7034-4130; A.U. 0000-0002-9991-3939

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ABSTRACT

Background and Aims: 1,25(OH)2D3 (vitamin D) is a pleiotropic hormone with anti-proliferative, pro-apoptotic, and pro-differentiation effects on various cell types, which suggest anti-cancer activity in addition to its classical regulatory action on calcium and phosphate metabolism.
Methods: We aimed to put forward the effects of vitamin D in various concentrations and time intervals on cell proliferation and invasion of human estrogen receptor-positive breast cancer (MCF-7) cells by real-time cell electronic sensing system (xCELLigence). A determined dose of the IC_{50} was applied on samples taken from cell lysates and analyzed the levels of the energy. We also aimed to clarify how vitamin D effects the activity of the protease uPA and their relations with each other.
Results: Vitamin D showed a cytotoxic effect on MCF-7 cells in a time and dose dependent manner, with dose of IC_{50} found to be 140 nM. ATP, ADP, and AMP levels, as well as uPA activities were respectively increased in vitamin D treatment group compared to the control group for the first 24 hours while decreasing at 48, 72, and 96 hours. We determined that 70 and 140 nM vitamin D were decreased in invasion of MCF-7 cells compared to control cells.
Conclusion: We observed that proliferation and invasion of breast cancer cells were inhibited by vitamin D treatment on a dose and time dependent manner, and also vitamin D supplementation decreased uPA activity and energy levels. Further studies on the mechanisms of vitamin D and the formulation of none-hypercalcemic analogues in featured are needed.
Keywords: Activity of uPA, Energy levels, Invasion, Proliferation, Vitamin D

Address for Correspondence:
Emine Nedime KORUCU, e-mail: nedimekorucu@hotmail.com

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INTRODUCTION

Breast cancer is the second most common cancer among cancer-related deaths in women. Growth factors, age, diet, genetic factors, and changes hormonal regulation play a role in breast cancer progression (Kamińska, Ciszewski, Łopacka-Szatan, Miotła, & Starosławska, 2015). Therefore, studies are ongoing to research the treatment of breast cancer. In recent years, there have been studies indicating that vitamin D can be taken as a supplement in cancer treatment. The active form of vitamin D not only regulates calcium metabolism (Anderson, 2017), but also has many functions, such as the regulation of immune response, cell proliferation, and differentiation in metabolism. It plays a critical role on many diseases, such as osteoarthritis, diabetes, cancer, cardiovascular diseases, and tuberculosis (Uitterlinden, Fang, Van Meurs, Pols, & Van Leeuwen, 2004). uPA, uPAR, plasminogen activator inhibitor-1 (PAI-1), and plasminogen activator inhibitor-2 (PAI-2) take place in the urokinase plasminogen activator (uPA) system. uPA system plays an important role in tumor invasion and metastasis by causing degradation of tumor stroma and basement membrane. It has been reported that a high activity of uPA in the primary tumor is associated with poor survival in breast cancer patients (Duffy & Duggan, 2004; Schmitt et al., 1997).

Cancer cells can proliferate rapidly and convert glucose into lactate in an anaerobic environment, since the amount of ATP obtained from glucose is not sufficient. This effect supports the accumulation of nucleosides and amino acids with increased glucose intake and therefore facilitates energy production (Pavlova & Thompson, 2016). In our study it was aimed to clarify the effects of vitamin D supplementation according to the metabolic critical points of MCF-7 breast cancer cells. ATP, ADP, and AMP levels were determined to evaluate the energy metabolism and the capacity of invasion, and uPA activity was demonstrated to explain the status of the invasion of the cancer cells. The difference of our study from current studies is that there are no cell culture experiments in breast cancer cells in which vitamin D supplementation and their capacity of uPA and invasion and energy status have been evaluated so far. Studies in which vitamin D is given externally make up the majority of clinical studies, therefore this aspect makes our studies important.

MATERIAL AND METHODS

Cell culture

MCF-7, human breast cancer cell line, gained from the ATCC (Manassass,VA, USA), was cultured in DMEM (Dulbecco's Modified Eagle Medium), containing 10% fetal bovine serum (FBS) and 1% penicilin/ streptomycin, respectively. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2–95% air.

Cell viability assay

1α,25-Dihydroxyvitamin D_3 (calcitriol) was purchased from Sigma Aldrich (Missouri, USA). It was dissolved in ethanol. The cytotoxic effect of vitamin D on breast cancer cells was determined by a real time cell analyzer (xCELLigence, ACEA Biosciences, Inc, CA, USA). The cells (10000/well) were seeded into 16-well plates for 24 h. After seeding, based on previous studies (Mathiasen, Lademann, & Jäättelä, 1999), a range for vitamin D dose has been established and in order to determine the proliferative and toxic range, with a total of eight different doses being applied in a wide range to attain the sigmoidal curve. The cells were thus treated with various concentrations of vitamin D (10, 25, 50, 125, 250, 500, 1000, and 2500 nM). MCF-7 cells were monitored every 15 min for a period of up to 81 h via xCELLigence system. The values of the electrode impedance were represented as the cell index. The concentration of vitamin D that inhibits 50% cell viability (IC_{50}) was determined according to sigmoidal curve.

Determination of protein concentration

The cells were seeded in a 5. 10^5 arrangement within the T-25 flasks. After 24 hours, cells were divided into two groups: 1-control group and 2- vitamin D treated group, which was formed according to the value of IC_{50} (140 nM) that we found in the assay of cell viability. Cells were washed with PBS, culture supernatant was removed, and ripa lysis buffer (Saint Louis.U.S.A.) was added to cell lysates to measure protein, uPA, and energy levels. The protein concentration was analyzed with Lowry protein assay method (Lowry, Rosebrough, Farr, & Randall, 1951). Bovine serum albumin (BSA) was used, which is a standard in protein analysis (Melbourne, Germany). This assay was performed at 700 nm against a reagent blank via colorimetric method with a spectrometer Perkin Elmer Lambda 25 UV / Vis, U.S.A.) Finally, the samples were calculated as mg/mL.

Determination of uPA enzyme activity

The samples (control and vitamin D treated groups), blank, and uPA standards were added into the well. For calibration and linearity studies of uPA (R&D systems, U.S.A.) standard curve and linearity equation of uPA standards were obtained at dose ranges of 10-2500 ng/ml. Firstly, Tris-HCl and plasminogen (R&D systems, U.S.A) were added to all samples, they were incubated at 37 ºC for 2 hours. Then plasminogen activator substrate (Chromogenix, Canada, U.S.A.) were added into the well, which was then shook at 37 ºC for 6 hours. The uPA values of the samples were measured at 405 nm via colorimetric method. In order to calculate the uPA activity as IU/mg protein, total protein concentration (mg/mL) was divided by uPA concentration (IU/mL).

Measurement of energy levels of cells

The energy levels of the cells was measured using the method established by a study conducted by Cimen et al. in 2004 (Cimen, Turkozkan, Unlu, & Erbil, 2005). Mobile phase was prepared by degassing the solution containing 160 mM KH_2PO_4 and 100 mM KCl, and energy values of the cells were measured in HPLC (AGILENT 1200, Santa Clara, U.S.A.) with GES C18 column (VertiSep™ 4,6x150 mm, 5 µm, Thailand). It was determined that ATP, ADP, and AMP peaks according to the retention times of ATP, ADP, and AMP standards, respectively (). ATP, ADP and AMP standards were prepared at different concentrations and were first injected into the system subsequently.

Determination of invasion capacity in vitamin D treated cells

Firstly, we added matrigel (BD Biosciences, Germany) to each well of the upper chamber of the 16-well cell invasion/mi-

gration (CIM)plate for 4 hours incubation. Then, DMEM was placed in the lower chamber, before the upper and lower chambers were combined. Into the upper chamber, we then added 20,000 cell/each well and a different concentration of plasminogen. The ideal amount of plasminogen for invasion of MCF-7 cells was determined from this experiment, and subsequent experiments were performed based on these values. In the second experiment of invasion assay, different doses of vitamin D (28-70-140 nM) with plasminogen were treated in MCF-7 cells. Changes in cell invasion capacity were observed on CIM-plates with the xCELLigence® device inside the incubator every 15 min for 72 hours.

Statistical analysis

Statistical analyzes were made with SPSS 18.0 package program. The Kolmogorov-Smirnov test was performed to examine whether the MCF-7 cell line proliferation, energy levels, uPA activity, and invasion capacity data, which were treated with various doses of vitamin D, fit the normal distribution. Accordingly, it was seen that the data showed a normal distribution. Then, One Way Anova analysis of variance was performed in repeated measurements to examine the differences between dose groups and times in an interactive fashion. The difference in times in each dose group was analyzed with the Post Hoc Dunnett test. Energy level, uPA activity, and invasion capacity data at different time intervals were evaluated with the "t" test. The statistical difference was accepted as $p \leq 0.05$.

RESULTS

Effect of vitamin D treatment on MCF-7 cell proliferation

The dose- and time-dependent effect of vitamin D on MCF-7 cell growth was analyzed and cell growth rates were monitored for 82 hours at 10, 25, 50, 125, 250, 500, 1000, and 2500 nM concentrations of vitamin D. It was observed that vitamin D inhibited cells compared with the control group in a dose and time dependent manner, as shown in Figure 1 below.

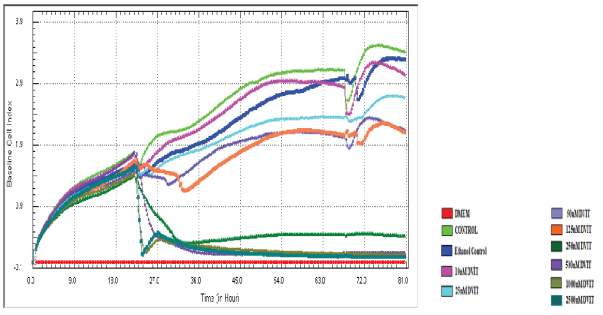

Figure 1. The effect of vitamin D treatment to MCF-7 at different concentrations and time dependent manner on cell viability.

The IC_{50} value was calculated as 140 nM (r^2= 0.99) from the proliferation curve graph of the vitamin D treatment on MCF-7 cells. This value was calculated by taking the logarithms of all administered dose groups at 48 hours, except the control and ethanol groups, and plotting a sigmoidal curve against the cell index value with this value.

ATP, ADP, AMP levels of vitamin D treatment cells

We evaluated the energy status effects of vitamin D in MCF-7 cells on according to various time intervals. The levels of ATP were higher at 24th hour forvitamin D application group compared to the control group, with decreases observed at 48, 72, and 96 hours ($p \leq 0{,}001$). ADP levels for vitamin-D-treated group were increased ($p \leq 0.05$) compared to the control group at 24 hours, and decreased respectively at 48, 72, and 96 hours ($p \leq 0.001$). AMP levels decreased at an application time of 48, 72, and 96 hours compared to the control group ($p \leq 0.001$). All of these values are provided in Figure 2 below.

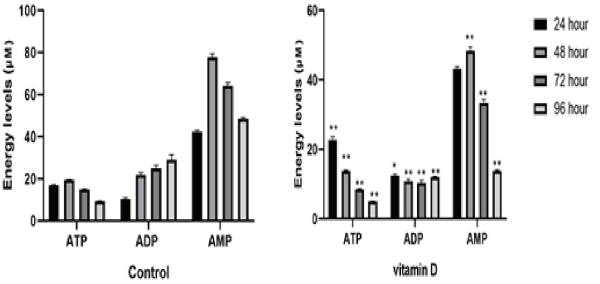

Figure 2. Comparison of the effect of 140 nM vitamin D treatment on energy level at different times (*$p \leq 0.05$ and **$p \leq 0.01$, treatment group vs control).

The measurement of uPA activity in vitamin D treated and control group cells

The effect of vitamin D treatment on uPA activity of MCF-7 cells was evaluated considering different times of application. In our study, when the vitamin D administered group was compared with the control group at 24th hour, the uPA level increased ($p \leq 0.05$), with decreases observed at the 48th, 72nd ($p \leq 0.05$), and 96th hours ($p \leq 0.001$). Comparison of the effect of vitamin D on uPA activity at different times is given in Figure 3 below.

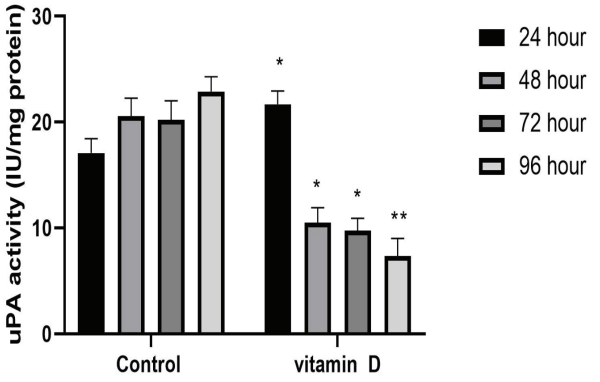

Figure 3. uPA activity of control and 140 nM vitamin D treated groups (*$p \leq 0.05$ and **$p \leq 0.001$, treatments groups vs control group).

Invasion capacity of vitamin D treated and control group cells

Since the invasion capacity of the MCF-7 cell line is low, we firstly increased the invasion capacity by giving different doses of plasminogen to the cells, as shown in Figure 4a. Following

this, we determined appropriate dose of plasminogen as 10 µg/mL.

The effect of different doses of vitamin D at the 72nd hour (48 hours after treatment of vitamin D) on the invasion of MCF-7 cells was compared with the control group. There was a significant difference between the dose groups (70 and 140 nM) compared to the control group ($p \leq 0.05$). As can be seen from Figure 4b, vitamin D decreases the invasion capacity of cells in a dose and time-dependent manner.

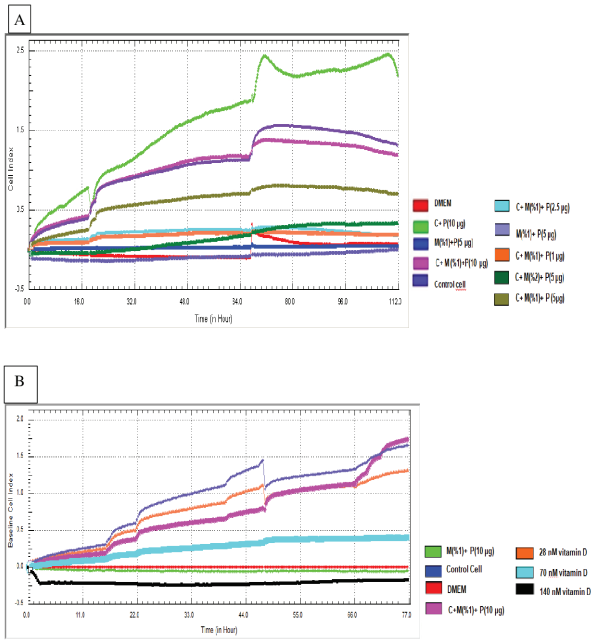

Figure 4. A. Time dependent invasion capacity of MCF-7 cells at different amounts of plasminogen and matrigel (C: cell, M: matrigel, P: plasminogen) B. the effect of vitamin D on invasion in MCF-7 cells.

DISCUSSION

The effects of vitamin D supplementation in cancer have been mostly shown in clinical studies. Supplementation of vitamin D constitutes an alternative to the use of chemotherapy and hormone therapy with anti-estrogens, especially in the treatment of breast cancer. Both in vitro and in vivo studies have shown that vitamin D compounds can inhibit breast cancer. Some evidence suggests that vitamin D deficiency enhances the risk of cancer development and/or progression (Welsh, 2021).

The first studies were conducted on epidemiological effect of vitamin D. It has been postulated that epidemiological studies on the relationships between breast cancer and vitamin D were primarily ecologically based (Robsahm, Tretli, Dahlback, & Moan, 2004).

Garland et al. (1991) showed the relationship between lowest area which receives intense sunlight versus the incidence and mortality of breast cancer in the USA. Therefore, they suggested that there is a relationship between vitamin D and sunlight. A strong inverse relationship was established between breast cancer mortality and sunlight ($r=-0.80$ $p \leq 0.0001$) (Garland et al., 2006) and also exposure to sunlight and breast cancer incidence (Lim et al., 2006; Porojnicu et al., 2007).

Furthermore, when the studies on receiving vitamin D as a supplement are evaluated, the research of Rossi et al. (2009) draws particular attention. The study was performed on 2,569 patients aged 23-74 years who were diagnosed with breast cancer as well as 2,588 healthy women aged 20-74 years, asking about their weekly diets for two years. The study determined that intake of vitamin D >3.57 µg or 143 IU appeared to have a protective effect against breast cancer. In addition, a study supported the protective effects of vitamin D on pre-menopausal women with breast cancer (Abbas, Chang-Claude, & Linseisen, 2009).

Veldhuis et al. (2011) included a total of 885 women in their study, 112 (12.7%) of which were found to have various types of cancer. The prevalence of breast (n = 56, 50%) cancer in women with low 25-Hydroxyvitamin D_3 (25-OHD) (≤50 nmol/L) was higher than in women with high 25-OHD levels (≥ 50 nmol/L). It has been indicated that the prevalence of breast cancer is increased in osteoporotic women with low 25-OHD (≤50 nmol/L) serum levels.

The cell culture experiments were carried out to explain the effectiveness of vitamin D based on a molecular perspective. In vitro study of MCF-7, the ratio of apoptosis to proliferation (A/P) was determined. It has been reported that 1.25 D_3 levels are related to an increased A/P (apoptosis/proliferation) rate (Veldhuis et al., 2011). Furthermore, mechanisms underlying the anti-proliferative actions of 1.25 D_3 have been identified. Some data support the concept that the anti-tumor effects of vitamin D_3 compounds on ER (estrogen receptor)-sensitive human breast cancer cells are associated with estrogen-mediated disruption of mitogenic and viable signaling. Flanagan et al. (2003) found that 100 nM vitamin D supplementation inhibited the cell number and invasion of breast cancer. Although the types of cell lines are different than those used in our studies, the results are similar from the view of the inhibited effects of vitamin D supplementation on invasion of breast cancer.

According to a study working in the same cell culture line as our study, three main vitamin D metabolic enzymes have been found in malignant breast tissue including 25-hydroxylase, 1alpha-OHase, and 24-OHase. The study revealed that MCF-7 cells are expressed 24-hydroxylase. The ability to form inactive vitamin D metabolite with the 24 OH'lase enzyme might be a major action that tumor cells will use to protect themselves against the antiproliferative and calcitriol induced-apoptosis (Diesing, Cordes, Fischer, Diedrich, & Friedrich, 2006).

The presence of VDR (vitamin D receptor) has been identified in the MCF-7 cell line in 2009 by Sertznig et al. In vivo, osteosclerotic metastasis developed more rapidly as vitamin D-deficient mice had extensive defects in their tibia compared with mice with adequate vitamin D levels. The tumor area has been shown to be increased by 55.8% in vitamin D-deficient mice. Also, MCF-7 cells express genes of VDR, 1α- and 24-hydroxylase and play critical roles in the vitamin D signaling pathway and metabolism (Ooi et al., 2010).

In a study comparing 25(OH)D_3 and 1,25-D_3, 10^{-7} -10^{-9} M 25(OH) D_3 caused insignificant growth inhibition in the proliferation of MCF-7 cells, whereas treatment of 25-D_3 with a concentration of 10^{-7} and 10^{-8} M was found to inhibit cell growth significantly (Friedrich et al., 2006).

In two metastatic subtypes of human MCF-7 breast tumor cells, QW-1624F2-2 (1,25-$(OH)_2D_3$ synthetic analogue) inhibited breast tumor cell proliferation and invasion, promoted cell differentiation, and induced apoptotic cell death. It is thought that QW-1624F2-2 affects the blocking of 24-hydroxylase, thus protecting 1,25-$(OH)_2D_3$ and its analogs from 24-hydroxylation, prolonging their biological life, facilitating the effective treatment of breast cancer, and enabling the use of chemotherapeutic agents at low levels (Sundaram et al., 2006).

Although the active form of vitamin D_3, 1,25-dihydroxy vitamin D_3, has anti-invasion and anti-migration properties in pre-clinical studies, it has not yet been fully implemented into clinical practice due to its hypercalcemic side effects. Therefore, vitamin D analogues have been developed to reduce hypercalcemia. In a study by Chiang et al. (2014), it was reported that MART-10, a vitamin D analog, is 1,000 times more active than vitamin D in suppressing MCF-7 cell growth. In addition to MART-10, MMP-13 are more active than vitamin D in preventing cell invasion and migration in MCF-7. The MCF-7 cells were treated with 10^{-7} and 10^{-6} M, with vitamin D inhibiting the invasion of MCF-7 cells by 46±5% and 62±6%, respectively. MCF-7 cells were treated with 10^{-8} and 10^{-7} M MART-10 was found to be at least 10 times more effective than vitamin D in preventing MCF-7 invasion. In our study, the invasion capacities of 28, 70, and 140 nM vitamin D at the 72nd hour were reduced by 29%, 78% and 94%, respectively, compared to the control group. When we compared our results with Chiang et al (2014), findings about the reduction of invasion capacity are similar with our results. The differences in the values may be due to the method used in analysis of invasion capacity in both studies.

In a study conducted on different cell lines, non-malignant MCF-12A and malignant MCF-7, MDA-MB-231 epithelial breast cells were treated for six days at increasing concentrations of 1.25-D_3. MCF-12A cells (growth inhibition 60% by 100 nM treatment for six days) were shown to be more sensitive than MCF-7 cells (growth inhibition 40% by 100 nM treatment for 6 days) ($p \leq 0.001$). Malignant MDA-MB-231 cells were not susceptible to growth inhibition on treatment with 1,25-D_3 ($p \leq 0.05$). The cell viability test was measured with the neutral red dye assay and the same results were obtained with the cell growth (Brosseau, Pirianov, & Colston, 2010).

Marchionatti et al. (2009) applied different doses of 1.25$(OH)_2D_3$±Menadione concentrations for 96 hours. While 1 or 10 nM D_3 alone or combined with 5 µM menadione did not inhibit MCF-7 cell growth, 100 nM D_3 alone or combined with different doses of menadione was found to inhibit MCF-7 cell growth. In our study, the appropriate dose of vitamin D was found to be close to the mentioned literature.

In a different vitamin D- combined study, to test whether resveratrol (RES) improves cellular sensitivity at lower doses of 1,25$(OH)_2D_3$, T47D cells were treated with vitamin D in the presence of 4nM RES or absence of RES for five days. It was observed that vitamin D at 1 nM did not alone reduce cell growth, but it reduced cell numbers by approximately 40% when combined with RES. Similarly, 10 nM vitamin D reduced cell numbers by 25% in the absence of RES and by 50% in the presence of RES. In the presence of RES, 10nM vitamin D alone was as effective as 100 nM vitamin D in growth inhibition (Wietzke & Welsh, 2003).

Proietti et al. (2011) treated MCF-7 breast cancer cells with combined vitamin D_3 and melatonin, and demonstrated the synergistical proliferative inhibition with the completion of cell growth for 144 hours.

Besides our proliferation study, we also analyzed uPa activity as an invasion marker. As it is well known that extracellular matrix proteases (such as uPa, ADAM, MMP, TIMP, RECK) are complex and heterogeneous enzymes play an important role in many pathological processes including cancer. They can alter various biological processes, such as angiogenesis, growth factor bioavailability, cytokine modulation, cell migration, proliferation, invasion, and apoptosis. Highly-invasive cancer is usually characterized by an abnormal activity of certain intracellular or extracellular molecules, such as protein kinases, phosphatases, transcription factors, and proteolytic enzymes. The expression of both urokinase-type plasminogen activator (uPA) and its receptor (uPAR) were correlated with an invasive cancer cell phenotype and a poor prognosis (Sliva, 2004). Duffy and Duggan (2004) stated that uPA and PAI-1 are among the strongest prognostic factors in node-negative patients, and a combined evaluation of these factors would make a more powerful prognostic criteria rather than alone. In a study comparing urokinase system factors, high uPA levels were associated with low efficacy of tamoxifen treatment (Meijer-van Gelder et al., 2004). In our study, uPA levels decreased on the 48th (10.5±1.41 versus 20.5±1.68 IU/mg protein), 72nd (9.75±1.17 versus 20.22±1.79 IU/mg protein), and 96th (7.34±1.66 versus 22.89±1.39 IU/mg protein) hours in the vitamin D-treated compared to the control groups. We demonstrated that the reduction of invasion by vitamin D treatment might be mediated with the reduction in uPA activity.

So et al. (2013) observed that Geminin (vitamin D analog) in MCF10DCIS cells inhibited MCF10DCIS xenograft tumor growth. They stated that the vitamin D analog is more effective in cell invasion than vitamin D. Kim et al (2014) investigated the effectiveness of pepper seed extract (PSE) on the invasion and migration of breast cancer cells using a Boyden chamber. PSE application suppressed the invasion of MDA-MB-231 and MCF-7 cells in a dose-dependent manner. They observed that the invasion of MDA-MB-231 and MCF-7 cells was reduced by 27% and 32.3%, respectively, at a concentration of 50µg/mL.

In a study with grape seed extract (GSE), it was observed that high concentrations of grape seed extract inhibited cell proliferation and apoptosis. Conversely, low GSE concentration inhibits the activity of uPA, MMP-2 (matrix metalloprotease-2) and MMP-9. Thus, it resulted in the reduction of cell migration and invasion in MCF-7 and MDA-MB- 231 cell lines (Dinicola et al., 2014).

Another remarkable aspect of our study is the results of energy charge with ATP, ADP, and AMP values, of which we evaluated the effect of vitamin D on energy metabolism at different time intervals. It is a critical point that the energy charge explains a sensitive intracellular mechanism of the cells through the regulation of enzymatic reactions in the utilization of ATP.

Kaur et al. (2013) examined 6-Mercaptopurine (6-MP) and dasatinib combination on changing ATP concentration in MCF-7 and MDA-MB-468 breast cancer cells, NCI-H23 and NCI-H460 non-small cell lung cancer cells, and A498 and 786-O kidney cancer cells. In their results, a significant decrease was observed in the ATP concentration of breast and lung cancer cells, while kidney cancer cells were resistant to this combination compound. Zoledronic acid (Zol), a bisphosphonate group compound, is an antitumoral compound used in the chemotherapeutic treatments of breast cancer patients with bone metastases. Fehm et al. (2012) reported that Zol significantly reduced the ATP concentration of breast cancer cells. One of the important semi-synthetic plant alkaloids, Vinorelbine, was used in the chemotherapeutic treatment of metastatic breast cancer. It was reported that Vinorelbine decreased the ATP concentration by 42%, and that MCF-7 cell lines were more sensitive to Vinorelbine supplementation than MDA-MB-435 (Ning et al., 2011).

Sucha et al. (2013) investigated the effect of α-Tomatin on the ATP concentration of MCF-7 human breast cancer cells. The study found that α-Tomatin significantly reduced the ATP concentration in MCF-7 cells compared to the control group, depending on dose and time interval.

In our study, there was a decrease in energy levels in the vitamin D administered group compared to the control group for 48 (13.76±0.25 versus 19.48±0.17 μM), 72 (8.46±0.21 versus 15.00±0.21μM), and 96 (4.97±0.13 versus 9.31±0.18 μM) hours. Although treatment differed between the studies, our findings have the same results as the mentioned studies in the terms of reduction of ATP levels.

CONCLUSION

We observed that vitamin D is anti-proliferative, causes a decrease in ATP, ADP, and AMP nucleotides, which indicates the energy level, and is anti-invasive and effective in the uPA system. We demonstrated the reductive effects of vitamin D on invasion via uPA. However, more extended studies are required to demonstrate how different proteases influence this process. Further studies are also needed to evaluate vitamin D analogues as an anticancer agent with strong anticancer effects and low calcemic activity.

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