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IN-VITRO ANTI-PROLIFERATIVE AND APOPTOTIC POTENTIAL OF VITAMIN D3 ON CANCER CELL LINES

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Abstract

Background: The role of vitamin D3 in calcium and mineral metabolism is known and it is an effective regulator of cell differentiation, cell growth and angiogenesis and recently it has been demonstrated to affect, tumor invasiveness and apoptosis. Though, many evidences from epidemiological studies show the potential involvement of vitamin D3 in the treatment and prevention of certain cancers, although it is not completely identified how vitamin D3 inhibits the growth of cancer cells. More recently numerous studies have demonstrated the known pro-apoptotic and anti-proliferative properties of vitamin D3 which are mediated by binding of a receptor called vitamin D receptor (VDR). Furthermore, it is also studied that vitamin D3 is a potent antioxidant and it reduces the reactive oxygen species (ROS) by preventing the growth of cancer cells. Though, it is currently unidentified whether vitamin D3 prompted cancer cell death which is intermediated by its effect on reactive oxygen species. Therefore, the efficacy of vitamin D3 for inhibiting the growth of HeLa and HepG2 cell lines was determined. **Methods:** In the present research, cancerous (HeLa and HepG2) and normal (BHK-21) cell lines were used to check the proliferation, angiogenesis, apoptosis and anti-oxidative activity. Cells were cultured and treated with 10^{-3}M, 10^{-4}M and 10^{-5}M of vitamin D3. **Results:** After the treatment of vitamin D3, ELISA with VEGF and p53 was performed to check angiogenesis and apoptosis respectively. Gene expression level was analyzed by using apoptotic and proliferative markers. Reduced proliferation via MTT, reduced angiogenesis via VEGF and increased apoptosis via p53 were observed in cancer cells after the treatment as compared to normal treated cells. Anti-oxidant (SOD, CAT, APOX, GSH and LDH) activity was increased and oxidative stress was reduced in treated cells as compared to normal cells after the treatment with vitamin D3. **Conclusion:** In short term

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vitamin D3 decreases the cell viability by apoptosis induction. While in long term it has anti-angiogenic effect that is independent to cell viability. Our data showed that vitamin D3 inhibited the growth HeLa and HepG2 cells in a dose dependent manner. However, more studies are required in this respect.

Keywords: Angiogenesis, Anti-Oxidant, Apoptosis, Cancer, Cell Lines, Proliferation, ROS, Vitamin D3

INTRODUCTION

Vitamin D is a class of fat-soluble secosteroid with ability to inhibit cancer cells proliferation and it is important for accumulative intestinal absorption of calcium, phosphate, zinc and several other biological metabolites [1]. In human the most vital complexes in this class are vitamin D3 and D_2 also called cholecalciferol and ergocalciferol respectively. Vitamin D3 is synthesized from 7- dehydrocholesterol in the skin and vitamin D which we intake from nutrition is biologically nonfunctional, an enzymatic change through the process of hydroxylation in the body (liver and kidney) is essential for the activation of vitamin D [2]. Mechanically, vitamin D3 binds to vitamin D receptor, which further activate the vitamin-D responsive elements (VDREs), thus directly regulate the transcription of numerous target genes such as regulation of cell cycle arrest, angiogenesis and differentiation [3-5]. Several epidemiological researches have recommended that vitamin D3 has a defensive effect against the progression of breast cancer and certain carcinogenesis [6-9]. The active form of vitamin D, possesses several properties including pro-differentiation, anti-angiogenesis, anti-proliferation, pro-apoptosis and immune regulation [10-13]. Vitamin D acts as membrane antioxidant and regulates the levels of ROS through its anti-inflammatory mechanism and cell-signaling pathways [14, 15]. The basic reason for the cancer prevention agent capacity of the Vitamin D and its metabolites is considered as far as their molecular linkage to vitamin D2 and cholesterol [16]. This study was designed to investigate the effect of vitamin D3 on cancer (HeLa and HepG2) and normal (BHK-21) cell lines. In this present study, cells treated with vitamin D3 showed reduced proliferation and angiogenesis, increased apoptosis and reduced oxidative stress.

MATERIALS AND METHODS

Sampling of cell lines

HeLa, HepG2 and BHK-21 cell lines were collected from the laboratory of cell culture established at The University of Lahore, Lahore, Pakistan. These cell lines were preserved in cryo vials and present in liquid nitrogen. For further processing cryo vials were revived.

Culturing of Cell Lines

The cryo vials of HeLa, HepG2 and BHK-21 were obtained and thawed. Cells were cultured in the culturing flask in which HG-DMEM (High Glucose Dulbacco Modified Eagle Medium, CAISSON Inc., USA) along with10% FBS (fetal bovine serum), (GIBCO, USA),

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streptomycin and penicillin (GIBCO, USA) was added. Cells were incubated and observed under the microscope.

Sub-Culturing of cells

When these cultivated cells achieved 70-80% confluence then these cells were sub-cultured and shifted to other culturing flasks. For sub-culturing, the cells were washed with PBS (Invitrogen Inc., USA), for 2-3 times and incubated with 0.5ml of 0.05% trypsin and 0.53 EDTA (Thermo Scientific, USA) until the cells detached from the surface of culturing flask. Flasks were observed under the microscope to confirm the detachment of the cells. HG-DMEM (High Glucose Dulbacco Modified Eagle Medium) with 10% FBS was added to it and mixed it well.

Treatment of Cells with Vitamin D3

After sub-culturing HeLa, HepG2, and BHK-21 cells were plated onto 96-well plate (Corning, USA) for cell proliferation and bio-chemical assays. HeLa, HepG2, and BHK-21 cell lines were divided into four groups for each cell line. One group of each was control. Remaining three groups of each cell line were named as, HeLa treated with vitamin D3, abbreviated as treated HeLa as (He-10^{-3}M, He-10^{-4}M and He-10^{-5}M). Treated HepG2 as (HG-10^{-3}M, HG-10^{-4}M and HG-10^{-5}M) and BHK-21 as (BHK-10^{-3}M, BHK-10^{-4}M and BHK-10^{-5}M). For 24 and 48 hours cultured cells were subjected to treatment.

Cytotoxicity Assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Invitrogen Inc., USA) assay was performed on cultured 96 well plates (Corning, USA) to compare proliferative potential of these cell lines for four different groups. Cell suspension of 200ul was added in each well and incubate at 37^{o}C and 5% CO2 for 24 and 48 hours. One group of each was control. Remaining three groups of each cell lines were treated with vitamin D3. Cells were washed with PBS (Phosphate Buffered Saline, Invitrogen Inc., USA), further cells were incubated in 100µl HG-DMEM) containing 25µl solution of MTT (Invitrogen Inc., USA) for 2 hours. In living cells MTT is changed into formazans (purple colored) which then solubilized with 10% SDS (Invitrogen Inc., USA) and absorbance was taken at 570 nm.

Enzyme Linked Immunosorbent Assay (ELISA)

Solid phase sandwich ELISA assay was performed on these cell lines for vascular endothelial growth factor (VEGF) (Santa Cruz Biotechnology, USA) onto a 96-well plate (Corning, USA). VEGF rabbit polyclonal anti-antibody (Santa Cruz biotechnology, USA) was diluted to a concentration of 2-10µg/ml in a coating buffer. Then 96-well plate was coated with 100µl of this diluted antibody and incubated it for 120 minutes. After incubation the capture antibody was removed from each well. Each well was washed with washing buffer (1X TBS-T for three times). BSA (blocking solution) of 200µl was added and incubated it for 30 minutes. After the removal of BSA, wells were washed with

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washing buffer. Medium collected of 200µl from all experimental groups was added to each well. The medium was removed after 30 minutes and each well was washed. HRP conjugated secondary anti-body (Santa Cruz biotechnology, USA) of 00µl was added to all the wells and incubated it for 60 minutes. After incubation, secondary antibody was removed and each well was washed. 100µl of TMB (Tetramethylbenzidine) (Invitrogen Inc., USA) is the most common chromogenic substrate, was added to all the wells for the detection of HRP, and incubated for 20 minutes. 100µl of stop solution 0.18M H_2SO_4 (RICCA Chemical company, USA) was added to stop the TMB reaction. Absorbance was taken at 450 nm and the graph was plotted.

Lactate Dehydrogenase Assay (LDH)

With the use of lactate dehydrogenase kit (AMP Diagnostics, Austria) this assay was performed. Briefly, the medium collected from all experimental groups of 5µl was mixed with working reagent of 100µl of LDH, in 96 well plate, and incubated it for 5 min and absorbance of solution was taken at 340nm.

Evaluation of Anti-oxidative Enzymes

Glutathione Reductase (GSH) Assay

Glutathione reductase assay was performed onto a 96-well plate with the reaction mixture of 200µl in each well. Reaction mixture was prepared by using 40mM EDTA, 20mM KH_2PO_4 buffer (pH 7.5), and 10mM oxidized glutathione. Medium collected from all the groups of cell lines were added in this mixture. 20mM NADPH was added at the end and absorbance was taken at 340nm.

Catalase (CAT) Assay

Catalase assay was performed onto 96-well plate which contained 31.25mM H_2O_2, 12.5mM KH_2PO_4 (pH 7.0) and medium taken from all group of experiments of cell lines. Keeping it in the light for 45 to 60 seconds, the absorbance was taken at 240 nm.

Superoxide Dismutase (SOD) Assay

Reaction mixture was prepared by the medium of different experiments groups mixed with 1.2 ml sodium pyrophosphate buffer (52mM, pH 8.3), 0.1ml phenazine methosulphate (180 1M) (Santa Cruz Biotechnology, USA) and 0.3ml nitro-blue tetrazolium (300 lM) (Invitrogen Inc., USA). With the addition of 0.2 ml nicotinamide adenine dinucleotide (750 lM) the reaction was started (Santa Cruz Biotechnology, USA). Plates were incubated for 90s at 30˚C, to stop the reaction 0.1 ml of glacial acetic acid (GAA) was added to it. The reaction mixture was stirred vigorously with the use 4.0 ml n-Butanol. The mixture was incubated for 10 minutes and centrifuged for 5 minutes at 2,000 rpm and absorbance was taken at 560 nm against the blank.

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Ascorbate per Oxidase (APOX) Assay

Reaction mixture was prepared by adding 2.5mM ascorbate, 25mM KH_2PO_4 buffer of pH 7.0, and 75mM H_2O_2. Medium collected from different experimental groups was added. After mixing it well, the mixture was kept in light for 3 minutes and absorbance was measured at 290 nm.

GENE EXPRESSION

RNA Isolation

According to the protocol by using TRIZOL (Invitrogen Inc., USA) total RNA from these cells was isolated. TRIZOL reagent of 400ul was added to wells and cell suspension was shifted to 1.5 ml centrifuge tubes. Chloroform of 200µl was added to cell suspension in order to separate the phases, after vigorous shaking, samples were incubated at room temperature for 10 minutes. These samples were centrifuged at 12,000 rpm for 15 min at 4^{o}C. The transparent layer that contains RNA was isolated and to precipitate the RNA, 500µl isopropanol was added. Again, the mixture was centrifuged at 4^{o}C for 10 minutes at 12,000 rpm. Pellet of RNA was obtained and washed it with 75% ethanol and again centrifuge for 5 minutes at 7500 rpm. Pellet of RNA was allowed to air dried partially and it was dissolved in diethyl pyro carbonate (DEPC) treated water. Total RNA was quantified by Spectrophotometer (Nano Drop, ND-1000).

Complementary DNA Synthesis

cDNA was synthesized by using 1µl primer (dT), 10µl of water and 1ug of RNA. All these were mixed and incubated at 65˚C for 5 min. In this mixture 1µl dNTPs (10mM), 4µl of 5 x reaction buffers and 1µl water were added, mixed it and incubated for 5 minutes at 37˚C.After the incubation 1µl M-MLV reverse transcriptase (RT) (Fermentas, USA) was added and again incubated for three steps: such as at 42˚C for 60 minutes, at 70˚C for 5 minutes and at 4˚C for 5 minutes. The volume of the total reaction was 20µl.

Primer Designing

Primer3 software (http://frodo.wi.mit.edu/primer3/) was used to design the specific gene primers. From NCBI and UCSC sequences were taken. The oligonucleotide sequences for the selected and specific genes corresponding to cervix, hepatic, pro-apoptotic and pro-survival markers are presented in Table as shown below.

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Table: 1

Gene	Primer sequence	Tm
Ki67 (F)	5-GAGGACTCGATCGGTGG-3	60°C
Ki67 (R)	5-GTCAAGTCGCACCCCAAAGTC-3	60°C
59BAX (F)	5-GAGTGACACCCCGTTCTGA-3	60°C
59BAX (R)	5-CGATGCGCTTGAGACACTC-3	60°C
P53 (F)	5-TTTCTTTGCTGCCGTCTTCC-3	59°C
P53 (R)	5-TGACTGCTTGTAGATGGCCA-3	59°C
TOP2A (F)	5-ACTGGGACACTCAGATCCA-3	58°C
TOP2A (R)	5-GCAAAGTACCTGGCAAGCA-3	58°C
PCNA (F)	5-TTAAACGGTTGCAGGCGTAG-3	59°C
PCNA (R)	5-GATGAGGTCCTTGAGTGCCT-3	59°C
CASPASE (F)	5-GGAGCCATGGTGAAGAAGC-3	59°C
CASPASE (R)	5-TCCAGTTCTGTACCATGGCA-3	59°C

RT- PCR

Total RNA was extracted from all the experimental groups of HeLa cell line by using TRIZOL reagent (Invitrogen, USA). cDNA was synthesized from 1mg of RNA sample using Reverse Transcription System (Fermentas, USA) according to manufacturer's protocol. Gene expression analysis was carried out by real time RT-PCR using SYBR Green PCR Super Mix (Fermentas) on BioRad System iQ5. For in vitro Tran's differentiation experiments, expression of GAPDH for optimization, CASPASE-3, BAX and p53 as apoptotic markers and KI67, PCNA and TOP2A as proliferation markers were used. GAPDH was used as internal control.

Analysis of Statistics

The data of all the experimental groups was mentioned as mean ± SEM in the experiments. Quantitative data obtained from different group of experiments was statistically analyzed through a software graph pad by using two-way ANOVA. For statistical analysis mean of all the groups were compared by Bonferroni's test and one-way ANOVA was applied to analyze the differences among the groups. From statistical analysis if $P > 0.05$ then the values were considered as significant.

RESULTS

Decreased Proliferative Activity of vitamin D3

By performing MTT assay, it had been determined that there were different proliferation rates when treating the cells with different concentrations of vitamin D3. Cancerous (HeLa, hepG2) and normal (BHK-21) cells, were treated with 10^{-3}M, 10^{-4}M and 10^{-5}M of vitamin D3 for two different timings 24 and 48 hours. In case of 24 hours decreased proliferation was observed in HeLa cells treated with vitamin D3 as compared to untreated cells. In HepG2 cells proliferation rate was downregulated while in BHK-21 cells proliferation rate was upregulated as shown in figure1. In case of 48 hours a significant difference was observed in treated cells with vitamin D3 as compared to untreated cells.

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Proliferation rate was downregulated in HeLa and HepG2 cells while up regulated in BHK-21 cells as shown in figure 1a. Values obtained from the plot of percentage viability on HeLa, HepG2 and BHK-21 cell lines for 24 and 48 hours are given in table 1 and 1a respectively. According to our results decreased proliferation rate was observed at different concentrations in cancerous cell lines. The given values were taken as mean ± SEM, which indicates the significant difference between untreated and treated cells.

Table 2: Values obtained from the plot of percentage viability on HeLa, HepG2 and BHK-21 cell lines for 24 hours

Hela Cell Line Groups	Values (±SEM)
10^{-3} M	82.67± 6.278
10^{-4} M	86.91± 3.959
10^{-5} M	72.86± 7.000
Control	100.0± 0.0000
HepG2 Cell Line Groups	
10^{-3} M	70.21± 7.017
10^{-4} M	77.92± 8.506
10^{-5} M	89.35± 5.050
Control	100.0± 0.0000
BHK-21 Cell Line Groups	
10^{-3} M	162.8± 22.15
10^{-4} M	141.2± 7.542
10^{-5} M	155.2± 4.966
Control	100.0± 0.0000

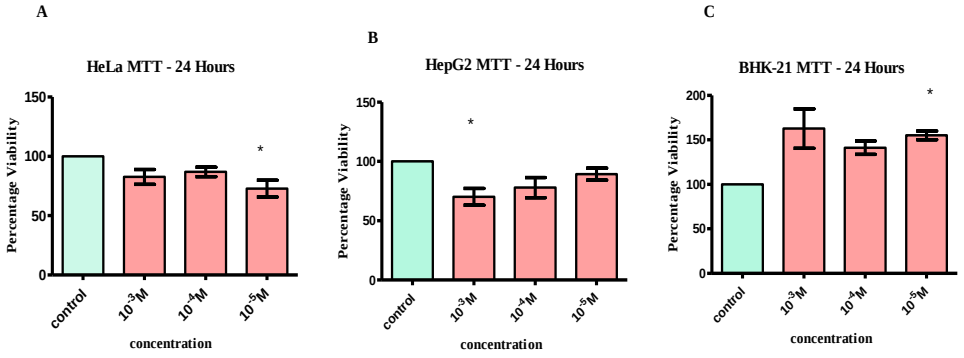

Figure 1: Vitamin D3 cytotoxicity in different cell lines as measured by MTT assay. Graph (A) (B) and (C) show proliferation rate of HeLa, HepG2 and BHK-21 cell lines after 24 hours treatment with vitamin D3 (*10^{-3}M, 10^{-4}M and 10^{-5}M*) respectively

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Table 2a: Values obtained from the plot of percentage viability on HeLa, HepG2 and BHK-21 cell lines for 48 hours

Hela Cell Line Groups	Values (±SEM)
10^{-3} M	88.81± 5.001
10^{-4} M	93.63± 12.62
10^{-5} M	94.96± 4.059
Control	100.0± 0.0000
HepG2 Cell Line Groups	
10^{-3} M	90.96± 2.838
10^{-4} M	90.54± 2.808
10^{-5} M	91.35± 10.84
Control	100.0± 0.0000
BHK-21 Cell Line Groups	
10^{-3} M	104.0± 6.329
10^{-4} M	189.4± 44.49
10^{-5} M	172.6± 21.94
Control	100.0± 0.0000

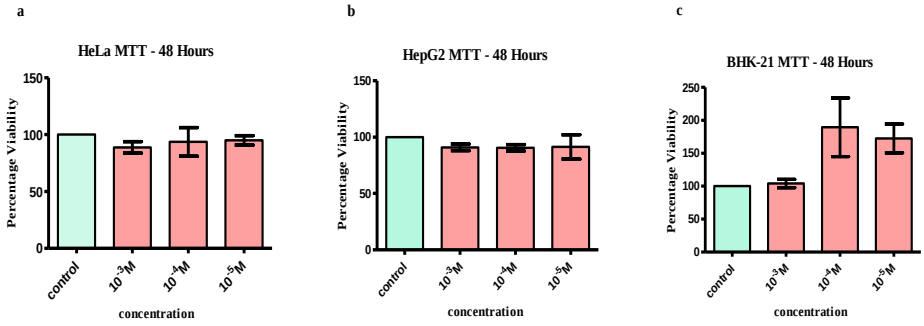

Figure 1a: Vitamin D3 cytotoxicity in different cell lines as measured by MTT assay. Graph (a) (b) and (c) show proliferation rate of HeLa, HepG2 and BHK-21 cell lines after 48 hours treatment with vitamin D3

ELISA (Enzyme linked Immunosorbent Assay) for Vitamin D3

ELISA was performed to evaluate apoptosis and angiogenesis between treated and untreated cells with vitamin D3. The decreased expression of VEGF in treated cells showed that there was decrease level of angiogenesis in cells treated with vitamin D3. In case of 24 hours angiogenesis level was significantly down regulated in HeLa and HepG2 cells treated with vitamin D3 as compared to untreated cells as shown in figure 2. In case of 48 hours angiogenesis level was also significantly downregulated in both HeLa and HepG2 cells as compared to 24 hours as shown in figure 2a. Values obtained from the plot of absorbance on HeLa and HepG2 cell lines for 24 and 48 hours are given in table 2 and 2a respectively. So according to our results angiogenesis level was decreased at different concentrations in HeLa and HeG2 cell lines. ELISA was performed to check the apoptosis level by using p53 and LDH in HeLa and HepG2 cell lines. According to our

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results it was observed that the expression of p53 and LDH in cells treated with vitamin D3 was high as compared to untreated cells. In case of 24 hours apoptotic level was significantly upregulated in HeLa and HepG2 cells as compared to untreated cells as shown in figure 3. While in case of 48 hours apoptotic level was also significantly upregulated in HeLa and HepG2 cells as compared to untreated cells as shown in figure 3a. Values obtained from the plot of absorbance on HeLa and HepG2 cell lines for 24 and 48 hours are given in table 3 and 3a respectively.

Table 3: Values obtained from the plot of angiogenesis absorbance on HeLa and HepG2 cell lines for 24 hours

Hela Cell Line Groups	Values (±SEM)
10^{-3} M	0.9390±0.03400
10^{-4} M	0.8805±0.01450
10^{-5} M	0.7620±0.07300
control	1.033±0.03800
HepG2 Cell Line Groups	
10^{-3} M	0.8665± 0.09950,
10^{-4} M	0.7605± 0.03350
10^{-5} M	0.8285±0.06850
Control	1.216± 0.2070

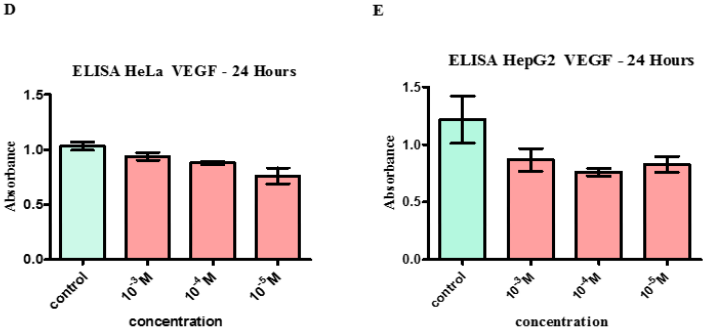

Figure 2: Rate of angiogenesis in different cell lines as measured by VEGF. Graph (D) and (E) show angiogenesis rate of HeLa and HepG2 cells after 24 hours treatment with vitamin D3

Table 3a: Values obtained from the plot of angiogenesis absorbance on HeLa and HepG2 cell lines for 48 hours

Hela Cell Line Groups	Values (±SEM)
10^{-3} M	0.7225±0.06750
10^{-4}M	0.7690±0.0740
10^{-5} M	0.7120±0.1230
control	0.8900±0.2080
HepG2 Cell Line Groups	
10^{-3} M	0.7760± 0.1190
10^{-4} M	0.8175± 0.07350
10^{-5} M	0.7315± 0.05250
control	1.466± 0.06300

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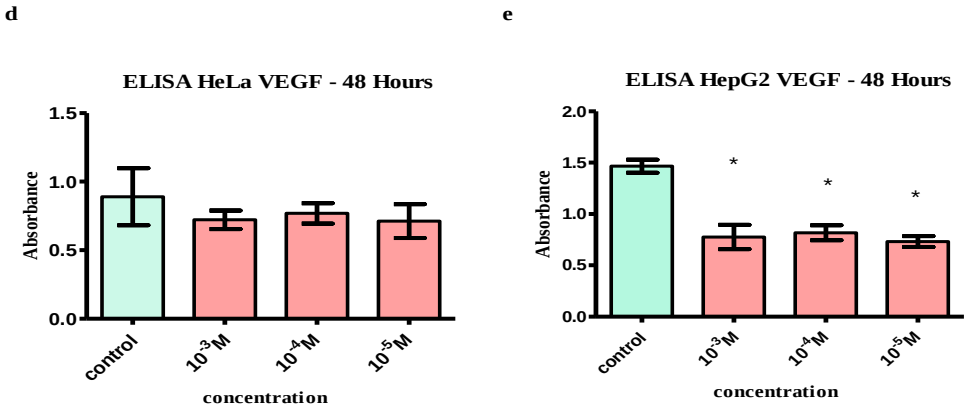

Figure 2a: Rate of angiogenesis in different cell lines as measured by VEGF. Graph (d) and (e) show angiogenesis rate of HeLa and HepG2 cells after 48 hours treatment with vitamin D3

Table 4: Values obtained from the plot of apoptosis absorbance on HeLa and HepG2 cell lines for 24 hours

HeLa Cell Line Groups (p53)	**Values (±SEM)**
10^{-3} M	0.9910±0.08500
10^{-4} M	0.9830±0.03400
10^{-5} M	0.9955±0.0395
Control	0.8890±0.09400
HepG2 Cell Line Groups	
10^{-3} M	0.8850±0.1190
10^{-4} M	0.9165±0.0485
10^{-5} M	0.9165±0.0485
control	0.7405±0.03550
HeLa Cell Line Groups (LDH)	
10^{-3} M	0.0515±0.005600
10^{-4} M	0.0407±0.0005000
10^{-5} M	0.04985±0.00915
control	0.0479±0.0139
HepG2 Cell Line Groups	
10^{-3} M	0.04495±0.004550
10^{-4} M	0.0481±0.003700
10^{-5} M	0.0471±0.002890
control	0.03905±0.005450

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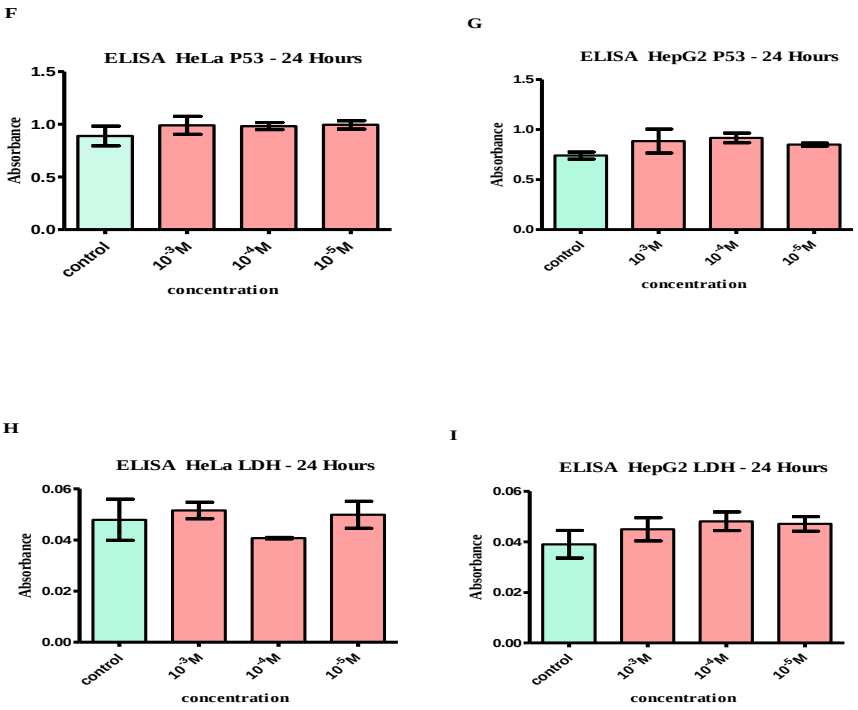

Figure 3: Rate of apoptosis in different cell lines as measured by p53 and LDH. Graph (F), (G), (H) and (I) show angiogenesis rate of HeLa and HepG2 cells after 24 hours treatment with vitamin D3

Table 4a: Values obtained from the plot of apoptosis absorbance on HeLa and HepG2 cell lines for 48 hours

HeLa Cell Line Groups (p53)	Values (±SEM)
10^{-3} M	0.9760±0.02600
10^{-4} M	1.059±0.03000
10^{-5} M	0.6340±0.2860
control	0.3865±0.05050
HepG2 Cell Line Groups	
10^{-3} M	0.9505±0.001500
10^{-4} M	1.011±0.008500
10^{-5} M	0.8895±0.1095
control	0.8640±0.03400
HeLa Cell Line Groups (LDH)	
10^{-3} M	(0.04965±0.009850
10^{-4} M	0.04415±0.005550
10^{-5} M	0.04435±0.002050
control	0.03805±0.01235
HepG2 Cell Line Groups	
10^{-3} M	0.04475±0.00175
10^{-4} M	0.04895±0.00495
10^{-5} M	0.0501±0.0096
Control	0.04015±0.00915

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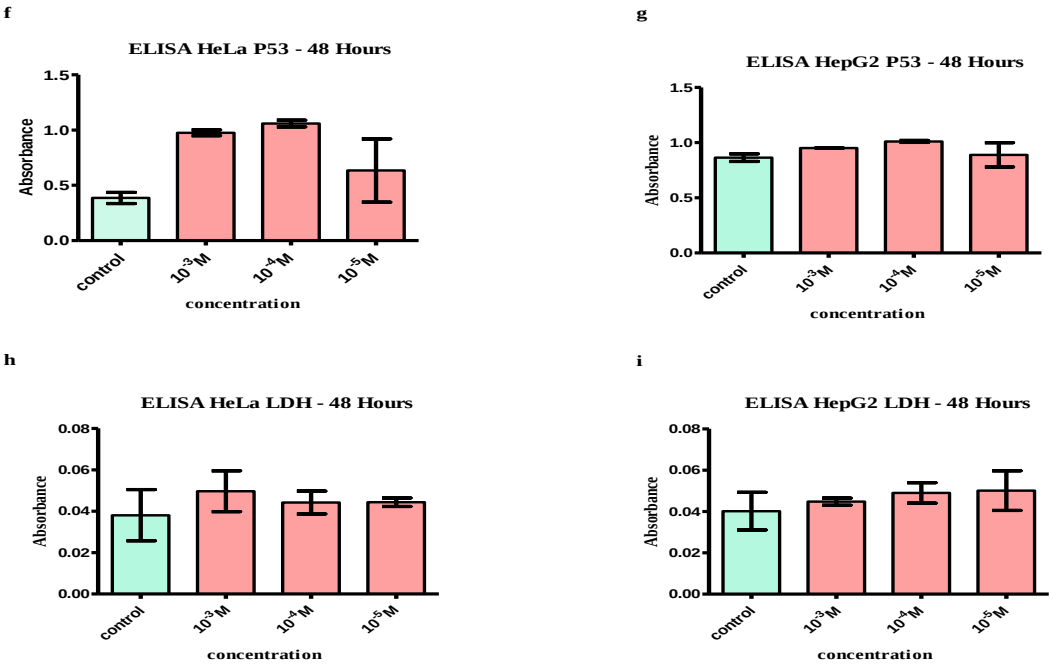

Figure 3a: Rate of apoptosis in different cell lines as measured by p53 and LDH. Graph (f), (g), (h) and (i) show angiogenesis rate of HeLa and HepG2 cells after 48 hours treatment with vitamin D3

Anti-oxidative Activity

To evaluate the anti-oxidative activity enzymes superoxide dismutase (SOD), ascorbate per oxidase (APOX), glutathione reductase assay (GSH) and catalase was performed. In case of 24 and 48 hours oxidative stress was decreased in treated cells while it was increased in untreated cells. It was observed that in case of glutathione (GSH) low level of ROS were produced in treated cells in 24 and 48 hours as shown in figure 4 and 4a respectively. Values obtained from the plot of anti-oxidative absorbance on HeLa, HepG2 and BHK-21cell lines for 24 and 48 hours are given in table 4 and 4a respectively.

Table 5: Values obtained from the plot of anti-oxidative absorbance on HeLa, HepG2 and BHK-21 cell lines for 24 hours

Hela Cell Line Groups	**Values (±SEM)**
10^{-3} M	0.3445±0.0195
10^{-4} M	0.3070±0.03200
10^{-5} M	0.3295±0.05950
Control	0.3175±0.02250
HepG2 Cell Line Groups	
10^{-3} M	0.3665±0.0705
10^{-4} M	0.3485±0.02250
10^{-5} M	0.3815±0.02250

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Control	0.3245±0.0315
BHK-21 Cell Line Groups	
10^{-3} M	1.460±0.1580
10^{-4} M	1.534±0.4370
10^{-5} M	2.097±0.5840
Control	1.331±0.2345

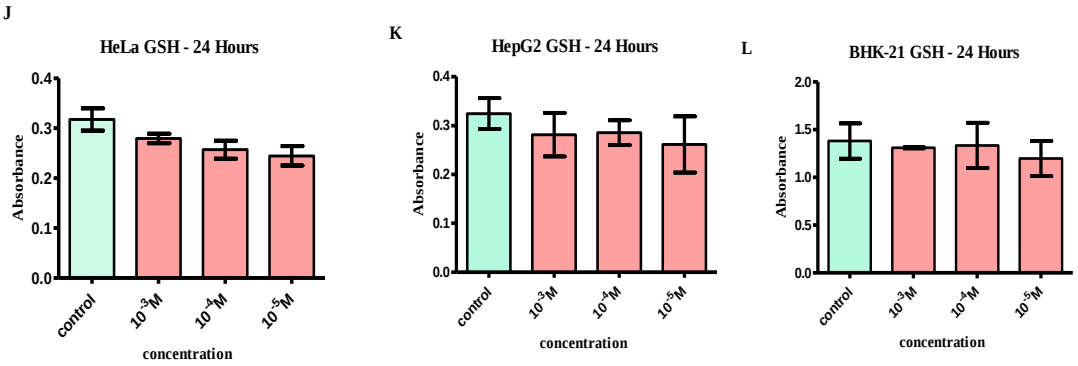

Figure 4: Anti-oxidative analysis in different cell lines as measured by GSH. Graph (J), (K) and (L) show decreased oxidative stress in treated cells of HeLa, HepG2 and BHK-21 cells after 24 hours

Table 5a: Values obtained from the plot of anti-oxidative absorbance on HeLa, HepG2 and BHK-21 cell lines for 48 hours

Hela Cell Line Groups	**Values (±SEM)**
10^{-3} M	0.3825±0.0385
10^{-4} M	0.3305±0.02750
10^{-5} M	0.2740±0.03300
Control	0.1150±0.0090
HepG2 Cell Line Groups	
10^{-3} M	0.4405±0.0235
10^{-4} M	0.3985±0.0165
10^{-5} M	0.4430±0.06900
Control	0.3305±0.02250
BHK-21 Cell Line Groups	
10^{-3} M	0.1925±0.008500
10^{-4} M	0.2615±0.1345
10^{-5} M	0.2810±0.07400
Control	0.3525±0.09950

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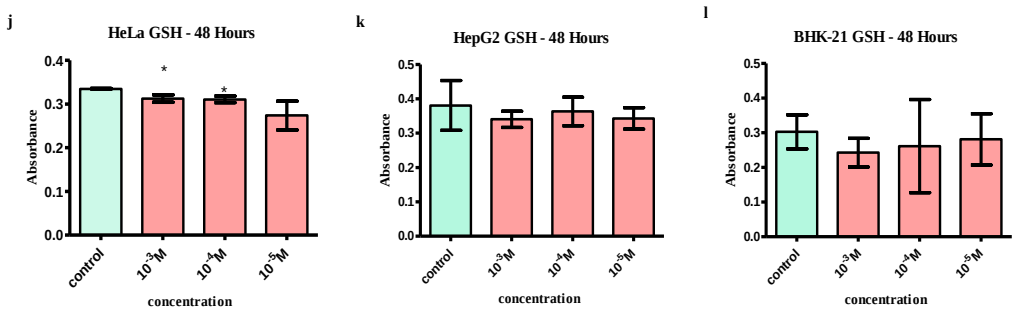

Figure 4a: Anti-oxidative analysis in different cell lines as measured by GSH. Graph (j), (k) and (l) show decreased oxidative stress in treated cells of HeLa, HepG2 and BHK-21 cells after 48 hours

Super oxide dismutase (SOD) Activity

According to our results it was observed that low levels of SOD indicated less reactive oxygen species were produced when the cells treated with vitamin D3 for 24 and 48 hours as shown in figure 5 and 5a respectively. Values obtained from the plot of anti-oxidative absorbance on HeLa, HepG2 and BHK-21cell lines for 24 and 48 hours are given in table 5 and 5a respectively.

Table 6: Values obtained from the plot of anti-oxidative absorbance on HeLa, HepG2 and BHK-21 cell lines for 24 hours

Hela Cell Line Groups	**Values (±SEM)**
10^{-3} M	2.216±0.1116
10^{-4} M	2.085±0.1334
10^{-5} M	1.978±0.1913
Control	1.897±0.2275
HepG2 Cell Line Groups	
10^{-3} M	1.796±0.4958
10^{-4} M	1.545±0.1403
10^{-5} M	0.2520±1.761
Control	1.470±0.2631
BHK-21 Cell Line Groups	
10^{-3} M	1.198±0.1885
10^{-4} M	1.092±0.2375
10^{-5} M	1.276±0.2375
Control	1.243±0.1940

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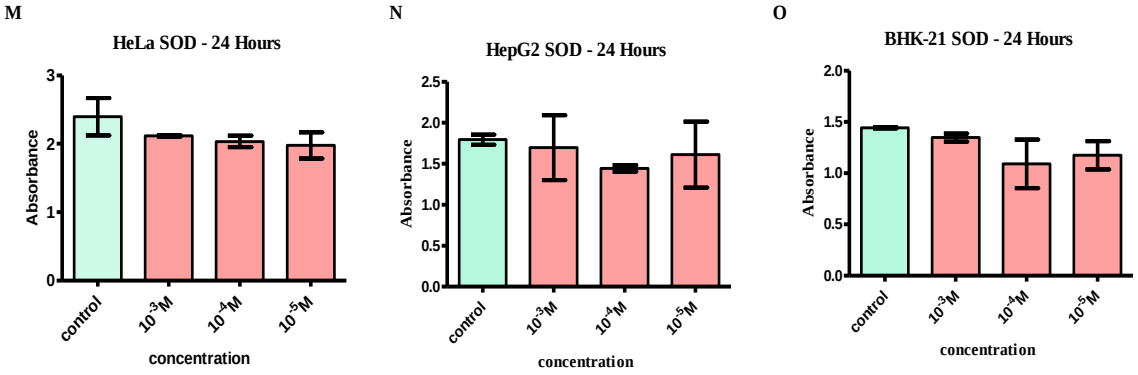

Figure 5: Anti-oxidative analysis in different cell lines as measured by SOD. Graph (M), (N) and (O) show decreased oxidative stress in treated cells of HeLa, HepG2 and BHK-21 cells after 24 hours

Table 6a: Values obtained from the plot of anti-oxidative absorbance on HeLa, HepG2 BHK-21 cell lines for 48 hours

Hela Cell Line Groups	Values (±SEM)
10^{-3} M	(1.913±0.1517
10^{-4} M	1.813±0.1424
10^{-5} M	1.794±0.1966
Control	1.334±0.2365
HepG2 Cell Line Groups	
10^{-3} M	1.294±0.8774
10^{-4} M	0.7223±0.4992
10^{-5} M	1.827±0.1359
Control	1.568±0.4636
BHK-21 Cell Line Groups	
10^{-3} M	1.986±0.1856
10^{-4} M	1.116±0.3882
10^{-5} M	1.502±0.4277
Control	1.588±0.4109

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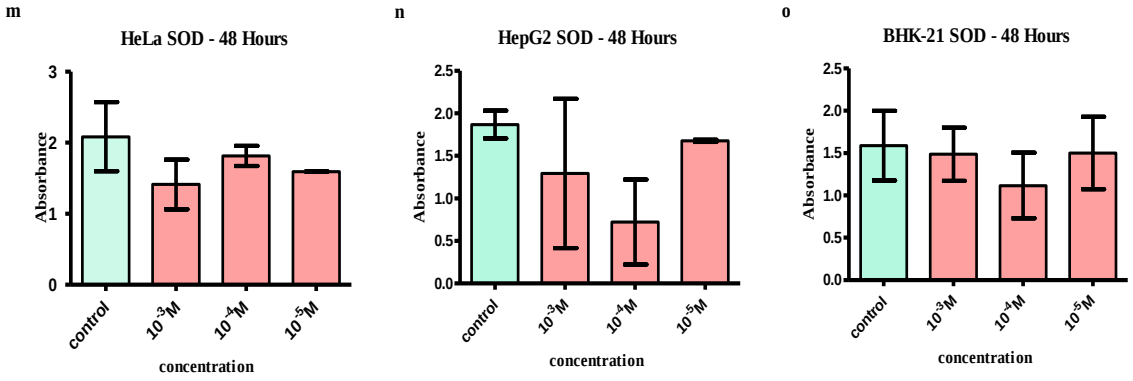

Figure 5a: Anti-oxidative analysis in different cell lines as measured by SOD. Graph (m), (n) and (o) show decreased oxidative stress in treated cells of HeLa, HepG2 and BHK-21 cells after 48 hours

Ascorbate per Oxidase (APOX) Activity

It was observed that APOX activity was decreased in treated cells showing the reduced oxidative stress after treatment of 24 and 48 hours as shown in figure 6 and 6a respectively. While in untreated cells APOX activity was increased. Values obtained from the plot of anti-oxidative absorbance on HeLa, HepG2 and BHK-21cell lines for 24 and 48 hours are given in table 6 and 6a respectively.

Table 7: Values obtained from the plot of anti-oxidative absorbance on HeLa, HepG2 and BHK-21 cell lines for 24 hours

Hela Cell Line Groups	**Values (±SEM)**
10^{-3} M	1.118±0.1125
10^{-4} M	0.9425±0.0535
10^{-5} M	0.8450±0.05200
Control	0.9060±0.08500
HepG2 Cell Line Groups	
10^{-3} M	0.8540±0.04700
10^{-4} M	0.7835±0.03350
10^{-5} M	0.7780±0.2140
Control	0.7495±0.04750
BHK-21 Cell Line Groups	
10^{-3} M	0.9070±0.2650
10^{-4} M	1.144± 0.09450
10^{-5} M	1.311± 0.1620
Control	1.017± 0.1275

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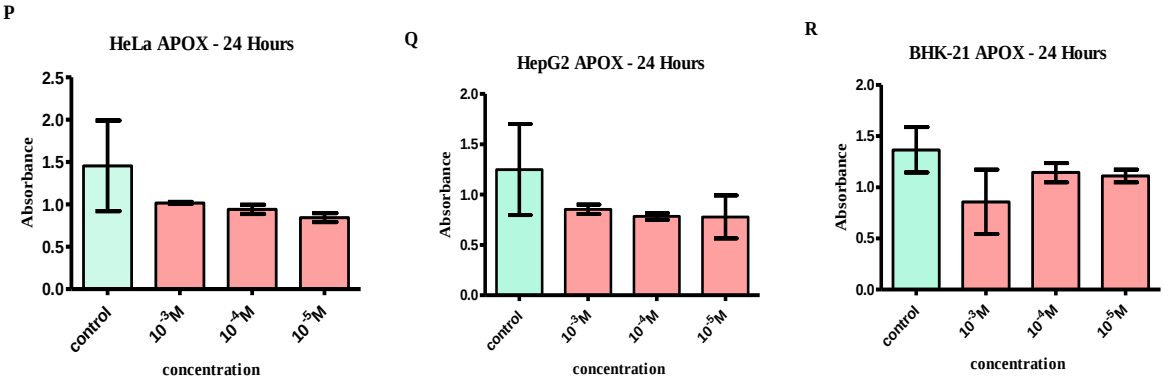

Figure 6: Anti-oxidative analysis in different cell lines as measured by APOX. Graph (P), (Q) and (R) show decreased oxidative stress in treated cells of HeLa, HepG2 and BHK-21 cells after 24 hours

Table 7a: Values obtained from the plot of anti-oxidative absorbance on HeLa, HepG2 and BHK-21 cell lines for 48 hours

Hela Cell Line Groups	**Values (±SEM)**
10^{-3} M	0.9835±0.08250
10^{-4} M	0.8910±0.0510
10^{-5} M	1.019±0.0325
Control	0.6980±0.04900
HepG2 Cell Line Groups	
10^{-3} M	0.8745±0.03150
10^{-4} M	0.7880±0.01500
10^{-5} M	0.7905±0.03350
Control	0.6585±0.1955
BHK-21 Cell Line Groups	
10^{-3} M	0.1625±0.0395
10^{-4} M	0.1540±0.0440
10^{-5} M	0.2400±0.02500
Control	0.2025±0.0645

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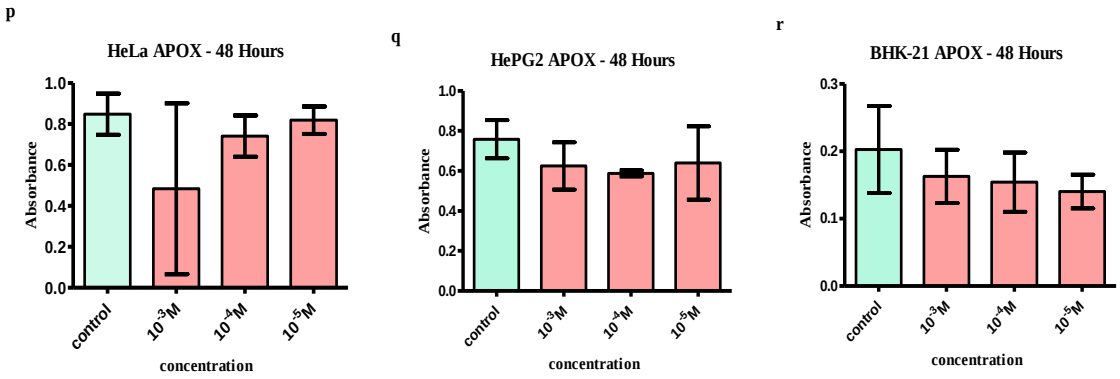

Figure 6a: Anti-oxidative analysis in cell lines as measured by APOX. Graph (p), (q) and (r) show decreased oxidative stress in treated cells of HeLa, HepG2 and BHK-21 cells after 48 hours

Catalase (CAT) Activity

In catalase assay low levels of ROS were produced in treated cells as compared to controlled group. In case of 24 and 48 hours results showing that catalase assay decreased the oxidative stress in treated cells as shown in figure 7 and 7a respectively. Values obtained from the plot of anti-oxidative absorbance on HeLa, HepG2 and BHK-21cell lines for 24 and 48 hours are given in table 7 and 7a respectively.

Table 8: Values obtained from the plot of anti-oxidative absorbance on HeLa, HepG2 and BHK-21 cell lines for 48 hours

Hela Cell Line Groups	Values (±SEM)
10^{-3} M	0.1380±0.005000
10^{-4} M	0.2115±0.004500
10^{-5} M	0.1735±0.0075
Control	0.2280±0.007000
HepG2 Cell Line Groups	
10^{-3} M	0.4315±0.01650
10^{-4} M	0.3230±0.01200
10^{-5} M	0.3480±0.02500
Control	0.2655±0.01950
BHK-21 Cell Line Groups	
10^{-3} M	1.391±0.03900
10^{-4} M	0.9570±0.2810
10^{-5} M	1.376±0.1625
Control	1.075±0.1860

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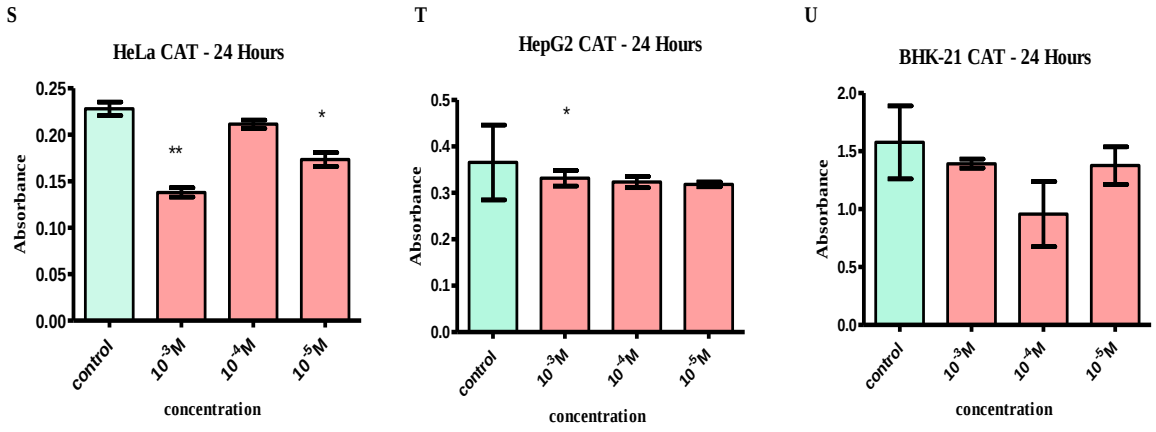

Figure 7: Anti-oxidative analysis in different cell lines as measured by APOX. Graph (S), (T) and (U) show decreased oxidative stress in treated cells of HeLa, HepG2 and BHK-21 cells after 24 hours

Table 8a: Values obtained from the plot of anti-oxidative absorbance on HeLa, HepG2 and BHK-21 cell lines for 48 hours

Hela Cell Line Groups	**Values (±SEM)**
10^{-3} M	0.2270±0.004000
10^{-4} M	0.1700±0.0170
10^{-5} M	0.2055±0.0005000
Control	0.0755±0.0055
HepG2 Cell Line Groups	
10^{-3} M	0.3775±0.0115
10^{-4} M	0.329±0.0285
10^{-5} M	0.3415±0.07850
Control	0.3395±0.0385
BHK-21 Cell Line Groups	
10^{-3} M	2.442±0.2225
10^{-4} M	1.930±0.6700
10^{-5} M	1.779±0.2780
Control	1.930±0.6700

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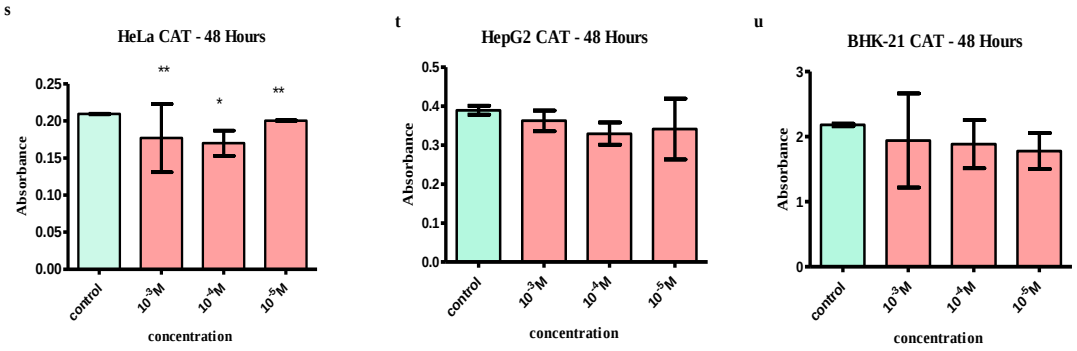

Figure 7a: Anti-oxidative analysis in cell lines as measured by APOX. Graph (s), (t) and (u) show decreased oxidative stress in treated cells of HeLa, HepG2 and BHK-21 cells after 48 hours

GENE EXPRESSION ANALYSIS

Analysis of Apoptosis and proliferation in Treated and Untreated HeLa BHK-21 Cells

RT-PCR for quantitative expression of HeLa cells

Expression level of proliferative genes (TOP2A, KI67 and PCNA) and apoptotic genes (BAX, P53 and CASPASE-3) was analyzed by using extracted RNA of HeLa and BHK-21 cells. According to our results apoptotic genes (BAX, P53 and CASPASE-3) were up regulated in HeLa cells as compared to proliferative genes. Expression of apoptotic markers such as BAX, p53 and CASPASE-3 was higher in treated HeLa cells as compared to untreated cells as shown in figure (23). By estimating the quantitative gene expression of proliferative markers (PCNA, TOP2A and KI67) it was observed that proliferation was down regulated in treated HeLa cells.

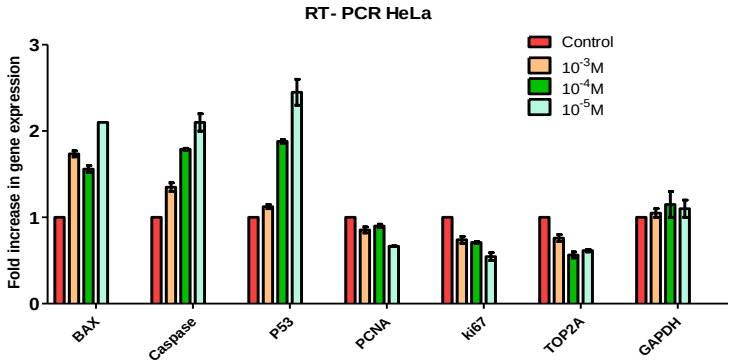

Figure 8: Quantitative analysis of percentage of cells expressing the specific genes

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RT-PCR analysis for the quantitative expression of apoptotic genes such as BAX, p53 and CASPASE-3 was up-regulated in treated HeLa cells as compared to control and expression of proliferative genes such as PCNA, KI67 and TOP2A was down regulated.

RT-PCR for quantitative expression of BHK-21 cells

According to our research work, the quantitative expression of BHK-21 cells in treated cells was nearly equal to untreated cells as shown in figure (24). The marker GAPDH was equally expressed in both treated and untreated cells.

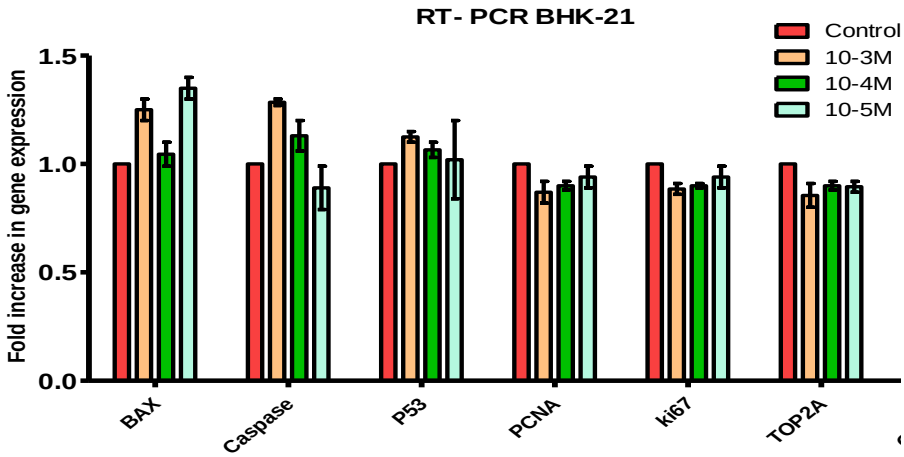

Figure 9: Quantitative analysis of percentage of cells expressing the specific genes

No significant difference was observed in RT-PCR analysis for the quantitative expression of apoptotic (BAX, p53 and CASPASE-3) and proliferative genes (PCNA, KI67 and TOP2A) in BHK-21 treated cells as compared to control group.

DISCUSSION

The purpose of the study is to observe the activity of vitamin D3 on cancerous and normal cells. Vitamin D is a class of fat-soluble secosteroids, which are changed into the body in numerous biologically active metabolites that act as precise hormones, circulating in the blood and controlling the actions of calcemic and non-calcemic cells [17]. The native production of vitamin D may be responsible for modulating up to 200 genes, may allow the several benefits of health that have been described for VD [18]. VD is not only an immunomodulatory but also the regulator of the calcium homeostasis and it induces differentiation in some kinds of hematopoietic cells and can exert anti-proliferative effects on many cells and these effects are triggered by vitamin D [19]. It has been found that Vitamin D receptor (VDR) involved in influencing cell differentiation and proliferation [20]. Numerous in vitro and in vivo study of vitamin D compounds has demonstrated the persuasive effects on the regulation of cancer cells [21]. Several in vitro

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studies have demonstrated that high concentrations of vitamin D exposure to tumor cells induce differentiation and inhibit their proliferation [22]. In our findings when we treated the cancer cells with vitamin D3 the proliferation was inhibited to some extant as compared to untreated cells but in normal cells there is no effect of vitamin D3 via MTT proliferation assay as shown in figure (1 to 3). VEGF is a key regulator of tumor angiogenesis, an active target for inhibition and control of malignant diseases such as solid tumors [23]. Vitamin D might also decrease the invasiveness of cancer cells, besides having anti-proliferative properties as well as acts as an anti-angiogenesis agent. All of these studies suggest that the properties of vitamin D might be explored for chemo-preventive and therapeutic purposes in cancer treatment [19]. Vitamin D and its metabolites decrease the incidence of certain types of cancer by inhibiting tumor angiogenesis, invigorating mutual adherence of cells, and upgrading intercellular correspondence through gap junctions [24]. According to our research work we found angiogenesis is inhibit in cancer cells treated with vitamin D3 but no effect was observed in normal cells as shown in figure (4 to 5). Our results showed that vitamin D3 has some factors which can inhibit angiogenesis but further studies are needed for confirmation. Apoptosis is a genetically programmed mechanism, where biochemical events lead to cell death. It also inhibits the probability of mutational changes, leading to distortion after the damage of DNA by the removal of such cells [17].

In some cell lines, there was up regulation of BAX which showed that vitamin D-prompted apoptosis was intervened through p53 pathway. These studies demonstrated that vitamin D-induced apoptosis differs with the cell type and can be intermediated by the p53-independent as well as dependent pathway [17]. In our own finding apoptosis level was increased in cancer cells treated with vitamin D3 as compared to untreated cells stained by p53 as shown in figure (6 to 7). So, vitamin D3 induces apoptosis in cancer cells. Vitamin D acts as a membrane antioxidant, superoxide dismutase (SOD) and glutathione are the most important anti-oxidative enzymes [25]. Thus Vitamin D3 and its metabolites inhibited iron-dependent lipid liposomal per-oxidation. 1, 25-dihydroxycholecalciferol and Cholecalciferol were all of comparative viability as inhibitors of lipid peroxidation yet were less compelling than 7-dehydrocholesterol [16]. Vitamin D could improve anti-oxidant defense against oxidative stress and it is an active factor. Though, no significant co-relation was found between activity of SOD and other antioxidants and the serum Vitamin D levels. Vitamin D supplementation could expand erythrocyte SOD and catalase activities in patients with atopic dermatitis. Furthermore, the study on the diabetic subject was a positive correlation between SOD activities, other anti-oxidative enzymes and vitamin D levels [25]. It is suggested by some researchers that vitamin D supplementation may be helpful in the treatment and deterrence of certain types of cancer, because of its anti-tumor activities [17]. In antioxidant assay we evaluated superoxide dismutase, glutathione reductase, catalase, ascorbate per oxidase and lactate dehydrogenase activity to observe the oxidative stress of injured cells. These antioxidative enzymes showed their role in reducing the oxidative stress of cells by removing the superoxide free radicals. In our present study when vitamin D3 was treated with HeLa and HepG2 and

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BHK-21 cells at different timings 24 and 48 hours same results were found. Oxidative stress was decreased by vitamin D3 treatment as shown in figure (8 to 21). vitamin D3, is putative anti-cancer agent interesting profile of induction of apoptosis, differentiation, and growth inhibition in tumor cells. Apoptosis is induced by vitamin D compounds via a novel caspases and p53-independent pathway [26]. On these cells we did not detect the VDR expression. In our findings it was observed that apoptosis genes such as p53, caspase-3 and BAX were unregulated. Quantitative gene expression of apoptotic genes was higher in treated cells as compared to untreated cells but expression of proliferative genes such as TOP2A, PCNA and KI67 was down regulated as shown in figure (21 to 23). It was demonstrated that vitamin D3 induces significantly more apoptosis in treated cells as compared to untreated cells. Similarly, vitamin D3 has same level of anti-proliferative activity in treated cells and significantly more anti-angiogenic ability in treated cells s compared to untreated cells.

FUTURE RECOMMENDATIONS

In the present study anti-proliferative activity of vitamin D3 has been investigated which indicates that vitamin D3 induced apoptosis and anti-angiogenic effects in cancer cell lines. Previously, the clinical trials and present study of vitamin D3 appear to be significant data, suggests that vitamin D3 should be used for treatment of cancer. It showed promising results against cancer cells growth. It could be further studied at molecular levels.

CONCLUSION

According to our research work, it was concluded that vitamin D3 induces significantly more apoptosis in treated cells as compared to untreated cells. Similarly, vitamin D3 has same level of anti-proliferative capacity in treated cells as compared to control and significantly more anti-angiogenic ability in treated cells as compared to control. Vitamin D3 affects the cell in two ways. In short term (24 hours) vitamin D3 decreases the cell viability by apoptosis induction. While in long term (48 hours) it only has anti-angiogenic effect that is independent to cell viability**.**

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