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RESEARCH ARTICLE

Cytotoxic Effects of Vitamin D_3 on Tumor Cell Lines

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ABSTRACT

Vitamin D_3 is a potent antiproliferative agent against various tumor cells *in vitro*. Here, the results of Vitamin D_3 study as a potential antitumor therapy *in vitro* are presented. Applying antiproliferative 3(4,5-dimethyl- 2-thiazolyl)-2,5-diphenyl-2H-terazolium bromide assays, the inhibitory effects of the Vitamin D were measured. The following cancer cell lines were employed: L20B (normal cell line) and RD (malignant rhabdomyosarcoma). Both cell lines were cultivated in 96-wells culture plates in the presence and absence of different doses of Vitamin D (10–6, 10–8, and 10–10 μg/ml) for 24 and 48 h. *In vitro* results of cytotoxic effects were variable on both cell lines, according to dose and exposure time, after 24 h exposure of RD, the highest concentration of Vitamin D3(10^{-6} μg/ml) treatment had significant effect in decreasing cell proliferation from O.D (0.4570 ± 0.0302) to (0.1540 ± 0.0017) as compared with negative control, with increasing concentrations the cytotoxicity is increased directly proportional; thus, the lowest cytotoxic effect was at the lowest concentration of both Vitamin D3 (10^{-12} μg/ml). While after 48 h, the same concentration of Vitamin D3 shows an increase in proliferation from 0.3710 ± 0.0023 to 0.4597 ± 0.0017 on the RD cell line. While a significant increase in L20B cell proliferation was observed after 24 h treatment at the concentration (10^{-6} μg/ml) from 0.3570 ± 0.0011 to 0.0330 ± 0.0017, when compared with the negative control. However, after 48 h treatment, a significant increases the proliferation of cells as shown from O.D 0.2927 ± 0.0008 to 0.4300 ± 0.0011, respectively. Thus, the present study was aimed to evaluate the antiproliferative property of Vitamin D and its relation to inhibition of cancer cell growth.

Keywords: 3(4,5-dimethyl- 2-thiazolyl)-2,5-diphenyl-2H-terazolium bromide; Tumor cell line; Vitamin D3, RD, L20B

INTRODUCTION

Cancer considered a major health problem and one of the main world's leading causes of death (Rani and Somasundaram, 2012). Cancer remains a leading cause of death globally, with approximately 17.5 million new cases and 8.7 million cancer related deaths in 2015. Incidents of cancer risen by 33% between 2005 and 2015, of which 12.6% were due to population development, 16.4% due to aging, and 4.1% due to age-specific incidence levels (Fitzmaurice et al., 2017). Due to high cancer related death rates and serious side effects of chemotherapy and radiation therapy, many cancer patients and scientists are looking at new alternative treatment techniques (Talib, 2011). There is currently a many efforts to find new therapeutic alternatives that can solve these problems. In the new investigations, chemoprevention, i.e. use of natural, synthetic or biological substances to reverse, and suppress or stop malignant disease development and progression (Davis and Wu, 2012).

Drug clinical use had a major effect on cancer diseases. However, due to their onset of chemoresistance, their therapeutic effectiveness is restricted. In this context, a consistent investigative body provides evidence of Vitamin D and its metabolites, (in addition to its involvement in homeostasis of calcium), also appears efficient in avoiding malignant transformation and the formation of different types of human tumors (Krishnan and Feldman, 2010; Vuolo et al., 2012).

The Vitamin D complex includes a group of fat-soluble prohormones that contribute to maintaining phosphate and calcium homeostasis (Bouillon et al., 2008). Evidence suggested that Vitamin D had affect on the regulation of other important biological processes such as cellular proliferation and differentiation (Gocek and Studzinski, 2009). Furthermore, experimental evidence showed that these effects were due to the antiproliferative, proapoptotic, and immunomodulatory activities (Vanoirbeek et al., 2011). Vitamin D compounds have also been shown to potentiate apoptosis caused by adriamycin, paclitaxel, radiation, and tamoxifen (Wang et al., 2004).

Colston et al. (1981) were first described the inhibiting effects of Vitamin D on the development of tumor cells, who showed for the 1st time a dose-dependent decrease of cell proliferation in melanoma cells treated

with 1,25(OH)2D3. These studies indicate the presence of specific receptors with high affinity for 1,25(OH)2D3 that appeared to be important for the growth inhibitory activity exerted by Vitamin D (Welsh, 2012). *In vitro* studies reported that antisense oligonucleotides, which reduces the intracellular levels of Vitamin D receptor (VDR), reduced the sensitivity of tumor cells to the antiproliferative effects of 1,25(OH)2D3 (Hedlund et al., 1996).

The aim of cancer studies is to find alternative therapeutic compounds with minimal side effects than the other used cytotoxic agents. This has contributed to studies on the efficiency of potential anticancer drugs of different naturally occurring agents.

MATERIALS AND METHODS

Maintenance of RD.and L20B.Cell Lines

RD and L20B cell lines were kindly provided by the Iraqi center for cancer and medical genetics research. Cells were grown in Roswell Park Memorial Institute-1640 with 10% fetal bovine serum, 0.5 ml penicillin and 0.5 ml streptomycin at 37°C.in a 5% CO_2 humidified environment. The cells were passed after the confluence. The cells were counted using a hemocytometer after being harvested from sterile culture flasks, and trypan blue exclusion determined the viability of the cells (Phelan, 1998). The cells were cultivated in 96-well plates (at a density of 30,000 cells/ml cells per well) in 200 µl culture medium. All cells were exposed.to Vitamin D after 70–80% confluence. Vitamin D was freshly dispersed in the cell with the culture medium and diluted to appropriate concentrations (10^{-6}–10^{-10} µg/mL). RD and L20B cells were cultured.in media containing different concentrations of Vitamin D for 24 and 48 h. Three wells of culture media and cell without Vitamin D used as the control in each experiment.

Cytotoxicity Assay for the 3(4,5-dimethyl- 2-thiazolyl)-2,5-diphenyl-2H-terazolium Bromide (MTT) Assays

Growth of tumor cells was measured by the ability of living cells to reduce the yellow dye MTT to a blue formazan product. MTT assay is an precise technique for assessing cell survival. This assay is based on the cleavage of yellow tetrazolium salt, MTT, forming a soluble blue formazan by mitochondrial enzymes, and the quantity of formazan generated is directly proportional to the number of living cells (Sylvester, 2011).

After incubation, 30 µl of MTT dye 0.5 mg/ml was added to each well, and plates were incubated at 37°C.in 5% CO_2/air for 3 h. The medium then carefully removed, and 200 µl of dimethyl sulfoxide was added for 15 min at room temperature. The liquid was then carefully aspirated. The optical density of each well in each plate was read using enzyme-linked immunosorbent assay. reader at a wavelength of 570 nm, then the data were represented as the mean±SE (Zamanian et al. 2012).

Statistical Analysis

For at least three autonomous triplicate determinations of each experimental points, the information are displayed as mean ± standard deviation. SPSS Statistics 22 Software was used to perform statistical analysis with *P*-value differences among the groups ($P < 0.05$).

RESULTS AND DISCUSSION

Antiproliferative activity of Vitamin D3 was determined by treating cancer cell lines (RD) with increasing the concentrations of Vitamin D for 24 h and 48 h, followed by counting the number of viable (living) cells using MTT assay as mentioned in materials and methods. The cytotoxic effects of Vitamin D were variable on changing the dose and time after 24 h, as shown in [Figure 1]. The effect of

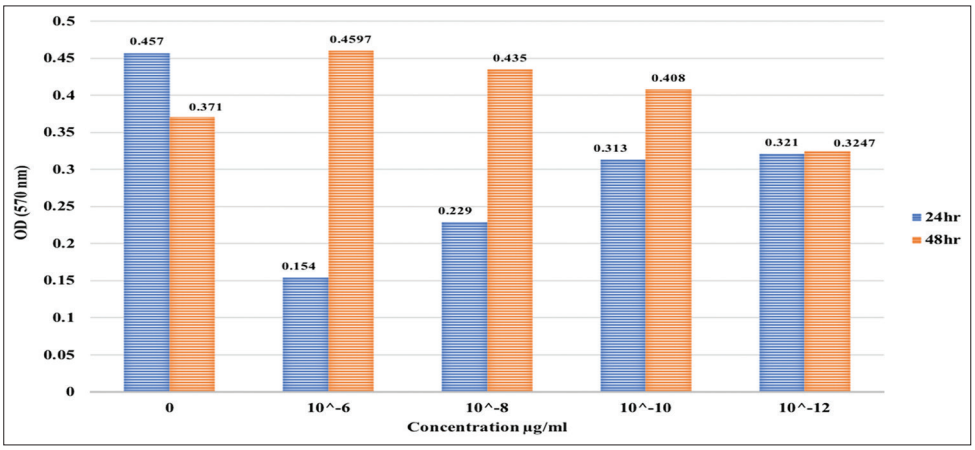

Figure 1: Vitamin D_3 inhibited cancer cell growth in a time and dose-dependent pattern: RD cells were treated with increasing concentrations of Vitamin D3 for 24 h, and 48 h and viability of cells determined using 3(4,5-dimethyl- 2-thiazolyl)-2,5-diphenyl-2H-terazolium bromide

different doses of Vitamin D_3 was significant ($P \leq 0.05$) on the proliferation of RD cell line over both periods (24 and 48 h) of treatment. After 24 h treatment, all concentrations (10^{-6}, 10^{-8}, 10^{-10}, and 10^{-12} µg/ml) were effective in decreasing the proliferation of RD cells as compared with the negative. control, which showed a complete confluent monolayer of cohesive malignant cells; the concentration of Vitamin D_3 was inversely related to the proliferation of RD cell lines as with increasing doses of Vitamin D_3, the proliferation decreased, as shown in [Figure 2].

Effects of Vitamin D3 on the proliferation of L20B cell line were highly significant ($P \leq 0.05$) according to different times and concentration doses, statistical analysis revealed that after 24 h treatment, decreasing cellular proliferation by increasing doses of Vitamin D3, the highest decrease in proliferation effect was at 10–6 µg/ml with 0.0330 ± 0.0017 and the lowest effect was at 10–12 µg/ml with 0.3420 ± 0.0011 as compared with the negative control (0.3570 ± 0.0011). However, after 48 h the effects were changed as compared with the effects after 24 h, whereby Vitamin D3 doses enhance cell proliferation significantly at doses (10–6, 10–8, and 10–10 µg/ml) with O.D (0.4640 ± 0.0020), (0.5147 ± 0.0018), and (0.5303 ± 0.0014), respectively, as compared with control (0.2927 ± 0.0008). The exception was at the concentration (10–12 µg/ml) causing a decrease in proliferation of L20B tumor cells significantly with O.D (0.2893 ± 0.0052), as shown in [Figures 3 and 4].

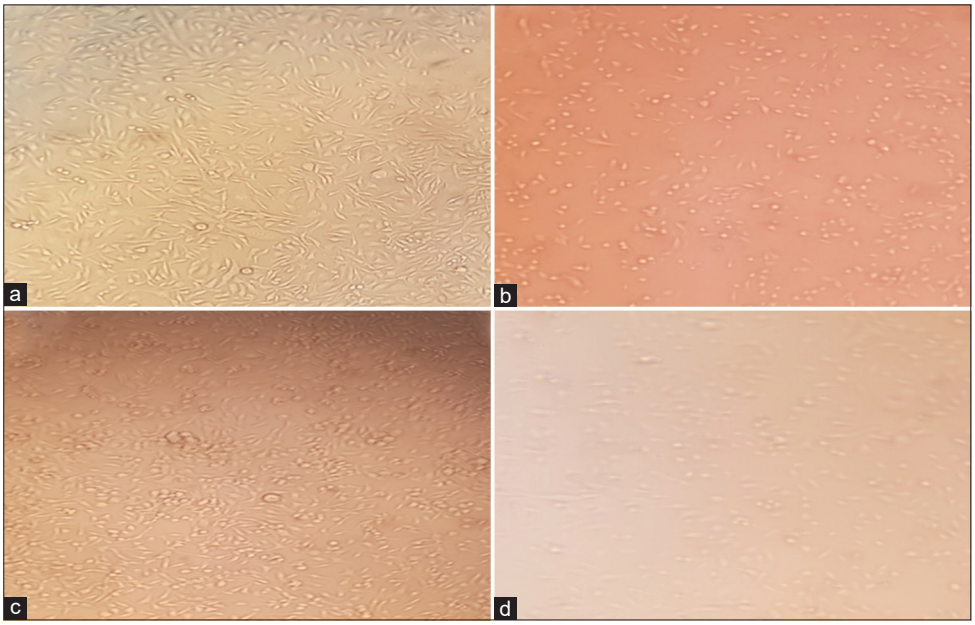

Figure 2: RD cell line treated with Vitamin D_3 at various concentrations: ×400: (a) Control; (b) with conc. 10^{-6} after 24 h; (c) conc. 10^{-6} after 48 h; and (d) conc. 10^{-12} after 24 h

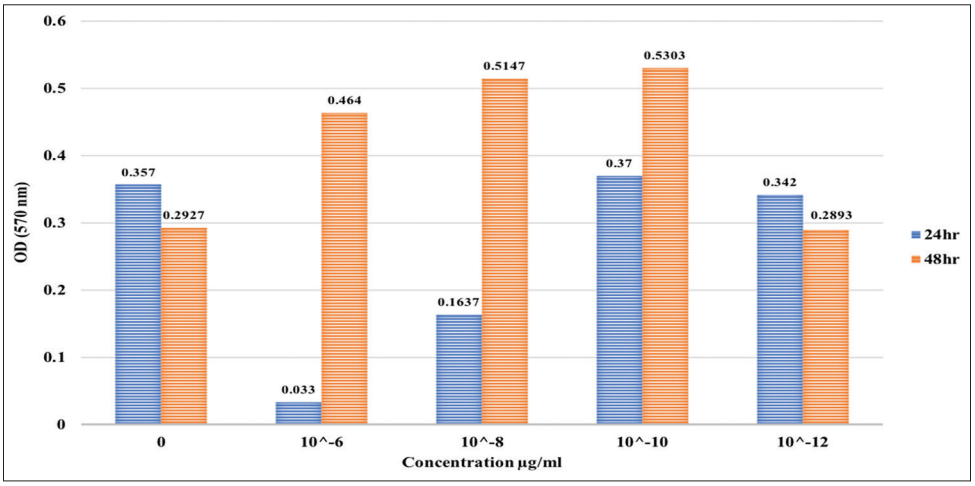

Figure 3: Vitamin D_3 inhibited cancer cell growth in a time and dose-dependent pattern: L20B cells were treated with increasing concentrations of Vitamin D3 for 24 h, and 48 h and viability of cells determined using 3(4,5-dimethyl- 2-thiazolyl)-2,5-diphenyl-2H-terazolium bromide

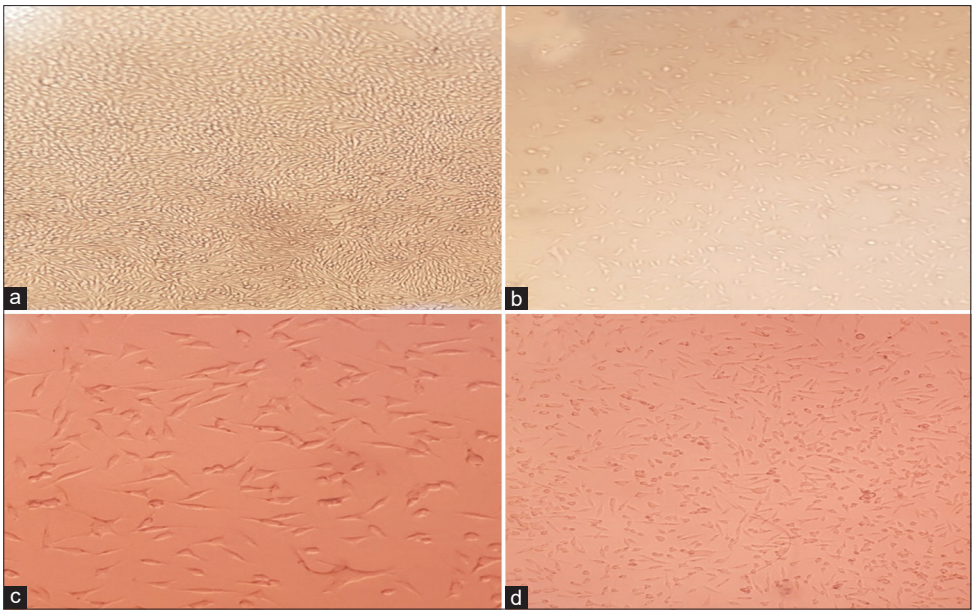

Figure 4: L20B cell line treated with Vitamin D_3 at various concentrations: (×400): (a) Control; (b) with conc. 10^{-6} after 48 h; (c) conc. 10^{-6} after 24 h; and (d) conc. 10^{-12} after 24 h

Cytotoxic effects of Vitamin D_3 after 24 h treatment on proliferation of L20B cell line were observed with O.D. (0.2396 ± 0.033); however, after 48 h Vitamin D_3 had lessened its cytotoxic effect in decreasing proliferation of RD cells lines and increasing proliferation was observed with O.D. (0.4261 ± 0.008) [Figure 4].

The results revealed that the RD tumor cell line was more sensitive than the L20B cell line to Vitamin D_3 concentrations. This sensitivity may be by the differences in membrane properties between RD and L20B. Lee et al. (2003); AL-Asady (2014) demonstrated that cell membrane receptors of the cell line vary in their response to different drugs or crude extracts during the chemotherapy treatments. Drag et al. (2009) showed significant differences in sensitivity between cell lines depending on the compound used.

Experimental observations suggest that the chemopreventive effects of Vitamin D appear to be mainly due to its activity on main biological functions such as cell differentiation, cell proliferation, signal transduction, growth factors gene expression, and apoptosis (Gocek and Studzinski, 2009; Samuel and Sitrin, 2008). Mechanisms of action of Vitamin D_3 are inducing apoptosis. Ko et al. (2004) *in vitro* study found that the absence of a Vitamin D in the maternal diet leads to raised mitosis and decreased apoptosis in rat brains. The antineoplastic activity of D_3 could be mediated by genomic pathways through classic VDR mechanisms (Wu et al., 2007).

Furthermore, other *in vitro* findings on prostate cancer cells indicate that the growth-inhibiting effects of Vitamin D on tumor cells appear to be associated with increased growth differentiation factor-15 (GDF-15) expression and secretion of the GDF-15, another member of the transforming growth factor-b (TGF-b) superfamily of growth factors (Lambert et al., 2006). Interestingly, the effects of long-term treatment with Vitamin D_3 on the mRNA encoding in different members of the TGF-b family also reported on squamous carcinoma cells or colorectal cancer cells (Lin.et al., 2002; Palmer et al., 2003).

Furthermore, Vitamin D_3 has been shown to enhance apoptosis in prostate cancer, breast cancer, and colon cancer (Gocek an Studzinski, 2009). In this context, *in vitro* investigations on colorectal cancer show that 1,25(OH)2D3 may promote apoptosis by a p53-independent mechanism. These studies show that these molecules may also stop apoptosis by downregulating anti-apoptotic expression and pro-survival proteins such as Bcl-2, Bcl-XL, or by increasing the expression of proapoptotic proteins such as Bax, Bak, and Bad (Diaz et al., 2000).

In summary, we demonstrate that Vitamin D_3 inhibits the proliferation of tumor cell lines in a dose-dependent manner and the normal cell lines are less sensitive to the inhibition proliferation effect of Vitamin D_3.

CONCLUSION

Vitamin D_3 metabolites have significant antineoplastic activity in clinical models. In medical studies, the impact of Vitamin D_3 has been reported in many different classes

of cancer. A low Vitamin D_3 activity was associated with an increase cancer risk and more aggressive tumor growth. The inhibitory effects of Vitamin D_3 can be achieved as a single therapeutic agent or in combination with other therapies.

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